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Influence of initial levels of carbohydrates, fructans, nitrogen, and soluble proteins on regrowth of *Lolium perenne* L. cv. Bravo following defoliation

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Abstract

An experiment was designed to evaluate the role of N and C reserves on regrowth of *Lolium perenne* cv. Bravo following defoliation. By using two nitrogen fertilization levels together with three photoperiodic conditions, plants with variable contents of water-soluble carbohydrates (43–216 mg g^{-1} DW in stubble) and contrasting amounts of nitrogen (7–49 mg g^{-1} DW) were obtained. Plants were severely defoliated and regrowth was followed for 28 d under the same environmental conditions. The yield of leaf dry matter at the end of the regrowth period was not related to the initial level of carbohydrate reserves. However, levels of fructan in leaf sheaths and in elongating leaf bases strongly influenced the shoot yield during the first 2 d following defoliation. Fructan exohydrolase activity increased 2–3-fold in sheaths and 3.5–5-fold in elongating leaf bases, suggesting that not only fructans from sheaths but also fructans from immature cells may be used as substrates for growth. In contrast, no direct relationship was found between shoot production and nitrogen or soluble protein accumulation in source organs during early regrowth. A significant correlation existed with the initial amount of soluble proteins in sheaths and in elongating leaf bases after only 6 d of regrowth.

Key words: Defoliation, regrowth, ryegrass, fructans, nitrogen reserve.

Introduction

It is generally accepted that frequent and severe defoliations, particularly if associated with high levels of nitrogen, lead to increasingly poor recovery and even death in various grass species, regardless of the most favourable climatic and soil environment (Davies, 1965; Richards, 1993).

Growth following defoliation depends first of all on the development of a photosynthetic surface (Richards, 1993). A critical role of carbohydrate and N reserves as substrates for refoliation has been advocated long ago (Graber *et al.*, 1927; Sullivan and Sprague, 1943). The hypothesis has been advanced that 'new top growths are initiated and developed largely at the expense of previously accumulated organic reserves, and that their quantity and availability limit the amount of growth that will occur' (Graber *et al.*, 1927). Indeed, extensive mobilization of non-structural carbohydrates and nitrogenous compounds (Alberda, 1957; Volenec, 1986; Ourry *et al.*, 1989; Prud'homme *et al.*, 1992) occurred in the residual parts of defoliated plants. However, it has been pointed out that a decrease in the amount of non-structural carbohydrates did not necessarily imply a causal role for these reserves in initiating and affecting regrowth (May, 1960). In experiments in which the level of carbohydrates have been modified by short-term exposures to darkness and raised temperatures, it has been shown that the yield of leaf material after cutting was related to the percentage of soluble carbohydrates in the stubble at the time of cutting and that once growth was fully established, the rate became independent of the pretreatments applied (Davies, 1965; Davidson and Milthorpe, 1966). Such a positive relationship between rate of leaf expansion and initial carbohydrate status of the stubble only arose if the carbohydrate level felt below a minimum value which has been estimated to be about 200 mg g^{-1} DW (Davies, 1965). An argument in favour of the reserve significance

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Abbreviations: C, carbon; FEH, fructan exohydrolase; N, nitrogen; WSC, water soluble carbohydrates.

for soluble carbohydrates accumulated in stubble was first suggested by Danckwerts and Gordon, who provided evidence that some of the reserve C, labelled with ^{14}C, was incorporated into new growth after defoliation (Danckwerts and Gordon, 1987). Recently, steady-state labelling of all pre-defoliation or post-defoliation C showed that before the third day of regrowth, C supply from reserves to meristematic zones was the most important C source of leaf dry matter accumulation. Thereafter, growth zones relied more on current assimilate supply as leaves emerging from stubble became photosynthetically active (De Visser *et al.*, 1997; Morvan-Bertrand *et al.*, 1999).

Adaptation to defoliation in many grass species also involved a capacity for mobilization of N compounds stored in perennial tissues such as roots or stubble, allowing N to be supplied to the growing zones despite the reduced N uptake by roots that usually occurred as a response to defoliation (Volenec *et al.*, 1996). The relative contribution of remobilization and root uptake in supplying nitrogen to new leaves after defoliation depends on the grass species (Thornton *et al.*, 1993). Moreover, the N status of the plant at the time of cutting is an important determinant of the extent to which mineral N uptake was down-regulated and of the subsequent dynamics of mobilization (Macduff *et al.*, 1989; Louahlia *et al.*, 1999*b*). Given that carbohydrates represent a source of energy for nutrient uptake and that the reserve-dependence of shoot growth lasts longer for N than for C (De Visser *et al.*, 1997), it is likely that interactions occur between carbohydrates and nitrogen supply for the growth of new leaves.

The specific objectives of this study were, firstly, to determine whether plant N and/or C status at the time of defoliation influences the rate of shoot production throughout the regrowth period; secondly, to evaluate the relative influence of N reserves (i.e. soluble proteins, the main N reserve of source organs in *Lolium perenne*; Ourry *et al.*, 1989) and C reserves (i.e. fructans, the main C reserve in *Lolium perenne*; Prud'homme *et al.*, 1992) and, thirdly, to analyse the fructan hydrolysis capacity of the tissues by measuring fructan exohydrolase (FEH) activity after defoliation.

Materials and methods

Plant material and culture

Seeds of *Lolium perenne* L. cv. Bravo were germinated and grown for 8 weeks in a controlled environment with a light intensity of 300 μmol photons $m^{-2} s^{-1}$, using a nutrient solution with 1 mM NH_4NO_3 (treatments A, B and C) or with 0.2 mM NH_4NO_3 (treatments A′, B′ and C′) as described previously (Gonzalez *et al.*, 1989; Prud'homme *et al.*, 1992). For 4 d before defoliation, plants were submitted to three different photoperiodic conditions. Plants were either placed in darkness (treatments A and A′), grown with a 16 h photoperiod (treatments B and B′) or maintained under continuous light with roots and shoot meristematic zones in a nutrient solution at 5 °C (treatments C and C′). Eighty-four plants were defoliated at 4 cm height and allowed to regrow under the same conditions of light (16 h photoperiod) with all plants given 1 mM NH_4NO_3 in the nutrient solution. The thermoperiod for regrowth was 23 °C (day) and 18 °C (night). The different treatments are summarized in Fig. 1. After defoliation, the residual material (stubble) was composed of leaf sheaths and bases of the elongating leaves. Plants were dissected into mature leaf sheaths, elongating leaf bases and new leaves that appeared above the cutting level, immediately following defoliation and 2, 6 and 28 d later. Twenty-one plants were harvested on each occasion. After 2, 6 and 28 d of regrowth, the production of leaf dry matter was estimated by summing the dry weight of elongating leaf bases and the dry weight of leaf tissue that had appeared above the cutting level. For the determination of nitrogen, carbon and fructan contents, plant tissues were frozen in liquid nitrogen, freeze-dried, and reduced to powder before analysis. Assays for enzyme activities were performed on fresh tissues. Weight of dry matter production was determined after freeze-drying.

Determination of total C and total N

Determination of total C and N contents were performed using a continuous flow isotope mass spectrometer (Twenty-twenty, Europa Scientific Ltd, Crewe, UK) linked to a C/N analyser (Roboprep CN, Europa Scientific Ltd, Crewe, UK).

Extraction and purification of water-soluble carbohydrates (WSC)

Freeze-dried tissues were dropped into boiling 80% ethanol and extracted under reflux for 1 h. The ethanol filtrate was evaporated to dryness under vacuum and the residue dissolved in boiling water and extracted under reflux for 1 h. The aqueous filtrate was evaporated to dryness under vacuum. The ethanol and aqueous extracts were dissolved in water, pooled and filtered with a 0.45 μm nylon membrane (Gelman Sciences, USA). Aliquots of the carbohydrate extracts were passed through a column containing cation exchange resin (Dowex 50W, H^+-form, Sigma, USA) and anion exchange resin (Amberlite IRA-416, Fluka, Germany) to remove charged compounds (Smouter and Simpson, 1991). The columns were eluted with water and samples were concentrated under vacuum and dissolved in water. Purified carbohydrates were separated by high-performance liquid chromatography (HPLC) on a Sugar-PAK column (300 × 6.5 mm, Millipore Waters, USA), eluted with 0.1 mM CaEDTA in water and, using mannitol as an internal standard, quantified using a refractive index detector (Guerrand *et al.*, 1996).

Soluble protein extraction and measurement

Plant tissue (5 g leaf sheath, 3 g elongating leaf bases) was ground at a ratio of 1 $cm^3 g^{-1}$ FW in 50 mM citrate-phosphate buffer (pH 5.7) at 4 °C containing 5 mM dithiothreitol (DTT). The homogenate was centrifuged at 20 000 *g* for 10 min and the supernatant was adjusted to a ratio of 2 $cm^3 g^{-1}$ FW. Protein was measured according to the procedure of Bradford, using a commercial Coomassie blue reagent (Biorad protein dye reagent concentrate) (Bradford, 1976). BSA grade V (Sigma) was used as a standard.

Fructan exohydrolase (FEH) assay and product analysis

An aliquot of the protein extract supernatant was desalted by passage through Sephadex G50, which also allowed the elimination of sucrose from the extract. This is of particular importance since inhibition of *in vitro* FEH activity by sucrose

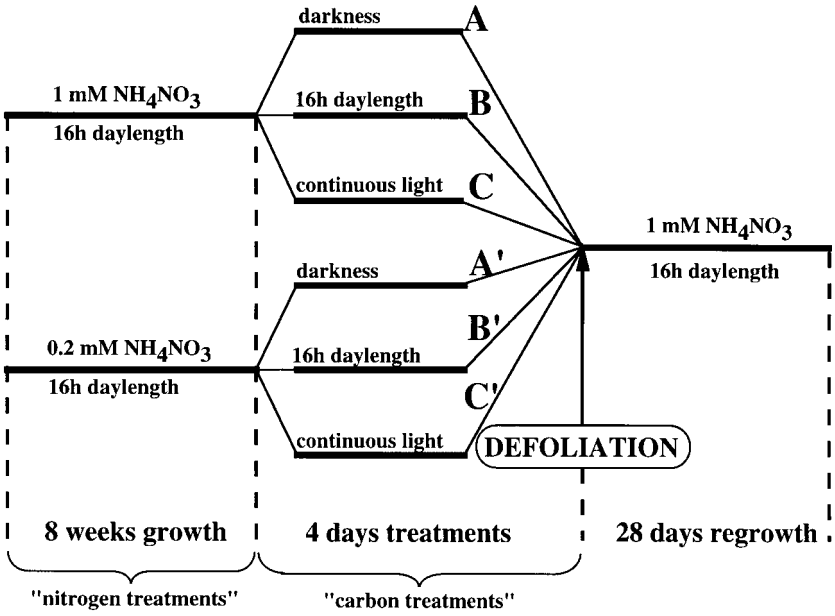

Fig. 1. Experimental system used to study the effects of different initial levels of N and C compounds on regrowth after defoliation in *Lolium perenne*. Plants were grown for 8 weeks on two nitrogen levels (A, B, C: 1 mM NH_4NO_3; A′, B′, C′: 0.2 mM NH_4NO_3). Plants were then submitted to three different photoperiodic conditions for 4 d before being defoliated (A, A′: darkness; B, B′: 16 h daylength; C, C′: continuous light and roots at 5 °C). For all the treatments, the regrowth occurred in a photoperiod of 16 h on a medium containing 1 mM NH_4NO_3.

has been reported for a range of species (Simpson and Bonnett, 1993; Marx *et al.*, 1997). The assay mixture consisted of 100 mm^3 enzyme extract and 100 mm^3 of a solution of high-molecular weight fructan (16 mg mm^{-3}) extracted from *Lolium perenne* (Morvan *et al.*, 1997). Triplicate samples were run together with duplicate enzyme blanks (no substrate). After incubation at 30 °C for 4 h, 100 mm^3 mannitol (1 g dm^{-3}) was added to the assay mixture and the reaction was stopped by boiling for 5 min. The samples were stored at −20 °C until further processing. Samples of 200 mm^3 were passed through a column filled with cation exchange resin (Dowex 50W, H^+ form), anion exchange resin (Amberlite IRA 416, formate form) and C18-modified silica (Millipore Waters, USA) to remove charged compounds, proteins, lipids, and pigments (Smouter and Simpson, 1991), as described previously (Guerrand *et al.*, 1996). Fructose in the assay mixture was purified and quantified by HPLC on the Sugar-PAK column under the conditions defined above for WSC analysis.

Statistical data analysis

The experiments consisted of two nitrogen levels and three photoperiodic treatments. Each treatment combination had three replicates including seven plants each. The treatment effects were analysed by ANOVA (StatView, 4.02). Significant differences are reported for $P<0.05$. The correlations between initial parameters and regrowth after defoliation were analysed by Spearman correlations (StatView, 4.02). A non-parametric correlation was used because the data were not normally distributed.

Results

Effects of C and N treatments on morphogenetic and biochemical parameters

Morphogenetic parameters: N treatments had an important effect on root dry matter without affecting significantly the dry matter of the remaining leaves (leaf sheaths and elongating leaf bases) at the time of defoliation (Table 1). Growth on low nitrogen nutrition (0.2 mM NH_4NO_3) resulted in plants with a root dry matter which was about 2 times higher than in plants grown on 1 mM NH_4NO_3. Consequently, the ratio of shoot to root dry matter was below 0.40 in the high N plants and over 0.53 in the low N plants at the time of defoliation. The level of N supply also modified the number of tillers with 15–18 per plant when grown on high nitrogen nutrition and 11–12 per plant when grown in low nitrogen conditions.

In contrast, the C treatments, applied for 4 d prior to defoliation, had no significant effect on root dry matter or on the number of tillers per plant. However, the mass of the elongating leaf base fraction increased with increasing photoperiod (Table 1). New shoot growth occurs in the immature base of the elongating grass leaf with most of the cells synthesized over a period of time similar to the C pretreatment period (Schnyder *et al.*, 1990). This is why leaf bases were affected by the short-term C supply treatments.

Biochemical parameters: Low N treatment prior to defoliation, decreased the total nitrogen concentration in all analysed tissues (Tables 2–4) and decreased the soluble protein content in elongating leaf bases (Table 2) and in leaf sheaths (Table 3). In contrast, the total concentration of WSC and fructans were higher in low N plants. This effect was particularly marked for fructans, which were between 2- and 10-fold higher and represented up to 100% of the increase in WSC. The C treatments also had

Table 1. *Amount of dry matter of roots, leaf sheaths and elongating leaf bases, shoot–root dry matter ratio, and number of tillers per plant of* Lolium perenne *after the treatments described in Fig. 1*

Values are means of three replicates. The significant level of differences (P) analysed by ANOVA is reported: n.s., non significant, $*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.001$, with $n = 18$.

Treatments		mg DW per tiller				Tiller number per plant
'Nitrogen'	'Carbon'	Roots	Sheaths	Elongating leaf bases	Shoot–root ratio	
1 mM	A	23.0	8.61	3.53	0.53	18.7
1 mM	B	14.7	9.22	3.57	0.88	15.4
1 mM	C	19.3	10.07	5.04	0.78	15.3
0.2 mM	A′	55.7	8.56	2.56	0.19	11.2
0.2 mM	B′	45.4	13.67	5.22	0.40	12.0
0.2 mM	C′	55.4	13.72	6.21	0.36	12.1
Source of variance						
'Nitrogen' treatment		***	n.s.	n.s.	***	**
'Carbon' treatment		n.s.	n.s.	***	n.s.	n.s.

Table 2. *Concentration (mg g^{-1} DW) of total nitrogen, soluble protein, total carbon, WSC, and fructans in elongating leaf bases of* Lolium perenne *after the treatments described in Fig. 1*

Values are means of three replicates. The significant level of differences (P) analysed by ANOVA is reported: n.s., non significant, $*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.001$, with $n = 18$.

Treatments		In elongating leaf bases (mg g^{-1} DW)				
'Nitrogen'	'Carbon'	Nitrogen	Soluble proteins	Carbon	WSC	Fructans
1 mM	A	87.2	35.1	352	32.3	15.6
1 mM	B	58.7	39.5	378	109	46.3
1 mM	C	54.4	43.5	395	146	88.6
0.2 mM	A′	41.7	20.3	382	100	28.8
0.2 mM	B′	13.7	22.6	407	231	165
0.2 mM	C′	18.2	31.0	408	242	163
Source of variance						
'Nitrogen' treatment		***	***	***	***	***
'Carbon' treatment		***	n.s.	***	***	***

Table 3. *Concentration (mg g^{-1} DW) of total nitrogen, soluble protein, total carbon, WSC, and fructans in leaf sheaths of* Lolium perenne *after the treatments described in Fig. 1*

Values are means of three replicates. The significant level of differences (P) analysed ANOVA is reported: n.s., non significant, $*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.001$, with $n = 18$.

Treatments		In leaf sheaths (mg g^{-1} DW)				
'Nitrogen'	'Carbon'	Nitrogen	Soluble proteins	Carbon	WSC	Fructans
1 mM	A	29.9	6.26	363	48.5	24.4
1 mM	B	33.0	8.60	358	69.0	40.7
1 mM	C	34.1	14.0	372	134	67.2
0.2 mM	A′	7.03	4.21	372	117	81.1
0.2 mM	B′	3.55	3.65	382	170	141
0.2 mM	C′	7.98	8.13	388	203	160
Source of variance						
'Nitrogen' treatment		***	***	***	***	***
'Carbon' treatment		*	**	***	***	***

a highly significant effect on the total concentrations of C, WSC and fructans in elongating leaf bases (Table 2), leaf sheaths (Table 3) and roots (Table 4). Compared to standard photoperiodic conditions (treatments B, B′), complete darkness (treatments A, A′) decreased the amounts of fructans by 1.6-fold in leaf sheaths, over 3-fold in elongating leaf bases, and between 1.3- and 2-fold in roots, irrespective of N nutrition. By contrast,

Table 4. *Concentration (mg g⁻ DW) of total nitrogen, total carbon, WSC, and fructans in roots of* Lolium perenne *after the treatments described in Fig. 1*

Values are means of three replicates. The significant level of differences (P) analysed by ANOVA is reported: n.s., non significant, $*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.001$, with $n = 18$.

Treatments		In roots (mg g^{-1} DW)			
'Nitrogen'	'Carbon'	Nitrogen	Carbon	WSC	Fructans
1 mM	A	32.2	408	8.49	6.21
1 mM	B	31.1	415	21.5	13.6
1 mM	C	37.3	405	59.7	32.3
0.2 mM	A′	17.7	412	78.9	66.5
0.2 mM	B′	14.3	416	118	89.6
0.2 mM	C′	15.9	416	150	74.9
Source of variance					
'Nitrogen' treatment		***	n.s.	***	***
'Carbon' treatment		*	n.s.	***	n.s.

continuous illumination (treatments C, C′) increased the fructan contents by 1.6-, 1.9- and 2.3-fold in leaf sheaths, elongating leaf bases and roots, respectively, in the high N plants only.

Effects of size of N or C reserve on soluble proteins, WSC, fructans, and FEH activity during regrowth

The two nitrogen treatments together with the three photoperiodic conditions resulted in plants with contrasting sizes of C reserve (24–160 mg g^{-1} DW fructans in leaf sheaths, 15–165 in elongating leaf bases, and 6–89 in roots) and N reserve (3–14 mg g^{-1} DW soluble proteins in leaf sheaths and 20–43 in elongating leaf bases). Plants were defoliated and regrowth was followed for 28 d in the same environmental conditions, i.e. plants received an optimal N supply and were subjected to a 16 h photoperiod.

There were marked differences between low N and high N plants in their soluble protein time-course as a response to defoliation (Fig. 2). In high N plants, soluble protein concentrations decreased in elongating leaf bases until day 2 and then increased. The changes in soluble protein concentrations were similar, irrespective of the pre-defoliation C treatment. In leaf sheaths of high N plants, soluble protein concentrations also decreased transiently indicating net hydrolysis, but only in plants that had been subjected to light before defoliation (treatments B and C). In contrast, soluble protein concentrations of low N plants were initially low and increased progressively over the 28 d recovery period in both leaf tissues.

WSC concentrations declined during the first 2 d (high N plants) or the first 6 d (low N plants) of regrowth in leaf bases (Fig. 3). The WSC concentrations increased thereafter. The same trend also occurred in leaf sheaths of the high N plants, but in low N plants, the WSC

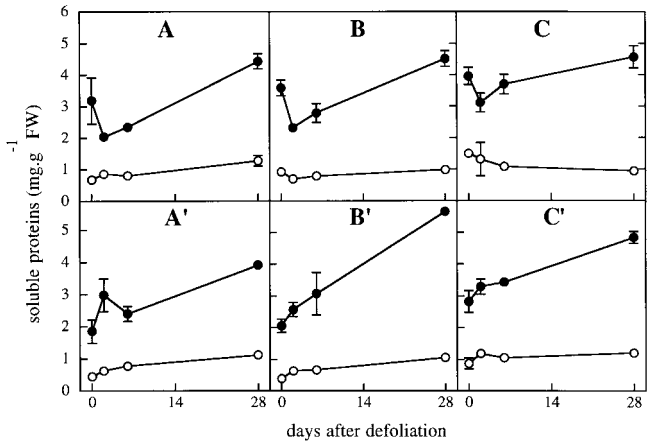

Fig. 2. Changes in the contents of soluble proteins (mg g^{-1} DW) in leaf sheaths (○) and in elongating leaf bases (●) of *Lolium perenne* during 28 d of regrowth. Plants were defoliated after being treated (A, B, C, A′, B′, C′) according to the conditions described in Fig. 1. Each value is the mean of three replicate determinations. Vertical bars indicate ±SE when larger than the symbol.

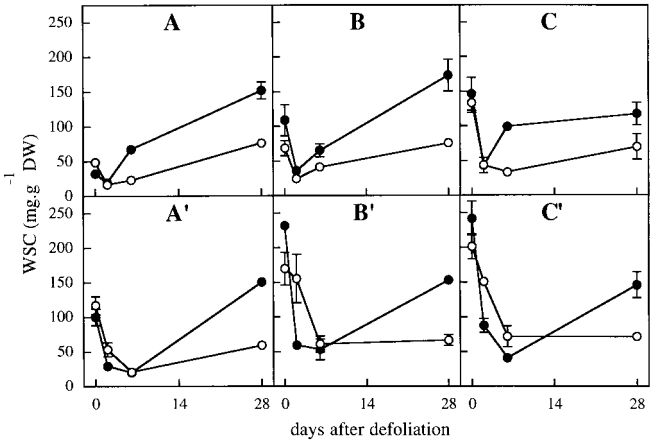

Fig. 3. Changes in the contents of WSC (mg g^{-1} DW) in leaf sheaths (○) and in elongating leaf bases (●) of *Lolium perenne* during 28 d of regrowth. Plants were defoliated after being treated (A, B, C, A′, B′, C′) according to the conditions described in Fig. 1. Each value is the mean of three replicate determinations. Vertical bars indicate ±SE when larger than the symbol.

concentration was not restored after the decline during the first 6 d.

Depletion of carbohydrates was essentially due to a decline in fructan concentrations (Fig. 4). At the time of defoliation, fructans represented 50–83% and 29–71% of the WSC in leaf sheaths and elongating leaf bases, respectively. During the first 2 d of regrowth, fructan contents decreased by 77±4% in both leaf tissues. The extent of their decline (mg g^{-1} DW) was then highly and positively correlated ($r = 0.999$; $P \leq 0.001$) with the initial fructan concentrations.

In contrast, restoration of fructan contents in leaf tissues occurred independently of the initial C status of the plants. The enhancement of fructans was approxi-

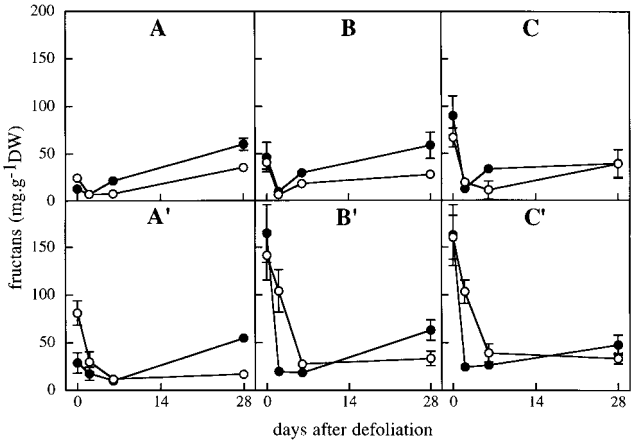

Fig. 4. Changes in the contents of fructans (mg g^{-1} DW) in leaf sheaths (○) and in elongating leaf bases (●) of *Lolium perenne* during 28 d of regrowth. Plants were defoliated after being treated (A, B, C, A′, B′, C′) according to the conditions described in Fig. 1. Each value is the mean of three replicate determinations. Vertical bars indicate ±SE when larger than the symbol.

mately 200% in leaf sheaths and 390% in elongating leaf bases. Therefore, at the end of the regrowth period, fructan concentrations reached similar values (31 mg g^{-1} DW in leaf sheaths and 54 mg g^{-1} DW in elongating leaf bases), irrespective of the treatment applied to the plants prior to defoliation.

The enzyme responsible for hydrolysis of fructan is the fructan exohydrolase (FEH), the activity of which increased after defoliation in both leaf sheaths (2.4) and elongating leaf bases (3.9) during the first days of regrowth and declined thereafter (Fig. 5), in contrast to the time-course of fructan content. However, the extent to which FEH increased was not related to the extent to which fructans were depleted (Fig. 4) but was correlated

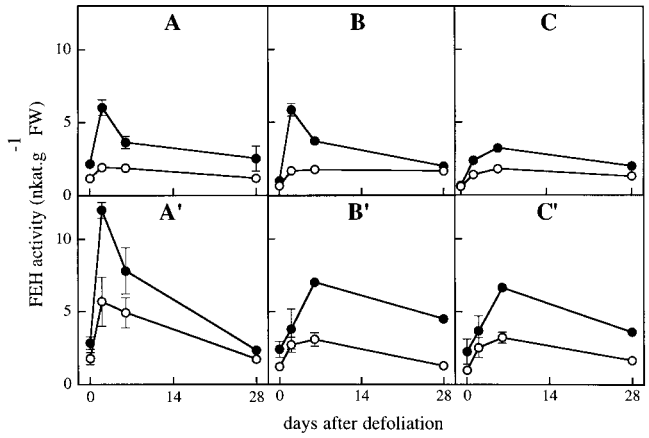

Fig. 5. Changes in FEH activity (nkat g^{-1} FW) in leaf sheaths (○) and in elongating leaf bases (●) of *Lolium perenne* during 28 d of regrowth. Plants were defoliated after being treated (A, B, C, A′, B′, C′) according to the conditions described in Fig. 1. Each value is the mean of three replicate determinations. Vertical bars indicate ±SE when larger than the symbol.

($r=0.797$, $P\leq0.01$) with the initial level of FEH activity in both leaf tissues.

Determinants of regrowth yield

Leaf dry matter production during regrowth is given in Table 5. The low N supply before defoliation did not affect the early leaf regrowth or the final shoot production (mg DW per tiller), but it decreased the leaf yield between the 2nd and the 6th days of regrowth. In contrast, the C pretreatment highly influenced leaf regrowth during the first 2 d following defoliation. Leaf production of plants C and C′ was almost 2-fold higher than shoot growth in plants A and A′ (darkness). This effect was also apparent on day 6 after defoliation. At the end of regrowth period, however, leaf dry matter yield could not be related any longer to the N and C treatments applied prior to defoliation.

In order to identify the determinants of regrowth, possible correlations were analysed between parameters describing the initial state of the plants at the time of defoliation (number of tillers per plant, dry weights, N, soluble protein, C, WSC, fructan content of source organs) and the production of leaf dry matter throughout the regrowth period (Table 6). Since N treatment applied prior to defoliation altered the shoot : root ratio (Table 1), the availability of stored compounds for regrowth was assessed by their total amount per tiller rather than by their concentration in a given organ. There were marked differences between the different phases of regrowth. During the first 2 d following defoliation, the shoot yield based on leaf dry weight was not related to initial nitrogen and soluble protein contents in roots, leaf sheaths and bases of elongating leaves and to the number of tillers per plant. WSC of roots also had no influence on the shoot yield. In contrast, a highly significant relationship was found between initial elongating leaf bases dry matter,

Table 5. *Production of leaf dry matter (mg DW per tiller) 2, 6, and 28 d after defoliation of* Lolium perenne *previously treated as in Fig. 1*

Values are means of three replicates. The significant level of differences (P) analysed by ANOVA is reported: n.s., non significant, $*P\leq0.05$; $**P\leq0.01$; $***P\leq0.001$, with $n=18$.

Treatments		Regrowth (mg DW per tiller)		
'Nitrogen'	'Carbon'	J2	J6	J28
1 mM	A	4.79	12.8	56.7
1 mM	B	7.17	17.2	59.8
1 mM	C	8.22	22.2	59.5
0.2 mM	A′	5.02	10.6	48.8
0.2 mM	B′	8.04	14.4	51.3
0.2 mM	C′	9.89	17.0	57.0
Source of variance				
'Nitrogen' treatment		n.s.	**	n.s.
'Carbon' treatment		***	***	n.s.

Table 6. *Correlations between the production of leaf dry matter during regrowth (mg DW per tiller) and (i) the number of tillers per plant, (ii) the amounts of dry matter in the tiller tissues at the time of defoliation, and (iii) the initial concentrations of nitrogen, soluble proteins, carbon, WSC, and fructan (mg per tiller)*

The correlation coefficient of Spearman ranks and the significant level of differences (P) are reported: n.s., non significant, $*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.001$, with $n = 18$.

	Production of leaf dry matter per tiller		
	J2	J6	J28
Initial number of tillers per plant	−0.239 n.s.	0.222 n.s.	0.222 n.s.
Initial DW per tiller			
in roots	−0.075 n.s.	−0.416 n.s.	−0.474 n.s.
in leaf sheaths	0.414 n.s.	0.240 n.s.	−0.001 n.s.
in elongating leaf bases	0.820***	0.558*	0.281 n.s.
Initial nitrogen per tiller			
in roots	−0.102 n.s.	−0.034 n.s.	−0.255 n.s.
in leaf sheaths	−0.026 n.s.	0.620*	0.536*
in elongating leaf bases	−0.245 n.s.	0.307 n.s.	0.371 n.s.
Initial soluble proteins per tiller			
in leaf sheaths	0.195 n.s.	0.595*	0.659**
in elongating leaf bases	0.158 n.s.	0.672**	0.449 n.s.
Initial carbon per tiller			
in roots	−0.069 n.s.	−0.418 n.s.	−0.445 n.s.
in leaft sheaths	0.474 n.s.	0.245 n.s.	−0.045 n.s.
in elongating leaf bases	0.853***	0.536*	0.273 n.s.
Initial WSC per tiller			
in roots	0.435 n.s.	0.071 n.s.	−0.296 n.s.
in leaf sheaths	0.618*	0.240 n.s.	−0.066 n.s.
in elongating leaf bases	0.812***	0.490*	0.134 n.s.
Initial fructans per tiller			
in roots	0.274 n.s.	−0.082 n.s.	−0.428 n.s.
in leaf sheaths	0.604*	0.133 n.s.	−0.103 n.s.
in elongating leaf bases	0.808***	0.513*	0.116 n.s.

as well as initial WSC and, more especially, fructan contents of leaf sheaths and elongating leaf bases. Therefore, under the conditions of these experiments where plants were grown after defoliation with no limiting factors such as light, temperature or mineral nutrition, the main factor limiting the early regrowth appeared to be the fructan content of leaf sheaths and of elongating leaf bases and the subsequent availability of C reserves.

After 6 d of regrowth, the shoot yield was not related any longer to the C status of leaf sheaths at the time of defoliation. A significant correlation was still apparent between leaf dry matter production and initial elongating leaf bases dry matter, C, WSC, and fructans. Interestingly, nitrogen remaining in leaf sheaths on the day of defoliation as well as soluble protein contents in leaf sheaths and elongating leaf bases became significantly correlated with the shoot yield.

The final shoot production was not determined by the level of C previously stored in root and leaf tissues. It was not influenced any longer by the initial dry matter of the elongating leaf bases, but a significant relationship was still found with nitrogen and soluble protein concentrations in leaf sheaths.

Discussion

Manipulating the amount of endogenous N and C in tissues remaining after defoliation demonstrated that regrowth yield is not only under the control of environmental and nutritional factors during regrowth, but also depended on the initial status of the plants (Ourry *et al.*, 1994, 1996). The present data confirm this to be true for *Lolium perenne* and also demonstrates that dependence on C and N reserves varies throughout regrowth after defoliation.

First phase of recovery from defoliation: day 0 to day 2

C and N treatments brought about before defoliation by means of short-term exposure to various photoperiods combined with long-term exposure to different levels of nitrogen nutrition, affected plant size and morphology, modifying the root dry matter, the elongating leaf bases dry matter, and the number of tillers per plant. Consequently, the shoot-root mass ratio was different from one treatment to another (Table 1). Despite these existing morphological differences at the time of defoliation, these data showed that the initial dry matter of elongating leaf bases was the only factor that influenced significantly the leaf production over the first 2 d of regrowth. More especially, evidence from the present experiment indicates that the amount of fructans stored in elongating leaf bases strongly influences shoot yield during the early phase of regrowth (Table 6). This result is in accordance with the findings of other researchers (Ward and Blaser, 1961; Davidson and Milthorpe, 1966;

Davies, 1965; Volenec, 1986; Busso *et al.*, 1990), who showed that when active meristems are present, the presence of high carbohydrate availability in stubble can increase the rate of refoliation. In the absence of active meristems, however, (i.e. in conditions when quiescent meristematic regions have to be activated), high concentrations of stored carbohydrates do not ensure a faster refoliation rate (Richards and Caldwell, 1985).

Most studies of defoliated grasses do not differentiate between the functionally different parts of the stubble. Stubble includes fully expanded leaf materials (sheaths) and basal immature parts of expanding leaves. The former serves as a source of carbon for roots and leaf meristem, while the latter generates the new foliage and is generally considered to behave as a sink for mobilized C. However, in *Lolium perenne*, the decrease in fructans in stubble after defoliation was not solely due to the decline of fructans in leaf sheaths. The fructan content of elongating leaf bases was also depleted. This has also been observed for tall fescue (Volenec, 1986). During undisturbed growth, fructan synthesis occurred principally in the elongation zone, which is confined to the basal part of the leaves surrounded by the sheaths of fully developed leaves (Schnyder *et al.*, 1988; Spollen and Nelson, 1988). After cell elongation ceased, fructans are hydrolysed and probably used for the secondary cell wall deposition (Allard and Nelson, 1991). Given that about 80% of the leaves that emerged from the stubble on the second day of regrowth was already present before defoliation in the whorl of mature sheaths, and that most of the leaf bases were newly synthesized (Morvan-Bertrand *et al.*, 1999), it follows that the decrease in fructan concentration occurring after defoliation in leaf bases could be due to a decrease in fructan synthesis, an increase in fructan degradation and/or a displacement of fructans from leaf bases where they have been synthesized to leaf tissues which are exposed above the cutting level. Steady-state labelling of all pre-defoliation C, separation of the sink and source tissues in stubble and carbohydrate analysis revealed that fructans in leaf bases were not recovered in emerged tissues (Morvan-Bertrand *et al.*, 1999). The present data show that the decline in fructan content was always associated with the increase of FEH activity (Figs 4, 5) indicating a mobilization of fructans as a response to defoliation. This, together with the positive link between rate of refoliation and initial size of fructan pool in leaf bases, strongly suggest that these fructans could act as storage carbohydrates, available for early regrowth after defoliation to sustain cell production and elongation (De Visser *et al.*, 1997; Morvan-Bertrand *et al.*, 1999).

This finding is of particular interest for an understanding of defoliation tolerance. Refoliation, i.e. restoration of active photosynthesis, is the crucial element of the plant's response to severe defoliation, reducing the time of dependence to stored C resources and allowing a rapid transition to current photosynthate. Utilization of carbohydrates which were already present in the immature zone at defoliation is considered to be one of the mechanisms that may facilitate a rapid refoliation, because investment of C from other sources (sheaths or roots) is decreased (Schnyder and De Visser, 1999). They also claim that transient dilution of structural biomass in immature zones may also contribute to leaf recovery by sustaining the exposure of photosynthetically active tissue at reduced C costs. In fact, the production of cells with thinner cell walls after defoliation might partly be a consequence of the preferential allocation of carbon from fructans to shoot meristems rather than to cell wall synthesis in differentiating cells.

Another source of C for regrowth could be provided by the sheaths that remained in stubble after defoliation and which were then converted from sink to source status (Volenec, 1986). Accordingly, evidence from the present experiment shows that the level of fructans previously stored in leaf sheaths influenced the shoot yield during the first 2 d of regrowth. Moreover, the amount of fructans degraded in sheaths over the first days of regrowth was not proportional to their initial contents (Fig. 4), suggesting that stored C in the plants was probably always in limiting concentrations to support the regrowth.

Relatively little is known about the regulation of FEH activity. Apart from a possible coarse control of FEH at the protein level (Yamamoto and Mino, 1989), FEH activity may also be directly inhibited by sucrose (Marx *et al.*, 1997). Indeed, the rapid decrease in sucrose concentration that occurred in leaf bases and in sheaths of *Lolium perenne* after defoliation (Morvan *et al.*, 1997), could increase the rate of hydrolysis of fructan by de-inhibition of FEH (Fig. 5). As far as the increase in FEH activity of low N plants is concerned, the effect of defoliation looked amplified by the effect of nitrogen enrichment of the culture solution. However, the maximum level of activity did not seem to correlate with the extent of fructan hydrolysis. This could either be attributed to the higher depletion of sucrose that might occur in low N plants or to an unknown signal associated with the modification in nitrogen nutrition.

In contrast to carbohydrates, no direct relationship was found between nitrogen or soluble protein accumulation in source organs and shoot production during the first 2 d of regrowth (Table 6). This agrees with the results of other experiment which also showed that plant N status at the time of defoliation was not a determinant of leaf growth production during the first days following defoliation (Ourry *et al.*, 1996; Louahlia *et al.*, 1999). Nevertheless, plant N status at the time of defoliation was an important factor influencing subsequent N mobilization and uptake, assimilation and translocation, i.e. the relative contribution of pre-defoliation and post-

defoliation N to the production of new foliage. Unlike lucerne (Ourry *et al.*, 1994), grasses, and more especially ryegrass, were able to sustain nitrogen uptake and translocation following defoliation for low N plants regrowing in non-limiting N conditions (Macduff *et al.*, 1989; Louahlia *et al.*, 1999). This capability presumably compensates for smaller N reserves resulting from the 'low N' pretreatment as already suggested (Louahlia *et al.*, 1999). This compensatory process might be the reason why significant correlations could not be found between initial N plant status and shoot production during early regrowth.

Soluble protein concentrations usually declined in stubble during the first few days following shoot removal (Ourry *et al.*, 1989). As already reported for other cultivars of *Lolium perenne* (Louahlia *et al.*, 1999) and due to nitrogen limitation prior to defoliation, protein content of the shoot was smaller in low N plants than in high N plants. After defoliation and transfer to non-limiting nutrient conditions, protein concentration of low N plants increased. This could be interpreted as an effect of nitrogen enrichment rather than attributed to defoliation *per se*.

Second phase of recovery from defoliation: day 2 to day 28

After the second day of regrowth, leaf dry matter yield was no longer related to the initial level of carbohydrate reserves in mature leaf sheaths and was less influenced by the initial C status of the elongating leaf bases than immediately after defoliation (Table 6). This lack or decrease of correlation might be due to the fact that photosynthesis had become a most important C source for leaf dry matter accumulation (De Visser *et al.*, 1997; Morvan-Bertrand *et al.*, 1999). Consequently, during the second phase of recovery, regrowth became more influenced by the environmental factors that control the photosynthesis rate (i.e. radiation level, air temperature) than by initial C reserve availability.

Between the second and the sixth days following defoliation, leaf production appeared to be limited by nitrogen compounds mobilized from shoot tissues. The final shoot yield after 28 d of regrowth was independent of the initial nitrogen and soluble protein levels of both roots and elongating leaf bases while a significant correlation still persisted with the amount of soluble proteins in sheaths at the beginning of regrowth (Table 6). This result could be explained by the delay that exists between the transition for C and N from reserve dependence to current assimilation for plant growth after defoliation. Indeed, this switch does not occur at the same time for C and N substrates, being longer for N (4.5 d) than for C (3 d) (De Visser *et al.*, 1997). Then, given that the leaf growth zone relied not only on pre-defoliation C, but also on pre-defoliation N at the beginning of regrowth, it follows that, in these experimental conditions, C but not N was most limiting for early regrowth.

Several factors are likely to influence the roles of mobilized C and N reserves. For example, species-specific responses in the use of N reserves for leaf regrowth have been found (Thornton *et al.*, 1993). The extent of defoliation might also influence the early production of leaf surface, by decreasing and even annihilating the reserves available for new growth (Ryle and Powell, 1975).

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