Influence of an elevated atmospheric co$_2$ concentration on source-sink relations during regrowth of Lolium perenne L. and Trifolium repens L.

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Publication Date:
1998

Permanent Link:
https://doi.org/10.3929/ethz-a-001895476

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INFLUENCE OF AN ELEVATED ATMOSPHERIC CO₂ CONCENTRATION ON SOURCE-SINK RELATIONS DURING REGROWTH OF *Lolium perenne* L. AND *Trifolium repens* L.

A dissertation submitted to the SWISS FEDERAL INSTITUTE OF TECHNOLOGY ZÜRICH for the degree of DOCTOR OF NATURAL SCIENCES

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Zürich, 1997
TABLE OF CONTENTS

ABBREVIATIONS AND SYMBOLS................................................................. 7
SUMMARY ........................................................................................................ 8
ZUSAMMENFASSUNG ..................................................................................... 10
1. GENERAL INTRODUCTION: HOW DO SOURCE–SINK RELATIONS
RESPOND TO ELEVATED $P_{CO_2}$ AND DEFOLIATION?.......................... 13
  1.1 Physiological effects of elevated $P_{CO_2}$............................................. 13
    1.1.1 Increased rates of $C_3$ photosynthesis......................................... 13
    1.1.2 Reduced stomatal aperture......................................................... 13
    1.1.3 Some evidence for reduced rates of respiration......................... 14
  1.2 What do plants do with the additionally available carbohydrate?........ 14
    1.2.1 Plants consist of source and sink regions .................................... 14
    1.2.2 Is the adjustment of source and sink characteristics mediated by
         sucrose?.................................................................................. 15
    1.2.3 What factors determine the responsiveness of growth to elevated
         $P_{CO_2}$? ........................................................................... 15
    1.2.4 Developmental and environmental constraints restrict the
         responsiveness of plant growth to elevated $P_{CO_2}$.................... 18
    1.2.5 Quantifying the responsiveness of growth to elevated $P_{CO_2}$.... 19
    1.2.6 Adjustments to maintain balanced source-sink relations.............. 20
  1.3 Increased responsiveness to elevated $P_{CO_2}$ during regrowth after defoliation of
      grassland plants? ................................................................. 21
  1.4 An integrated study on the response of two important grassland species to
      elevated $P_{CO_2}$ in the field .................................................... 22
    1.4.1 The ETH FACE project.............................................................. 22
    1.4.2 Regrowth after defoliation of L. perenne and T. repens............. 23
  1.5 Objectives and outline of the present thesis ...................................... 23
2. SOURCE–SINK RELATIONS IN Lolium perenne L. AS REFLECTED
   BY CARBOHYDRATE CONCENTRATIONS IN LEAVES AND PSEUDO-
   STEMS DURING REGROWTH IN A FREE AIR CARBON DIOXIDE
   ENRICHMENT (FACE) EXPERIMENT .............................................. 25
  2.1 Abstract ......................................................................................... 25
  2.2 Introduction .................................................................................... 25
  2.3 Materials and methods .................................................................... 26
<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.4.3</td>
<td>Morphology</td>
<td>45</td>
</tr>
<tr>
<td>3.4.4</td>
<td>Components of specific leaf weight and maximum carboxylation velocity</td>
<td>51</td>
</tr>
<tr>
<td>3.4.5</td>
<td>Water-soluble carbohydrate (WSC) concentrations</td>
<td>52</td>
</tr>
<tr>
<td>3.5</td>
<td>Discussion</td>
<td>52</td>
</tr>
<tr>
<td>3.5.1</td>
<td>Stimulation of seedling growth by elevated $p_{CO_2}$ in <em>L. perenne</em></td>
<td>54</td>
</tr>
<tr>
<td>3.5.2</td>
<td>Initial regrowth after defoliation not accelerated at elevated $p_{CO_2}$</td>
<td>54</td>
</tr>
<tr>
<td>3.5.3</td>
<td>Similar relative growth rates after recovery from defoliation</td>
<td>56</td>
</tr>
<tr>
<td>3.5.4</td>
<td>Maintaining balanced source and sink activities at elevated $p_{CO_2}$</td>
<td>59</td>
</tr>
<tr>
<td>3.5.5</td>
<td>Sink limited growth response to elevated $p_{CO_2}$ in <em>L. perenne</em></td>
<td>61</td>
</tr>
<tr>
<td>3.5.6</td>
<td>Conclusions</td>
<td>63</td>
</tr>
<tr>
<td>4.0</td>
<td>CARBOHYDRATES IN <em>TRIFOLIUM REPENS</em> L. IN RELATION TO REGROWTH AFTER DEFOLIATION AT ELEVATED $P_{CO_2}$</td>
<td>65</td>
</tr>
<tr>
<td>4.1</td>
<td>Abstract</td>
<td>65</td>
</tr>
<tr>
<td>4.2</td>
<td>Introduction</td>
<td>66</td>
</tr>
<tr>
<td>4.3</td>
<td>Materials and Methods</td>
<td>67</td>
</tr>
<tr>
<td>4.3.1</td>
<td>Growth chamber experiment</td>
<td>67</td>
</tr>
<tr>
<td>4.3.2</td>
<td>Field experiment</td>
<td>69</td>
</tr>
<tr>
<td>4.3.3</td>
<td>Chemical analysis</td>
<td>70</td>
</tr>
<tr>
<td>4.3.4</td>
<td>Photosynthesis measurements</td>
<td>72</td>
</tr>
<tr>
<td>4.3.5</td>
<td>Statistical analysis</td>
<td>72</td>
</tr>
<tr>
<td>4.4</td>
<td>Results</td>
<td>73</td>
</tr>
<tr>
<td>4.4.1</td>
<td>Growth chamber experiment</td>
<td>73</td>
</tr>
<tr>
<td>4.4.2</td>
<td>Field experiments</td>
<td>87</td>
</tr>
<tr>
<td>4.5</td>
<td>Discussion</td>
<td>91</td>
</tr>
<tr>
<td>4.5.1</td>
<td>Analysis of response mechanisms in controlled environment chambers</td>
<td>91</td>
</tr>
<tr>
<td>4.5.2</td>
<td>Validity of these interpretations under field conditions</td>
<td>102</td>
</tr>
<tr>
<td>4.5.3</td>
<td>Conclusions</td>
<td>105</td>
</tr>
<tr>
<td>5.0</td>
<td>GENERAL DISCUSSION: LIMITATIONS IN THE RESPONSE OF REGROWTH AND GROWTH TO ELEVATED $P_{CO_2}$ - A COMPARISON OF TWO SPECIES</td>
<td>107</td>
</tr>
<tr>
<td>5.1</td>
<td>Limited or no relative growth response in controlled environments</td>
<td>107</td>
</tr>
<tr>
<td>5.1.1</td>
<td>No stimulation of initial regrowth after defoliation</td>
<td>107</td>
</tr>
</tbody>
</table>
5.1.2 Growth stimulation depended on an enhanced production of new growing points................................................................. 107

5.1.3 Insufficient information available on growing point densities in the field. 108

5.1.4 What factors may have limited the growth response to elevated $p_{\text{CO}_2}$ in L. perenne and T. repens?.......................................................... 109

5.1.5 Sink development rarely carbon limited................................................................. 112

5.2 No acceleration of the recovery of carbohydrate reserves after defoliation...... 113

5.2.1 Reduced rate of carbohydrate depletion linked to residual leaf area? ...... 113

5.2.2 No shortening of the mobilisation phase............................................................. 114

5.2.3 Control of reserve carbohydrate accumulation and mobilisation - a speculative model ........................................................... 114

5.3 Sink limitation reflected in greatly increased leaf carbohydrate concentrations at elevated $p_{\text{CO}_2}$ and in rates of apparent carbohydrate export.... 117

5.4 Species differed in the contribution of various morphological and physiological adjustments to the balancing of C relations.................... 117

5.4.1 Similar morphogenetic responses to elevated $p_{\text{CO}_2}$ in both L. perenne and T. repens................................................................. 118

5.4.2 Different tendencies towards photosynthetic acclimation......................... 118

5.4.3 Were there alterations in the specific respiration rate?............................ 119

5.4.4 Species differences in the adjustment of carbon assimilation to relative growth rate ................................................................. 120

5.5 Conclusions ........................................................................................................ 121

5.6 Outlook............................................................................................................... 122

5.6.1 Can limitations in the plants’ response to elevated $p_{\text{CO}_2}$ be overcome by evolutionary adaptation? .................................................. 122

5.6.2 Promising directions for future research....................................................... 123

REFERENCES ............................................................................................................. 125

ACKNOWLEDGEMENTS.......................................................................................... 141

CURRICULUM VITAE............................................................................................... 143
ABBREVIATIONS AND SYMBOLS

A  assimilation rate \([\mu \text{mol m}^{-2} \text{s}^{-1}]\)

ABA  abscisic acid

C  carbon

\(C_i\)  internal (mesophyll) \(\text{CO}_2\) concentration \([\mu \text{mol mol}^{-1}]\)

d  day(s)

DM  dry mass \([\text{g}]\)

FACE  free air carbon dioxide enrichment

\(\Gamma_*\)  \(\text{CO}_2\) compensation point in the absence of day respiration \([\mu \text{mol mol}^{-1}]\)

\(K_c\)  Michaelis constant of RuBisCO to \(\text{CO}_2\) \([\mu \text{mol mol}^{-1}]\)

\(K_o\)  Michaelis constant of RuBisCO to \(\text{O}_2\) \([\text{mmol mol}^{-1}]\)

LAR  leaf area ratio (ratio of leaf area to total plant dry mass) \([\text{cm}^2 \text{g}^{-1}]\)

LWR  leaf weight ratio (ratio of leaf blade to total plant dry mass) \([\text{g g}^{-1}]\)

N  nitrogen

\(O_i\)  internal leaf \(\text{O}_2\) concentration \([\text{mmol mol}^{-1}]\)

\(\pi\)  average rate of gross photosynthesis as dry matter production per unit of leaf area and time \([\text{g cm}^{-2} \text{d}^{-1}]\)

PPFD  photosynthetically active photon flux density \([\mu \text{mol m}^{-2} \text{s}^{-1}]\)

\(p_{\text{CO}_2}\)  partial pressure of \(\text{CO}_2\) \([\text{Pa}]\)

\(\rho\)  average specific ‘dark’ respiration rate as loss of dry matter per unit of time \([\text{g g}^{-1} \text{d}^{-1}]\)

R:S  root to shoot ratio \([\text{g g}^{-1}]\)

\(R_d\)  day respiration \([\mu \text{mol m}^{-2} \text{s}^{-1}]\)

RGR  relative growth rate (rate of dry mass increase relative to current dry mass) \([\text{g g}^{-1} \text{d}^{-1}]\)

RuBisCO  ribulose-1,5-bisphosphate carboxylase/oxygenase

SLA  specific leaf area (ratio of leaf area to leaf dry mass) \([\text{cm}^2 \text{g}^{-1}]\)

SLW  specific leaf weight (ratio of leaf dry mass to leaf area) \([\text{g g}^{-1}]\)

\(T\)  leaf temperature \([\text{°C}]\)

TNC  total non-structural carbohydrate

\(V_{c_{\text{max}}}\)  maximum carboxylation velocity \([\mu \text{mol m}^{-2} \text{s}^{-1}]\)

WSC  water-soluble carbohydrate

yr  year
SUMMARY

Rising atmospheric partial pressures of CO$_2$ ($p_{\text{CO}_2}$) tend to enhance the availability of assimilates in plants through a stimulation of photosynthesis and an inhibitory effect on respiration. Even though an increased biomass is typically observed in plants grown at elevated $p_{\text{CO}_2}$, average relative growth rates (RGRs) are generally increased to a much smaller extent than rates of photosynthesis per unit leaf area. Both the availability of essential resources – such as nutrients – and environmental and developmental constraints may limit the extent to which growth rates depend on carbon (C) availability, thus restricting the responsiveness of plant growth to elevated $p_{\text{CO}_2}$. It was therefore hypothesised that an enhanced responsiveness to elevated $p_{\text{CO}_2}$ may be observed in systems with limiting assimilate availability, such as managed grassland during early regrowth after defoliation – particularly at ample nutrient supply. In addition, an enhanced assimilate availability at elevated $p_{\text{CO}_2}$ may reduce the depletion and favour an earlier replenishment of carbohydrate reserves after defoliation. These hypotheses were tested in the context of a co-ordinated research project on the effect of elevated $p_{\text{CO}_2}$ on two important species of temperate grasslands, *Lolium perenne* L. (perennial ryegrass) and *Trifolium repens* L. (white clover). The effect of elevated $p_{\text{CO}_2}$ on carbohydrate availability and regrowth after defoliation was studied in a combination of field experiments – using the Free Air Carbon Dioxide Enrichment (FACE) technology – and detailed studies in controlled environments.

In all experiments, elevated atmospheric $p_{\text{CO}_2}$ generally led to an enhanced availability of C building blocks as indicated by a pronounced accumulation of non-structural carbohydrates in both *L. perenne* and *T. repens*. A pronounced $p_{\text{CO}_2} \times \text{N}$ (nitrogen) interaction was observed in *L. perenne* in the field, even around the time of minimal carbohydrate concentrations. Here, water-soluble carbohydrate concentrations were typically around 0.5 mol kg$^{-1}$ (as hexose equivalents) while they were about 1 mol kg$^{-1}$ at elevated $p_{\text{CO}_2}$ in combination with low N supply. Starch concentrations in *T. repens* at elevated $p_{\text{CO}_2}$ were frequently at least twice as high as concentrations at ambient $p_{\text{CO}_2}$ (at elevated $p_{\text{CO}_2}$, leaf starch concentrations after apparent recovery from defoliation were typically in the range of 0.4–0.8 mol kg$^{-1}$). Together with a stimulation of the production of structural tissue components, the carbohydrate accumulation resulted in a roughly 30 % higher dry mass per growing point, as observed in the growth chamber in both species. In fact, this increase in dry mass per growing point was the principal growth response to elevated $p_{\text{CO}_2}$ in the growth chamber.

In contrast to expectations, during the first 4 d after defoliation, no stimulation of regrowth was observed in either *L. perenne* or *T. repens*. Nevertheless, total plant biomass remained higher in plants growing at elevated $p_{\text{CO}_2}$ throughout the experiments. Apparently, the vigour of initial regrowth depended more directly on the vigour of meristems present at the time of defoliation than either on the availability of carbohydrate reserves or on current rates of photosynthesis. Only about one week after defoliation, a transient
stimulation of growth – as hypothesised – was observed in *T. repens*. Thus, it appeared that regrowth was temporarily co-limited by C availability after a severe defoliation of *T. repens*. However, the growth stimulation (18 %) remained small in comparison to the persistent effect of elevated $p_{\text{CO}_2}$ on the dry mass accumulated per growing point. In contrast, in *L. perenne*, C availability never appeared to contribute to the control of the rate of regrowth. In contrast to expectations, N supply did not affect the responsiveness of growth and the production of new sinks to the increased carbohydrate availability at elevated $p_{\text{CO}_2}$ in either species. Thus, it is suggested that other – environmental or inherent – limitations restricted the responsiveness of sink development, and that only in *T. repens*, this restriction was briefly released after defoliation. In both species, patterns of carbohydrate concentrations in leaves and storage tissue observed in the field were essentially similar to those found in the growth chamber, indicating that similar processes were governing source-sink relations in both systems. Therefore, both in the growth chamber and in the field, the higher leaf carbohydrate concentrations and the enhanced accumulation of carbohydrate reserves towards the end of regrowth reflected an apparent sink limitation of the growth response to elevated $p_{\text{CO}_2}$. The $p_{\text{CO}_2} \times$ N interaction in *L. perenne* in the field suggested that this sink limitation was particularly pronounced at low N supply. Further, a sink limitation of carbohydrate utilisation was indicated by the pattern of apparent night-time carbohydrate export from leaves of *L. perenne* in the field. It is therefore suggested that not only differences between $p_{\text{CO}_2}$ treatments in total plant biomass in the growth chamber, but also differences in yield in the field may have been caused primarily by an accumulation of more dry matter per growing point. An enhanced accumulation of non-structural carbohydrates and an increased production of structural tissue components at the expense of additional leaf area led to a decrease in leaf area ratio (LAR) and appeared to aid in maintaining balanced source–sink relations in *T. repens* growing at elevated $p_{\text{CO}_2}$. In contrast, *L. perenne* also exhibited a pronounced acclimation of photosynthesis.

Even though carbohydrate availability was generally increased at elevated $p_{\text{CO}_2}$, recovery from defoliation as assessed by the resumption of growth of all plant tissues and/or the start of replenishment of carbohydrate reserves was not accelerated. Rates of carbohydrate depletion from storage tissue after defoliation were apparently not dependent on carbohydrate availability, but may have been related to the residual leaf area after defoliation. However, as hypothesised, the rate at which carbohydrate reserves were replenished after recovery was enhanced at elevated $p_{\text{CO}_2}$. It is therefore suggested that plants grown at elevated $p_{\text{CO}_2}$ may be more tolerant to further defoliation than plants grown at ambient $p_{\text{CO}_2}$ from about one week after defoliation. However, such an advantage may be only significant in cases of very severe defoliation and is likely to disappear when plants growing at ambient $p_{\text{CO}_2}$ have also restored their reserves to a non-limiting level.
ZUSAMMENFASSUNG

Durch eine Stimulierung der Photosynthese und eine hemmende Wirkung auf die Atmung führt der stetig steigende CO\(_2\)-Partialdruck (\(p_{\text{CO}_2}\)) in der Atmosphäre bei Pflanzen im allgemeinen zu einer gesteigerten Verfügbarkeit von Assimilaten. Obwohl in den meisten Versuchen mit erhöhtem \(p_{\text{CO}_2}\) eine größere Biomasse beobachtet wird, ist die mittlere Zunahme der relativen Wachstumsrate (RGR) meist wesentlich geringer als die Steigerung der Photosyntheserate pro Blattflächeneinheit. Sowohl die Verfügbarkeit essentieller Ressourcen (wie z. B. Nährstoffe), als auch umwelt- und entwicklungsbedingte Limitierungen beschränken wahrscheinlich das Ausmass, in dem das Pflanzenwachstum von der Verfügbarkeit von Kohlenstoff (C) abhängt und damit auch die Reaktion des Pflanzenwachstums auf einen erhöhten \(p_{\text{CO}_2}\). Es wurde deshalb die Hypothese aufgestellt, dass Systeme mit eingeschränkter Verfügbarkeit von Assimilaten, wie z. B. bewirtschaftetes Grünland, eine ausgeprägtere Reaktion auf einen erhöhten \(p_{\text{CO}_2}\) zeigen, besonders wenn ausreichend Nährstoffe zur Verfügung stehen. Ausserdem wäre zu erwarten, dass eine gesteigerte Verfügbarkeit von Assimilaten bei erhöhtem \(p_{\text{CO}_2}\) zu einer reduzierten Auslagerung von Kohlenhydratreserven nach einem Schnitt führt und eine frühere Wiedereinlagerung begünstigt. Im Rahmen eines breit abgestützten Forschungsprojektes zur Wirkung eines erhöhten \(p_{\text{CO}_2}\) auf zwei wichtige Arten des Grünlandes gemäßigter Klimazonen – *Lolium perenne* L. (Englisch Raigras) und *Trifolium repens* L. (Weissklee) – wurden diese Hypothesen experimentell überprüft. Die Wirkung eines erhöhten \(p_{\text{CO}_2}\) auf die Verfügbarkeit von Kohlenhydraten und den Wiederaustrieb nach einer Entblätterung wurde in einer Kombination von Feldversuchen – unter Verwendung der “Free Air Carbon Dioxide Enrichment” (Freiluft-CO\(_2\)-Begasungs-, oder kurz: FACE-) Technologie – und detaillierten Studien in Klimakammern untersucht.

In allen Versuchen führte ein erhöhter \(p_{\text{CO}_2}\) im allgemeinen zu einer gesteigerten Verfügbarkeit von C, wie aus einer deutlichen Anhäufung von nicht-strukturbildenden Kohlenhydraten sowohl bei *L. perenne* als auch bei *T. repens* hervorgeht. Eine ausgeprägte \(p_{\text{CO}_2} \times \text{N} \) (Stickstoff) Interaktion wurde bei *L. perenne* im Feld beobachtet, sogar zu dem Zeitpunkt, als die Konzentrationen wasserlöslicher Kohlenhydrate ein Minimum erreichten und im allgemeinen im Bereich um 0.5 mol kg\(^{-1}\) (als Hexose-Äquivalente) lagen, während sie bei der Kombination von erhöhtem \(p_{\text{CO}_2}\) und geringer N-Versorgung im Bereich um 1 mol kg\(^{-1}\) lagen. Stärkekonzentrationen in *T. repens* unter erhöhtem \(p_{\text{CO}_2}\) waren häufig mindestens doppelt so hoch wie bei heutigen \(p_{\text{CO}_2}\) (Stärkekonzentrationen in Blättern nach der Erholung von einer Entblätterung lagen unter erhöhtem \(p_{\text{CO}_2}\) meistens im Bereich von 0.4–0.8 mol kg\(^{-1}\)). Zusammen mit einer Stimulierung der Bildung struktureller Gewebebestandteile resultierte diese Anhäufung von Kohlenhydraten in einer um ca. 30 % grösseren Trockensubstanz pro Wachstumspunkt, wie in der Klimakammer bei beiden Arten beobachtet wurde. Diese Zunahme der Trockensubstanz pro Wachstumspunkt war sogar die hauptsächliche Wachstumsreaktion auf einen erhöhten \(p_{\text{CO}_2}\) in der Klimakammer.

Trotz einer im allgemeinen gesteigerten Verfügbarkeit von Kohlenhydraten unter erhöhtem $p_{\text{CO}_2}$ erholten sich die Pflanzen – nach der Wiederaufnahme des Wachstums aller Pflanzenteile und/oder dem Beginn der Kohlenhydrateinlagerung zu urteilen – nicht schneller von der Entblätterung. Die Geschwindigkeit der Auslagerung von Kohlen-
hydraten aus dem Speichergewebe hing offenbar nicht von deren Verfügbarkeit für die Pflanze ab, sondern stand möglicherweise im Zusammenhang mit der Restblattfläche nach dem Schnitt. Hingegen wurden die Kohlenhydratreserven nach der Erholung, wie postuliert, unter erhöhtem $p_{\text{CO}_2}$ schneller wiederaufgefüllt. Es wird daher vermutet, dass Pflanzen unter erhöhtem $p_{\text{CO}_2}$ ab ca. einer Woche nach dem Schnitt eine weitere Entblätterung besser ertragen als Pflanzen unter heutigem $p_{\text{CO}_2}$. Allerdings ist ein solcher Vorteil wahrscheinlich nur nach einem sehr starken Schnitt von Bedeutung und verschwindet vermutlich nach dem Zeitpunkt, bei dem Pflanzen unter heutigem $p_{\text{CO}_2}$ eine für einen uneingeschränkten Wiederaustrieb ausreichende Menge an Kohlenhydratreserven eingelagert haben.
1. GENERAL INTRODUCTION: HOW DO SOURCE–SINK RELATIONS RESPOND TO ELEVATED $p_{\text{CO}_2}$ AND DEFOLIATION?

Since the beginning of the industrial age, the burning of fossil fuels, massive deforestation and cement production have led to an ongoing rise in the atmospheric partial pressure of carbon dioxide ($p_{\text{CO}_2}$) at an unprecedented rate of over 0.1 Pa yr$^{-1}$ (Lashof and Tirpak 1990). A partial pressure of twice the present 36 Pa may be reached within the 21st century. It has been suggested that such high levels of $p_{\text{CO}_2}$ may have a profound impact on the global climate. Rising temperatures and changes in precipitation patterns may shift vegetation zones. However, the extent of changes in the global climate is still highly debated. In contrast, there is no doubt that the rising $p_{\text{CO}_2}$ by itself has profound effects on the physiology of plants.

1.1 Physiological effects of elevated $p_{\text{CO}_2}$

1.1.1 Increased rates of $C_3$ photosynthesis

Probably the most important direct effect of elevated $p_{\text{CO}_2}$ is a stimulation of photosynthesis in plants with the $C_3$ photosynthetic pathway (Mott 1990). RuBisCO (ribulose-1,5-bisphosphate carboxylase/oxygenase), the primary carboxylating enzyme in this group of plants, has a $K_m$ for $\text{CO}_2$ of about 8–25 mmol m$^{-3}$ compared to estimated concentrations of dissolved $\text{CO}_2$ in the chloroplast stroma in the range of 4–8.5 mmol m$^{-3}$ at current levels of atmospheric $p_{\text{CO}_2}$ (Bowes 1991). Thus, RuBisCO is not $\text{CO}_2$ saturated at current $p_{\text{CO}_2}$. Further, RuBisCO accepts $O_2$ as a competing substrate, leading to a loss of previously assimilated $\text{CO}_2$ in a process termed photorespiration. Elevated atmospheric $p_{\text{CO}_2}$ therefore stimulates carboxylation rates and competitively inhibits photorespiration, thus increasing the quantum yield of photosynthesis (the quantity of $\text{CO}_2$ assimilated relative to the amount of light energy absorbed). This leads to a higher efficiency of photosynthesis (Long et al. 1996). Stitt (1991) estimated that a doubling of $p_{\text{CO}_2}$ from 35 to 70 Pa can be expected to cause a stimulation of photosynthesis in the range of 23–78 %, depending on which biochemical process is limiting the rate of photosynthesis. Only if end-product (carbohydrate) synthesis is limiting, then no stimulation can be expected.

1.1.2 Reduced stomatal aperture

Another direct response of plants to elevated $p_{\text{CO}_2}$ is a reduction in stomatal conductance (Long et al. 1996). In fact, this may be the principal reason why an increase in water use efficiency (the amount of water transpired per unit of assimilated carbon) and growth is often also observed in plant species with the $C_3$ photosynthetic pathway, even though their rates of photosynthesis are not limited by $p_{\text{CO}_2}$. In many plant species, stomata have been found to close with increasing atmospheric $\text{CO}_2$, keeping the ratio of internal to external $p_{\text{CO}_2}$ approximately constant (Mott 1990). This results in a reduced transpiration
rate at the expense of some of the stimulatory effect of elevated $p_{\text{CO}_2}$ on photosynthesis (Long et al. 1996).

1.1.3 Some evidence for reduced rates of respiration

Finally, there is an increasing body of evidence for a short-term inhibition of dark respiration in plants, even at moderately elevated levels of $p_{\text{CO}_2}$ (Wullschleger et al. 1994; González-Meler et al. 1996a). This rapid and reversible effect appears to be due to an inhibition of cytochrome c oxidase and succinate dehydrogenase, but not of the alternative oxidase (González-Meler et al. 1996b).

1.2 What do plants do with the additionally available carbohydrate?

These short-term effects tend to increase the carbon (C) gain from photosynthesis and to reduce C and water losses due to respiration and transpiration. Do these short-term effects also represent long-term responses of plants growing at elevated $p_{\text{CO}_2}$? Intuitively, one might expect that an increased supply of C should result in enhanced growth as a more or less direct consequence. However, since key processes of plant metabolism are affected, a variety of physiological and developmental responses may be elicited. Complex interactions between the three primary effects and secondary responses, such as physiological adjustments and alterations of tissue composition and morphology, will determine the overall effect of elevated $p_{\text{CO}_2}$ on plant growth.

Which are the main factors determining the overall response of plants to elevated $p_{\text{CO}_2}$? For the following considerations, I shall focus on effects of elevated $p_{\text{CO}_2}$ on C metabolism.

1.2.1 Plants consist of source and sink regions

Leaf tissue ceases extension growth before or shortly after it starts to export carbohydrate to other parts of the plant. It is therefore clear that increased rates of net C assimilation can only be sustained in mature leaf tissue if the additional assimilates can be exported to some other part of the plant. Otherwise, carbohydrate would rapidly accumulate beyond the storage capacity of the leaf.

Therefore, the processes which regulate the transport of C between sources – regions of net C export – and sinks – regions of net C import – and the co-ordination of assimilation with C utilisation are of primary importance in determining the long-term response of plants to elevated $p_{\text{CO}_2}$. While ‘sources’ and ‘sinks’ can be defined in a similar manner for any resource which is transported within a plant (nutrients are often also of interest), in the following, the exchange of assimilated C (typically as sucrose) will be implied if nothing else is specified.
1.2.2 *Is the adjustment of source and sink characteristics mediated by sucrose?*

Based on evidence that the growth of barley roots is stimulated by an enhanced sucrose supply, a model for the integration and co-ordination of C assimilation in source leaves and C utilisation in sink tissues has been proposed by Farrar and Williams (1991) and elaborated by Farrar (1992). According to the pressure flow hypothesis of phloem transport originally proposed by Münch in 1928, the flow of sucrose is driven by an osmotic pressure gradient from sources to sinks. Thus, the steady-state phloem sucrose concentration in sources and sinks integrates information about the availability and the demand for assimilates. It is suggested that sucrose concentrations in the phloem are related to sucrose concentrations in (cytosolic and apoplastic) transport pools in sources and sinks. The Farrar model proposes that rates of sink growth and metabolism are controlled in a feed-forward mechanism by the sucrose concentration in a sink. Similarly, the capacity for photosynthesis and sucrose synthesis in the source is regulated in a feed-back mechanism by the sucrose concentration in the source.

There is now considerable evidence that high sugar concentrations reduce the expression of many genes involved in photosynthesis (Koch 1996) and mediate an acclimation of photosynthesis which is frequently observed in plants exposed to elevated $P_{\text{CO}_2}$ (Webber *et al.* 1994). Similarly, sugars have been shown to modulate the expression of many genes involved in C metabolism in sinks (Koch 1996). However, there is much less evidence for sugar effects on sink development (Pollock and Farrar 1996), even though enhanced carbohydrate availability (at elevated $P_{\text{CO}_2}$ or as a consequence of genetic alterations) has been shown to stimulate the growth of new sinks in some cases (Stitt 1991; Stitt and Schulze 1994).

While it is easy to see how a regulation of sink metabolism by carbohydrate availability may confer a selective advantage, it is also very likely that more complex regulatory mechanisms may be required for a more specific developmental response to a variety of environmental factors, such as the availability of essential resources (light, water, nutrients) or the presence of competing neighbour plants.

1.2.3 *What factors determine the responsiveness of growth to elevated $P_{\text{CO}_2}$?*

While several surveys have found an average increase in plant dry mass or yield at elevated $P_{\text{CO}_2}$ of about 30–40% (Kimball 1983; Cure 1985; Poorter 1993), relative growth rates (RGRs) were only increased by 7% averaged over 60 C$_3$ species (Poorter *et al.* 1996). This stimulation of growth is thus considerably less than what might be expected from the effect of elevated $P_{\text{CO}_2}$ on photosynthesis. Further, a stimulation of RGR was typically most pronounced in seedlings or at the beginning of exposure to elevated $P_{\text{CO}_2}$ and declined with increasing plant age and prolonged exposure (Stitt 1991; Poorter 1993).
These findings suggest that growth rates only depend to a limited extent on carbohydrate availability. What factors may restrict the responsiveness of growth to elevated $p_{\text{CO}_2}$? It is often argued that the responsiveness of growth to elevated $p_{\text{CO}_2}$ may be reduced if the availability of essential resources limits growth.

1.2.3.1 Nutrient uptake

Under conditions of steady-state exponential growth, a stimulation of relative growth rate (RGR, the rate dry mass increase relative to current dry mass) requires that the relative rate of nutrient uptake (the rate of nutrient uptake relative to the current nutrient content in the plant) be increased, irrespective of any changes in steady-state nutrient concentrations. Unless nutrient availability to roots is high and the specific rate of nutrient uptake can be readily increased, more biomass and a greater proportion of nutrients have to be invested into a larger root system. This re-allocation potentially reduces the rate of C assimilation of the shoot – and thus RGR – relative to plants which can increase the specific rate of nutrient uptake of their roots. Thus, a more pronounced relative growth response to elevated $p_{\text{CO}_2}$ may be expected in plants with a non-limiting nutrient supply than in plants with a limiting nutrient supply.

Indeed, there are a number of reports of a higher responsiveness of plant growth to elevated $p_{\text{CO}_2}$ at ample compared to limiting nutrient supply. Thus, it is generally accepted that nutrient availability may affect the responsiveness of plant growth to elevated $p_{\text{CO}_2}$ (e.g. Bazzaz 1990; Farrar and Williams 1991; Stitt 1991). However, it is important to compare relative rather than absolute growth responses to elevated $p_{\text{CO}_2}$. In an evaluation of published experiments on *Gossypium hirsutum*, *Triticum aestivum* and *Eucalyptus grandis*, Conroy (1992) always found an increase in the absolute growth response to elevated $p_{\text{CO}_2}$ with increasing N supply, however, the relative growth response was only increased in *G. hirsutum*. In a comparison of a large number of studies, Poorter (1996) found a substantial correlation between the relative growth response to elevated $p_{\text{CO}_2}$ and the extent to which growth was reduced at low relative to high N supply. On average, there was almost no growth response to elevated $p_{\text{CO}_2}$ in studies with highly limiting N treatments. However, a very large amount of scatter indicates that this represents only a general tendency and the extent to which nutrient availability affects the relative growth response to elevated $p_{\text{CO}_2}$ may depend on the particular species or other conditions. Similar tendencies have also been observed for other nutrients, e.g. phosphorus.

In contrast to the effects of low nutrient availability, a limiting water supply may actually enhance the responsiveness of plant growth to elevated $p_{\text{CO}_2}$ at least in relative terms. Under conditions of limiting water availability, the increased water use efficiency at elevated $p_{\text{CO}_2}$ would allow for an enhanced productivity (Pearcy and Björkman 1983). Further, the higher water use efficiency at elevated $p_{\text{CO}_2}$ may allow plants to maintain both active photosynthesis and vitality further into a drought period when exposed to elevated $p_{\text{CO}_2}$ than at ambient $p_{\text{CO}_2}$ (e.g. Nijs et al. 1989a).
1.2.3.2 Developmental restrictions

Apart from the availability of essential resources, there are also developmental constraints which may restrict the responsiveness of growth to elevated $p_{CO_2}$. It appears suitable to consider individual growth processes separately. Each partial growth process may respond to a different extent to an enhanced carbohydrate availability and may have a distinct potential to promote whole plant RGR.

A. Growth response of individual modules

In herbaceous plants, where secondary growth does not take place, all growth depends on the production of new morphological units which subsequently enlarge until a final size is reached. An increase in the final size (either through greater cell numbers or greater cell expansion) or mass (through the accumulation of more dry matter per unit volume) of plant organs may lead to an increased plant size and/or mass. Plants grown under elevated $p_{CO_2}$ have often been found to produce larger leaves and to accumulate more dry mass in most of their organs (Baker and Enoch 1983). However, an enhancement of final organ size at elevated $p_{CO_2}$ only affects RGR as long as the average size of plant organs keeps increasing. Thus, an increase in mean organ dry mass is likely to contribute only to a transient stimulation of RGR during an acclimation of plants which have been switched from ambient to elevated $p_{CO_2}$, but not to long-term effects of elevated $p_{CO_2}$ on RGR.

Irrespective of effects on final organ size, it is also conceivable that an enhanced availability of C might increase the rate of development of plant organs after their initiation. Again, such a response would only transiently increase RGR until a new, stable size distribution of developing organs is established. However, in special cases, e.g. during the regrowth of defoliated plants, an acceleration of organ development may allow for a faster restoration of leaf area and thus allow for an earlier recovery. In several experiments, elevated $p_{CO_2}$ has been found to increase the rate of leaf expansion (Baker and Enoch 1983). Similarly, an increased rate of leaf elongation in a grass (Oryza sativa) has been observed at elevated $p_{CO_2}$ (Seneweera et al. 1994).

B. A persistent stimulation of RGR requires an enhanced production of new growing points

Clearly, a sustained stimulation of RGR requires that the rate at which new plant parts are produced is increased. The rate of initiation of new leaf primordia at an apical meristem largely determines the rate at which new leaves and their nodes appear, although the two processes need not be strictly linked (e.g. Skinner and Nelson 1995). Further, plants typically produce one bud in the axil of each leaf. Thus, the rate of leaf production also determines the rate at which buds with new apical meristems can be produced. However, the actual rate at which new shoots are produced also depends on the percentage of buds which become active and grow out into a new shoot. The activation and continued outgrowth of buds appears to be under strong developmental control in many
plant species. Often, the presence of a nearby active apex has been found to inhibit lat-
eral bud outgrowth, a phenomenon termed apical dominance and often ascribed to the
action of plant hormones (auxins). However, other factors can also be of great impor-
tance, e.g. the presence of nodal roots in *Trifolium repens* (Thomas 1987; Lötscher and
Nösberger 1996).

In plants grown at elevated $p_{\text{CO}_2}$, both a reduction of the plastochron (the interval be-
tween the initiation of two successive leaves at an apex) or the phyllochron (the interval
between the appearance of two successive leaves) (e.g. Baker *et al.*. 1989; Cure *et al.*.
1989; Baker *et al.*. 1990) and a stimulation of branching (Stitt 1991 and references
therein) have been reported, indicating that both of these processes may respond to an
enhanced C availability under suitable conditions.

1.2.3.3 Growth response of plants within a canopy in the field

For plants growing in a canopy and exposed to competition, further restrictions on
growth apply and other processes may contribute to effects of elevated $p_{\text{CO}_2}$. Most im-
portantly, the spatial expansion of plants is physically restricted by the presence of
neighbour plants. Many plant species ‘anticipate’ this restriction and respond to changes
in light quality (a reduced red/far-red ratio due to the absorption of red light by chloro-
phyll and the reflection of far-red light by neighbouring plants) with an altered growth
pattern. This usually involves increased shoot elongation at the expense of the activation
of lateral buds. While such a strategy may be useful for evading the risk of being out-
grown and overtopped by competitors, this response potentially restricts the ability of a
species to increase growth in response to an enhanced C availability. However, there is
evidence that the effect of various light signals also depends on carbohydrate availabil-
ity (Koch 1996).

1.2.4 Developmental and environmental constraints restrict the
responsiveness of plant growth to elevated $p_{\text{CO}_2}$

Thus, the growth processes which offer a potential for a sustained relative growth re-
response to elevated $p_{\text{CO}_2}$ are subject to a variety of environmental and developmental con-
straints. Different kinds of sinks can be expected to have different uptake characteristics
for sucrose and thus the partitioning model discussed above may explain a great propor-
tion of the observable patterns of partitioning. However, additional inputs are most
likely required to control differential behaviour of similar sinks (e.g. bud activation) by
modifying their uptake characteristics and/or the conductance of the transport path
linking them to a source. Such constraints on the responsiveness of sink growth and de-
velopment to C availability, in combination with limiting resource availability, may ex-
plain why observed relative growth responses are often much lower in the long term
than what should be expected from the stimulatory effect of elevated $p_{\text{CO}_2}$ on photosyn-
thesis.
It may be dangerous to focus research on agricultural crops. These varieties have been selected for high partitioning to sinks (seeds, storage organs) to increase the harvest index. Therefore, constraints on sink development may be less pronounced and growth may be more often source limited than in natural populations. This may explain why the growth stimulation in response to elevated $p_{CO_2}$ was generally more pronounced in crops than in wild species (Poorter et al. 1996). Apparently, a stimulation of the growth of wild species by elevated $p_{CO_2}$ is most pronounced during seedling development (Poorter 1993) and may completely disappear thereafter. Further, the increase in the amount of dry mass accumulated in most tissues may explain to a large extent the transient stimulation of RGR often observed in plants shifted from ambient to elevated $p_{CO_2}$. It should be kept in mind that an acceleration of development may completely obscure any stimulation of RGR at elevated $p_{CO_2}$ if RGR decreases during plant development, as it appears to be the case in various plant species (Poorter 1993). Even though RGR at elevated $p_{CO_2}$ may still be enhanced relative to plants of similar developmental stage at ambient $p_{CO_2}$, RGR may actually be equal to or lower than that in the physiologically younger plants at ambient $p_{CO_2}$.

1.2.5 Quantifying the responsiveness of growth to elevated $p_{CO_2}$

In most systems, rates of individual processes are not directly proportional to single factors, but a multitude of environmental signals may contribute to the control of a process. Response coefficients have therefore been proposed as an adequate means to describe the extent to which fluxes or other measurable rates depend on a given change in environmental conditions (Jones and Lynn 1994). Thereby, a response coefficient of 1 corresponds to a response proportional to the stimulus while a response coefficient of 0 corresponds to no response. This concept has been successfully applied to compare the dependence of growth rates on leaf area in two plant species at different levels of $p_{CO_2}$ (Farrar 1996). It was concluded that dependence of growth on leaf area was much lower at elevated $p_{CO_2}$ than at ambient $p_{CO_2}$. Thus, the old concepts of a ‘source limitation’ or a ‘sink limitation’ of growth must be viewed as quantitative concepts.

No attempt has been made to calculate response coefficients in the present study, as $p_{CO_2}$ is only indirectly linked to growth so that a response coefficient for growth with respect to $p_{CO_2}$ would be difficult to interpret. On the other hand, a response coefficient with respect to ‘C availability’ would be of great interest. Sucrose concentrations at the site of phloem loading might be taken as a suitable measure for carbohydrate availability; however, there is no way to obtain a quantitative estimate for this parameter in routine measurements. Instead, whole tissue water-soluble carbohydrate (WSC) concentrations have been used as an indirect indicator for carbohydrate availability. Thus, only the absence (corresponding to a response coefficient of 0 and designated as ‘sink limitation of growth’) or presence of a relative growth response (reflecting a response coefficient larger than 0) to an increased carbohydrate availability has been assessed.
1.2.6 Adjustments to maintain balanced source-sink relations

In a narrow sense, Stitt and Schulze (1994) define growth as the “formation of those components of biomass that themselves directly promote further acquisition and transport of resources, with the exception of growing structures for storage”. Temporarily, C which cannot be utilised for growth in this narrow sense may be stored as carbohydrate. Stitt and Schulze (1994) distinguish between ‘accumulation’ and ‘reserve formation’. Reserve formation is defined as the production of a storage compound at the expense of growth for which the same resources could have been used at the same time. Any other production of storage compounds is termed accumulation. While reserve formation may also be stimulated by elevated $p_{\text{CO}_2}$, the production of carbohydrates which is often observed at elevated $p_{\text{CO}_2}$ is generally considered as accumulation. Some local accumulation of carbohydrate is observed in most experiments, even when only small leaf sections are exposed to elevated $p_{\text{CO}_2}$ (Körner and Würth 1996), and may simply reflect a higher ‘back-pressure’ of increased fluxes though the transport path. In contrast, high concentrations of carbohydrate throughout the plant indicate that no other use for additional assimilates at elevated $p_{\text{CO}_2}$ is available. However, there exist upper limits to the amount of carbohydrate that can be accumulated. Carbohydrate concentrations exceeding 50% of dry mass are rarely observed. Therefore, carbohydrate accumulation represents only a transient solution, particularly with high rates of photosynthesis at high light intensities.

Differences between the response of growth and short-term physiological effects of elevated $p_{\text{CO}_2}$ on photosynthesis therefore clearly necessitate some adaptations so that C fluxes within the plant remain balanced. For growth defined in a broad sense as total dry mass accumulation, the usefulness of the following relationship has been pointed out by Causton and Venus (1981):

$$\text{RGR} = \pi \text{LAR} - \rho$$

with RGR and $\rho$ in g g$^{-1}$ d$^{-1}$ (the specific ‘dark’ respiration rate as loss of dry matter per unit of dry matter and time), $\pi$ in g cm$^{-2}$ d$^{-1}$ (the rate of gross photosynthesis as dry matter production per unit of leaf area and time) and LAR (leaf area ratio, the ratio of leaf area to total plant dry mass) in cm$^2$ g$^{-1}$.

1.2.6.1 Increase in dry mass vs. increase in leaf area

If a plant were to increase linearly in size in response to elevated $p_{\text{CO}_2}$, LAR would decrease in proportion to the cubic root of the mass increase. However, a much more pronounced decrease in LAR is a very typical response to elevated $p_{\text{CO}_2}$ in C$_3$ plant species (Poorter et al. 1996) indicating that there must be more specific alterations. In fact, much of the decrease in LAR may be simply the consequence of carbohydrate accumulation in the absence of a similar increase in leaf area, leading to a higher proportion of non-productive biomass in the plant. Increased partitioning of biomass to roots (Stulen
and Den Hertog 1993) may further decrease LAR. In principle, a sufficient reduction of LAR may fully compensate for an increased rate of photosynthesis at elevated $p_{\text{CO}_2}$.

1.2.6.2 Acclimation of photosynthesis

If the reduction of LAR does not suffice to restore balanced source-sink relations, a plant may reduce its photosynthetic capacity. Photosynthetic acclimation is thought to be due mostly to a reduced expression of genes which encode enzymes involved in photosynthesis, e.g. RuBisCO (Webber et al. 1994). For a long time, it has been speculated that photosynthetic acclimation may be related to carbohydrate accumulation (Stitt 1991 and references therein). Indeed, recent findings suggest that the expression of photosynthesis-related genes is related directly to carbohydrate metabolism (Sheen 1994). However, there is evidence that photosynthetic acclimation under elevated $p_{\text{CO}_2}$ occurs mainly in response to a pronounced nutrient limitation or restricted root growth in small pots (Pettersson and McDonald 1994; Sage 1994; Long et al. 1996).

1.2.6.3 Respiration

Since the major component of respiration, oxidative phosphorylation, serves primarily to provide cells with metabolic energy, long-term effects of elevated $p_{\text{CO}_2}$ on respiration rate can be expected to reflect the demand for metabolic energy as affected by metabolic and/or structural changes in response to elevated $p_{\text{CO}_2}$. In addition, an increased flux of electrons through the alternative oxidase pathway may provide a means to dissipate excess carbohydrate at elevated $p_{\text{CO}_2}$ (González-Meler et al. 1996a).

Thus, changes in both dry matter partitioning and in respiration rates at elevated $p_{\text{CO}_2}$ can lead to a lower responsiveness of RGR than of photosynthesis; alternatively, the rate of photosynthesis itself may be adjusted after a prolonged exposure of plants to elevated $p_{\text{CO}_2}$. If large amounts of carbohydrate are accumulated, it may be of interest to calculate relevant growth parameters both on a total and on a structural dry matter basis (as it has been done e.g. in Wong 1990). This allows to distinguish between effects of carbohydrate accumulation and changes in the partitioning and the relative growth rate of plant structural dry matter. In addition, it provides further insight into effects of elevated $p_{\text{CO}_2}$ on the rate and pattern of plant development. However, such an approach can get very laborious if many plant fractions are to be distinguished.

1.3 Increased responsiveness to elevated $p_{\text{CO}_2}$ during regrowth after defoliation of grassland plants?

If the responsiveness of growth to elevated $p_{\text{CO}_2}$ is often restricted by the fact that C availability is not limiting growth rates, then a more pronounced response to elevated $p_{\text{CO}_2}$ might be expected in systems where the availability of C has been reduced. Managed grassland after a cut represents such a system. Defoliation by cutting involves a drastic loss of assimilatory surface and consequently a severe reduction of photosynthetic C gain. Carbohydrate reserves are depleted until sufficient leaf area has been re-
stored to supply C for maintenance and growth from current photosynthesis. Typically, C partitioning after defoliation is found to favour leaf growth at the expense of root growth and activity. Thus, leaf growth may not be C limited after moderate defoliation or if sufficient reserves are available. However, if insufficient C is available from both photosynthesis of residual leaf area and mobilisation of carbohydrate reserves, initial regrowth after a severe defoliation may become C limited (Davies 1988; Richards 1993). Consequently, an accumulation of more carbohydrate reserves before defoliation and a stimulation of photosynthesis by residual leaf area at elevated $p_{\text{CO}_2}$ may enhance regrowth (Nijs et al. 1988b; Nijs et al. 1988a). A higher carbohydrate availability and particularly a potential stimulation of the rate of leaf expansion and/or leaf appearance may lead to an earlier recovery. Further, elevated $p_{\text{CO}_2}$ may lower the impact of defoliation on roots and other low priority sinks. It may even reduce the rate at which stored carbohydrate is depleted after defoliation and may favour a faster rate of replenishment of carbohydrate reserves after recovery. Thus, managed grassland provides an interesting system to study the interactions between elevated $p_{\text{CO}_2}$ and source-sink relations in plants.

1.4 An integrated study on the response of two important grassland species to elevated $p_{\text{CO}_2}$ in the field

1.4.1 The ETH FACE project

In a set of co-ordinated research projects at the Institute of Plant Sciences of the Swiss Federal Institute of Technology (ETH), the effect of elevated $p_{\text{CO}_2}$ on the dry matter production and distribution of managed grassland on a fertile soil was to be characterised. Research focused on *Lolium perenne* L. and *Trifolium repens* L., the most important and best characterised grass and legume species, respectively, of temperate grassland. The two species provide an interesting basis for comparisons as they differ in their primary storage carbohydrate, in their growth habit and in their dependence on soil N. *L. perenne*, a fructan accumulating species, grows in dense tufts and is highly responsive to mineral N supply. In contrast, *T. repens* accumulates mainly starch. It can explore large surface areas with its stolon system and can grow independently of mineral N availability due to a symbiotic association with the N$_2$ fixing soil bacterium *Rhizobium leguminosarum* bv. *trifolii*.

Using these two species, different projects were set up to identify essential processes determining the overall response to $p_{\text{CO}_2}$ at the level of the canopy (competition) and at the level of the individual plant (N and C relations). A field experiment using the Free Air Carbon Dioxide Enrichment (FACE) technology developed by the Brookhaven National Laboratory (Lewin et al. 1994) allowed to study the response of established canopies under conditions comparable to those observed in agricultural practice and in the absence of artefacts related to microclimatic parameters such as temperature and irradiation.
1.4.2 Regrowth after defoliation of L. perenne and T. repens

Reflecting the agricultural importance of these two species, there already exists a considerable body of literature on their growth response to elevated $p_{\text{CO}_2}$. However, in spite of a number of studies on the effects of elevated $p_{\text{CO}_2}$ on yield as affected by season and the presence of competitors, there is only little information on effects of elevated $p_{\text{CO}_2}$ on regrowth and recovery from defoliation.

Several studies on the regrowth of *L. perenne* at elevated $p_{\text{CO}_2}$ have focused mainly on the yield response of canopies of varying complexity without closely considering carbohydrate dynamics and source-sink relations (e.g. Newton *et al.* 1994; Clark *et al.* 1995; Sæbø and Mortensen 1995; Schenk *et al.* 1996; Clark *et al.* 1997; Schenk *et al.* 1997; review by Newton 1991). Based on differences in aboveground biomass between $p_{\text{CO}_2}$ treatments 3 d after defoliation, Nijs *et al.* (1988b) concluded that early regrowth may have been stimulated at elevated $p_{\text{CO}_2}$. However, since initial biomass data were not given, this conclusion has to be treated with caution and more detailed investigations are necessary.

Similarly, most studies on the regrowth of *T. repens* only examined final yield (e.g. Newton *et al.* 1994; Clark *et al.* 1995; Sæbø and Mortensen 1995; Clark *et al.* 1997; Schenk *et al.* 1997; review by Newton 1991). Ryle and Powell (1992) concluded that a persistent growth response to elevated $p_{\text{CO}_2}$ had been obtained in ramets of *T. repens* maintained at an approximately constant mass by a continuously removing newly expanded leaves. However, this growth response depended largely on the production of larger stolon systems at elevated $p_{\text{CO}_2}$ during an initial phase of acclimation to the treatments. It would therefore be of interest to examine if a stimulation of regrowth in *T. repens* can also be observed in plants already adapted to elevated $p_{\text{CO}_2}$ and subjected to a less stringent defoliation regime. Such a response has been suggested by Nijs *et al.* (1988a) based on results from swards of *T. repens* exposed to elevated $p_{\text{CO}_2}$ in the greenhouse. However, as in their *L. perenne* experiment, no data were collected before defoliation or during the first few days of regrowth to further support this suggestion. In a study with single ramets of *T. repens*, Scheidegger and Nösberger (1984) did not find a stimulation of the production of new growing points either during adaptation to elevated $p_{\text{CO}_2}$ or during regrowth after moderate defoliation.

1.5 Objectives and outline of the present thesis

The objective of the present thesis was therefore to compare the response of source–sink relations to elevated $p_{\text{CO}_2}$ during regrowth after defoliation of *L. perenne* and *T. repens* and to relate the results to the growth response of these species to elevated $p_{\text{CO}_2}$.

The principal hypothesis was that elevated $p_{\text{CO}_2}$ would alleviate the impact of defoliation on source-sink relations and thus potentially stimulate regrowth. Further, the hypothesis was tested that any stimulatory effect of elevated $p_{\text{CO}_2}$ on regrowth would be more pronounced at high N supply (high growth rates) than at low N supply (reduced growth
Irrespective of regrowth patterns, the stimulation of photosynthesis at elevated $p_{\text{CO}_2}$ should allow for an accelerated recovery of carbohydrate reserves after defoliation. Source-sink relations during regrowth in the field were characterised from carbohydrate dynamics in source and storage tissues and related to effects of elevated $p_{\text{CO}_2}$ on yield and nutrient status (Parts 2 & 4). To obtain further insight into processes leading to the patterns of source-sink relations observed in the field, detailed analyses of regrowth were performed in controlled environment chambers. Potentially limiting processes were to be identified from a detailed comparison of growth processes and carbohydrate dynamics (Parts 3 & 4).

Studies of the long-term responses of yield in relation to nutrient supply (Hebeisen 1997; Zanetti 1997) indicated that the aboveground growth of *T. repens* was stimulated to a much greater extent than that of *L. perenne*. Therefore, differences in the effect of elevated $p_{\text{CO}_2}$ on source-sink relations in *L. perenne* and *T. repens* and their importance for the difference between these two species in their growth response to elevated $p_{\text{CO}_2}$ will be discussed in a final chapter (Part 5).
2. SOURCE–SINK RELATIONS IN *Lolium perenne* L. AS REFLECTED BY CARBOHYDRATE CONCENTRATIONS IN LEAVES AND PSEUDO-STEMS DURING REGROWTH IN A FREE AIR CARBON DIOXIDE ENRICHMENT (FACE) EXPERIMENT

2.1 Abstract

The effect of an elevated partial pressure of CO$_2$ ($p_{CO_2}$) on carbohydrate concentrations in source leaves and pseudo-stems (stubble) of *Lolium perenne* L. (perennial ryegrass) during regrowth was studied in a regularly defoliated grass sward in the field. The free air carbon dioxide enrichment (FACE) technology enabled natural environmental conditions to be provided. Two levels of nitrogen (N) supply were used to modulate potential plant growth. Carbohydrate concentrations in source leaves were increased at elevated $p_{CO_2}$, particularly at low N supply. Elevated leaf carbohydrate concentrations were related to an increased structural carbon (C) to N ratio and thus reflected an increased C availability together with a N-dependent sink limitation. Immediately after defoliation, apparent assimilate export rates (differences in the carbohydrate concentrations of young source leaves as measured in the evening and on the following morning) showed a greater increase at elevated $p_{CO_2}$ than at ambient $p_{CO_2}$; however, replenishment of carbohydrate reserves was not accelerated. Distinct, treatment-dependent carbohydrate concentrations in pseudo-stems suggested an increasing degree of C-sink limitation from the treatment at ambient $p_{CO_2}$ with high N supply to that at elevated $p_{CO_2}$ with low N supply. During two growing seasons, no evidence of a substantial change in the response of the carbohydrate source in *L. perenne* to elevated $p_{CO_2}$ was found. Our results support the view that the response of *L. perenne* to elevated $p_{CO_2}$ is restricted by a C-sink limitation, which is particularly severe at low N supply.

2.2 Introduction

When plants are exposed to elevated partial pressures of CO$_2$ ($p_{CO_2}$), their photosynthesis is stimulated (Bowes 1993). This stimulation generally persists and leads to enhanced growth, provided that there is an adequate supply of nutrients and water, and that growth is not limited by environmental, developmental or morphological constraints (Baker and Enoch 1983; Goudriaan and de Ruiter 1983; Wardlaw 1990). However, if assimilate production exceeds the carbohydrate demand for growth and respiration, the difference between assimilate use and assimilate production is generally stored as carbohydrate in the plant. Thus, carbohydrate concentrations reflect carbon (C) availability. Low concentrations of carbohydrates indicate a limiting rate of C supply (source limitation), and high concentrations indicate a limiting rate of C use (sink limitation) (Wareing and Patrick 1975; Farrar and Williams 1991).

In grassland, management by defoliation, and thus loss of assimilatory surface, repeatedly leads to reductions of photosynthetic C gain. Carbohydrate reserves are depleted
and may even become limiting for initial regrowth after a severe defoliation (Davies 1988; Richards 1993). Consequently, increased C availability at elevated $p_{\text{CO}_2}$ is expected to stimulate regrowth (Nijs et al. 1988b). Similarly, elevated $p_{\text{CO}_2}$ may reduce depletion and favour an earlier replenishment of carbohydrate reserves after defoliation. Several studies on the regrowth of the prominent grassland species *Lolium perenne* L. (perennial ryegrass) at elevated $p_{\text{CO}_2}$ have focused mainly on the yield response of canopies of varying complexity without closely considering carbohydrate dynamics and source-sink relations (e.g. Newton et al. 1994; Clark et al. 1995; Sæbø and Mortensen 1995; review by Newton 1991). Further, all these studies have been performed in greenhouses, in growth cabinets or other enclosures which influence environmental conditions.

Our objective was to characterise the effects of elevated $p_{\text{CO}_2}$ on C source performance and carbohydrate storage in *L. perenne* during regrowth after defoliation. Two contrasting levels of mineral nitrogen (N) supply were used to modulate growth and, hence, sink demand for carbohydrates. At the ETH free air carbon dioxide enrichment (FACE) facility near Zürich, we tested the following hypotheses in the field under natural micrometeorological conditions: In Experiment I, the main hypothesis was that elevated $p_{\text{CO}_2}$ would increase C availability in source leaf blades. This would allow for increased rates of export to satisfy the high sink demand after defoliation and/or lead to earlier and faster accumulation of excess carbohydrates in source leaves. In Experiment II, the main hypothesis was that pseudo-stems of *L. perenne* would accumulate more carbohydrate reserves at elevated $p_{\text{CO}_2}$, which, after defoliation, would be depleted to a lesser extent at elevated than at ambient $p_{\text{CO}_2}$. Finally, in both experiments the hypothesis was tested that carbohydrate concentrations in source tissues would rise as a consequence of N-limited growth and, hence, reduced carbohydrate demand.

### 2.3 Materials and methods

#### 2.3.1 Plant material

Monocultures of *Lolium perenne* L. cv. Bastion were grown in the field at Eschikon near Zürich, Switzerland as described for the frequent defoliation treatment in Hebeisen et al. (1997). Briefly: Swards were established at ambient $p_{\text{CO}_2}$ in August 1992. From the end of May 1993, a FACE system (Free Air Carbon Dioxide Enrichment; Lewin et al. 1994) was used to increase $p_{\text{CO}_2}$ from ambient (35 Pa) to 60 Pa. The swards were defoliated at approximately 5 cm above ground at regular intervals of four to six weeks. N fertilizer was applied after each cut as ammonium nitrate solution. N doses for the regrowth period under investigation in 1993 were 1.5 g m$^{-2}$ and 6 g m$^{-2}$, applied three days after the cut in the low- and high-N treatment, respectively (10 g m$^{-2}$ yr$^{-1}$ and 42 g m$^{-2}$ yr$^{-1}$); these were increased to 1.7 g m$^{-2}$ and 7 g m$^{-2}$, applied on the day after the cut in 1994 (14 g m$^{-2}$ yr$^{-1}$ and 56 g m$^{-2}$ yr$^{-1}$). The experiments were set up as a split plot design with three blocks (replications) on the basis of the crops grown in previous years and
with $p_{\text{CO}_2}$ as the main plot factor and N supply as sub-plot factor (for details see Hebeisen et al. 1997).

Experiment I. In 1993, from mid-July to mid-August, growing leaves (no part of the sheath visible yet) were sampled at intervals throughout regrowth, starting immediately before defoliation. On each occasion, one set of approximately 40 leaves per plot was sampled on the previous evening (17h - 21h) and another set on the morning (6h - 10h) of the day indicated in the figures. The part of the blade extending from the ligule of its surrounding sheath was divided into a proximal 5 cm section and a distal section of up to 5 cm in length (where available).

Experiment II. In 1994, from the beginning of August to the beginning of September, vegetative tillers were sampled at intervals throughout regrowth, starting immediately before defoliation. Each time, at least 20 tillers per plot were divided into blades of growing leaves (defined as above), blades of first fully grown leaves (the next older leaf on each tiller) and pseudo-stems (stubble; essentially leaf sheaths and enclosed parts of the growing leaf); older leaf blades and apparently dead (dry) leaf sheaths were discarded. In both experiments, the samples were frozen in liquid N$_2$ immediately after processing, stored at -20 °C, lyophilised and finely ground. All harvests were done by replication. Thereby, treatment effects were unaffected by trends in carbohydrate concentrations during the sampling interval.

During both experimental periods, there was sufficient water supply from rainfall (Experiment I: 135 mm rainfall vs. 112 mm potential evaporation; Experiment II: 145 mm rainfall vs. 70 mm potential evaporation). Experiment II started at the end of a drought period, but some rainfall (5, 8 and 6 mm, respectively) had occurred on each of the two days before defoliation and on the day of defoliation itself (for details see Hebeisen 1997). While the previous drought period had led to reduced growth and thus affected the proportion of leaf material removed at defoliation, it is assumed that regrowth was not seriously restricted by drought. Daily mean temperatures ranged from 12 to 22 °C for both experiments, a range close to optimal for the growth of *L. perenne* (Mitchell 1956a). Mean daily radiation sums were 17.5 MJ m$^{-2}$ for Experiment I and 15.1 MJ m$^{-2}$ for Experiment II. Meteorological factors apparently had no consistent effect on our experimental results.

### 2.3.2 Extraction and analysis of water-soluble carbohydrates

Approximately 10 mg of sample material were sequentially extracted with 1 cm$^3$ of 80 % (v/v) ethanol in water at 80 °C for 30 min and with 1 cm$^3$ of water in a sonicator at approximately 45 °C for 15 min. The combined supernatants, as well as the pellets, were stored at -20 °C.

Carbohydrates in the extract (i.e. water-soluble carbohydrates, comprising hexoses, sucrose, fructan, as well as a, probably insignificant, water-soluble fraction of starch) were determined in duplicate using the anthrone method (Dreywood 1946); a reagent similar to that described by Deriaz (1961) was used (350 cm$^3$ of water, 735 cm$^3$ of concentrated
H₂SO₄ (ρ=1840 kg m⁻³), 10 g of thiourea and 865 mg of anthrone). Five cm³ of ice-cold anthrone reagent were added to samples containing up to 1 µmol of hexose equivalents of water-soluble carbohydrate in a volume of 500 mm³, mixed vigorously and incubated at 96.2 °C for 40 min. After 30 min resting time at room temperature and re-mixing, the absorption at 625 nm was measured. Carbohydrate concentrations were expressed as hexose equivalents. The reaction conditions had been optimised for the simultaneous, quantitative determination of glucose and fructose, as well as other carbohydrates composed thereof (e.g. fructan, Thomas 1977). Using sucrose as a standard, maximum deviations for reference samples consisting of either pure glucose or fructose were below 5 %. Therefore, fructan is expected to be determined within a similar relative precision.

2.3.3 Starch determination

Starch was determined as glucose equivalents after enzymatic hydrolysis (Schweizer 1986). The starch in the pellets remaining after the extraction of water-soluble carbohydrate was digested sequentially with 5 mm³ of dialysed Termamyl 120 L (a heat-stable α-amylase from Bacillus licheniformis, Novo Industri A/S, Copenhagen, Denmark) in 600 mm³ of water at 95 °C for 15 min and subsequently with 4.8 units of amyloglucosidase (from Aspergillus niger, Boehringer Mannheim GmbH, Mannheim, Germany) in 1.18 cm³ of 125 mol m⁻³ sodium acetate buffer (final pH 4.6) at 60 °C for 30 min. Samples were then made up to exactly 10 cm³ with water and filtered (filter LS 14, Schleicher & Schuell, Dassel, Germany). Glucose in these extracts was determined enzymatically using hexokinase and glucose-6-phosphate dehydrogenase. The assays contained up to 200 nmol of glucose in a final volume of 1505 mm³, as modified from Farrar (1993).

2.3.4 Elemental analysis

Approximately 20 mg or more of sample material were analysed for nitrogen (N) and carbon (C) using an elemental analyser (LECO CHN-1000, LECO Corp., St. Joseph, MI, U.S.A.) calibrated against an acetanilide standard for elementary analysis (Merck, Darmstadt, Germany). Results were expressed as C to N ratios corrected for the C contribution of water-soluble carbohydrates (structural C/N-ratios).

2.3.5 Statistical data analysis

The experiment was analysed as a split-plot (Gomez and Gomez 1984) design with \( p_{\text{CO}_2} \) as main plot factor and N supply as sub-plot factor and three replications. Statistical analyses were carried out using the GLM (general linear model) procedure of the SAS statistical analysis package (SAS institute, Cary, NC, USA). All tests were carried out at the 95 % confidence level. Standard errors of means were calculated from the residual mean square error for each harvest.
2.4 Results

In Experiment I, morning starch concentrations were negligible compared to watersoluble carbohydrate concentrations, but similar $p_{CO_2}$ and N effects were observed (Fig. 2-1a). Diurnal starch concentration differences changed with time and in response to the $p_{CO_2}$ treatments in a manner similar to the diurnal concentration differences of watersoluble carbohydrate (Fig. 2-2a). Differences in starch concentrations amounted at most to approximately one fourth of the differences in water-soluble carbohydrate concentrations. Therefore, water-soluble carbohydrate concentrations can be taken as a relative measure for the total non-structural carbohydrates in leaves – both for the absolute concentrations (Figs 2-1 & 2-3) and for the diurnal concentration differences (termed ap-

![Figure 2-1](image-url)

**Figure 2-1.** Carbohydrate concentrations in the morning were measured to estimate carbohydrate availability in (a) proximal 5 cm sections and (b) distal sections of blades of growing leaves of *L. perenne* from monocultures in the ETH-FACE array (Experiment I; July-August, 1993). Experimental plots were subjected to two $p_{CO_2}$ treatments (△ □: ambient $p_{CO_2}$; ▲ ■: elevated $p_{CO_2}$) and two N treatments (△ ▲: low N supply; □ ■: high N supply). Solid lines: water-soluble carbohydrate; dashed lines: starch (not measured in distal sections). Carbohydrate concentrations are expressed as moles of hexose equivalents per unit dry weight. Error bars indicate the standard error of means (n=3) for each harvest.
parent night-time export; Fig. 2-2). Similarly, starch concentrations in pseudo-stems never exceeded 5% of the total non-structural carbohydrate concentration; therefore, only the water-soluble carbohydrate concentrations are presented in Fig. 2-4.

2.4.1 Characterisation of source leaf activity during regrowth (Experiment I)

Throughout the regrowth period from July to August 1993, carbohydrate concentrations in proximal sections of growing leaves were higher in plants grown at elevated $p_{\text{CO}_2}$ than

![Figure 2-2. Apparent night-time export of carbohydrate was estimated as the differences in the carbohydrate concentrations as measured in the evening and on the following morning in (a) proximal 5 cm sections and (b) distal sections of blades of growing leaves of *L. perenne* from monocultures in the ETH-FACE array (Experiment I; July-August, 1993). Experimental plots were subjected to two $p_{\text{CO}_2}$ treatments (O: ambient $p_{\text{CO}_2}$; : elevated $p_{\text{CO}_2}$) and two N treatments; however, because of the lack of significant N effects, averaged results over the N treatments are shown. Solid lines: water-soluble carbohydrate; dashed lines: starch (not measured in distal sections). Carbohydrate concentration differences are expressed in moles of hexose equivalents per unit dry weight. Error bars indicate the standard error of means (n=6) for each harvest.]


at ambient $p_{\text{CO}_2}$ (significant overall and for days 3, 9, 30; Fig. 2-1a). Carbohydrate concentrations after defoliation dropped sharply in all treatments, reaching a minimum after approximately 3 to 9 d. Concentrations initially decreased faster in controls ($p_{\text{CO}_2} \times$ harvest interaction significant for days 0–3), but the overall decrease from day 0 to day 5 was similar in all treatments.

Carbohydrate concentrations were higher at low than at high N supply, particularly during the first part of the regrowth period (significant for days 0, 3, 5, 9; Fig. 2-1a). The minimum carbohydrate concentration reached during regrowth at elevated $p_{\text{CO}_2}$ with low N supply was distinctly higher than the minima reached in the other treatments ($p_{\text{CO}_2} \times$ N interaction significant for day 9). Towards the end of the regrowth period, carbohydrate concentrations increased again. Due to a slightly higher rate of increase in the high-N than in the low-N treatment, purely $p_{\text{CO}_2}$-dependent final carbohydrate concentrations were reached. Elevated $p_{\text{CO}_2}$ had no significant effect on the time it took carbohydrate concentrations to recover (Fig. 2-1a).

Carbohydrate concentrations in the distal sections of growing leaves showed treatment effects similar to those in the proximal sections (Fig. 2-1b); however, the increase from day 9 to day 30 was smaller in the distal sections.

The disappearance of carbohydrates from source leaves during the night is due to assimilate export to sinks and to leaf respiration. Respiration rates are either roughly constant or positively correlated to carbohydrate export (Ho 1978; Bouma et al. 1995). Therefore, the difference in the carbohydrate concentration of source leaves between evening and the following morning is closely related to night-time carbohydrate export and designated as 'apparent night-time export' in this paper.

Apparent night-time export rates from leaves were not significantly affected by the N supply; therefore, in Fig. 2-2 the average of the N treatments is shown for each $p_{\text{CO}_2}$ treatment. Apparent night-time export from the proximal sections of growing leaves rose from near zero to a maximum about 5 d after defoliation (Fig. 2-2a). Significantly higher values were reached at elevated $p_{\text{CO}_2}$ than at ambient $p_{\text{CO}_2}$ for day 5. Thereafter, apparent night-time export decreased gradually. While it dropped back to near zero at elevated $p_{\text{CO}_2}$, a significant apparent night-time export persisted at ambient $p_{\text{CO}_2}$ ($p_{\text{CO}_2}$ effect significant for day 30).

Apparent night-time export from distal sections of growing leaves (Fig. 2-2b) showed a trend similar to that from proximal sections (Fig. 2-2a). However, towards the end of regrowth, apparent night-time export from distal sections was similar in both $p_{\text{CO}_2}$ treatments and was in a range similar to that from proximal sections at ambient $p_{\text{CO}_2}$.

### 2.4.2 Comparison of carbohydrate availability in different tissues during regrowth (Experiment II)

During the regrowth from August to September 1994, carbohydrate concentrations in growing leaves, as well as their rates of change, were similar in all treatments except
that at elevated $p_{\text{CO}_2}$ with low N supply (Fig. 2-3a; $p_{\text{CO}_2}$ effect significant overall and for days 0, 4 and 25; N effect significant overall and for days 4 and 25). Leaves of the latter treatment consistently had the highest carbohydrate concentrations ($p_{\text{CO}_2} \times \text{N interaction}$ significant overall and for days 4 and 25). Recovery from defoliation appeared complete by day eight after defoliation with carbohydrate concentrations remaining almost stable thereafter.

The pattern and absolute values of carbohydrate concentrations in first fully grown leaves (Fig. 2-3b) were largely similar to those in growing leaves. However, the decrease in carbohydrate concentration after defoliation was generally smaller and even absent in the first fully grown leaves from the treatment at elevated $p_{\text{CO}_2}$ with low N supply.

The carbohydrate concentration in pseudo-stems was always significantly higher at elevated $p_{\text{CO}_2}$ than in the corresponding treatment at ambient $p_{\text{CO}_2}$ (Fig. 2-4). It was also

![Graph](image)

**Figure 2-3.** Concentrations of water-soluble carbohydrate were measured to estimate carbohydrate availability in blades of (a) growing and (b) first fully grown leaves of *L. perenne* from monocultures in the ETH-FACE array (Experiment II; August-September, 1994). Experimental plots were subjected to two $p_{\text{CO}_2}$ treatments (△□: ambient $p_{\text{CO}_2}$; ▲■: elevated $p_{\text{CO}_2}$) and two N treatments (△▲: low N supply; □■: high N supply). Carbohydrate concentrations are expressed as moles of hexose equivalents per unit dry weight. Error bars indicate the standard error of means (n=3) for each harvest.
consistently higher in the low-N treatments than in the corresponding high-N treatments (significant overall and for days 0, 4, 14 and 25). No significant $p_{\text{CO}_2} \times N$ interactions were observed. An initial decrease in carbohydrate concentration after defoliation was observed in all treatments, but the magnitude of the decrease was smaller in plants grown at elevated $p_{\text{CO}_2}$ ($p_{\text{CO}_2} \times$ harvest interaction significant for days 0–4) and at low N supply ($N \times$ harvest interaction significant for days 0–4). By the end of the regrowth period, reserves were replenished to near starting levels.

2.4.3 Relationship between water-soluble carbohydrate and structural C/N-ratios

There was a highly significant correlation between water-soluble carbohydrate concentrations in the growing leaves of both experiments and structural C/N-ratios, which accounted for most of the variation in water-soluble carbohydrate concentrations (Fig. 2-5). In comparison, there were only minor contributions by other factors.

Figure 2-4. Concentrations of water-soluble carbohydrate were measured to estimate carbohydrate availability in pseudo-stems of *L. perenne* from monocultures in the ETH-FACE array (Experiment II; August-September, 1994). Experimental plots were subjected to two $p_{\text{CO}_2}$ treatments (△ ■ ambient $p_{\text{CO}_2}$; ▲ ■ : elevated $p_{\text{CO}_2}$) and two N treatments (△ ■ : low N supply; ▲ ■ : high N supply). Carbohydrate concentrations are expressed as moles of hexose equivalents per unit dry weight. Error bars indicate the standard error of means (n=3) for each harvest.
2.5 Discussion

For the first time, the effect of elevated $p_{CO_2}$ on the shoot carbohydrate concentration of a perennial grass has been studied during regrowth. The temporal pattern of carbohydrate mobilisation and accumulation in leaves and pseudo-stems after the defoliation of *L. perenne* remained unaffected by elevated $p_{CO_2}$ in the field. However, carbohydrate concentrations were consistently higher at elevated $p_{CO_2}$, particularly in the leaves of plants grown with low N supply. After defoliation, the rate of apparent night-time carbohydrate export from leaves was higher at elevated than at ambient $p_{CO_2}$.

2.5.1 Carbohydrate availability in source leaves

Consistently higher carbohydrate concentrations (Figs 2-1 & 2-3) suggest that there was a persistent stimulation of C assimilation at elevated $p_{CO_2}$ throughout the regrowth period. This interpretation was supported by periodic measurements of leaf gas-exchange rates, which indicated a significant stimulation of daytime net C assimilation at elevated $p_{CO_2}$ to the extent of 16 % and 20 % in the high and low N treatments, respectively, in 1993, and of 59 % and 61 % in 1994 (Phillip A. Davey, University of Essex, personal communication; Bryant 1994). Similarly, Nijs, Impens and Behaeghe (1989b) observed...
a persistent stimulation of canopy net C uptake during regrowth at elevated $p_{\text{CO}_2}$ in an experiment using acrylic enclosures with *L. perenne* in boxes. In our experiment, carbohydrate availability was further enhanced at elevated $p_{\text{CO}_2}$, as well as at low N supply because the proportion of leaf dry matter remaining after defoliation was greater (Tab. 2-1, Experiment I). Thus, in addition to retaining the largest proportion of leaf material, the plants at elevated $p_{\text{CO}_2}$ and low N supply had a relatively high potential for C assimilation. Similarly, in an open top chamber experiment during one growing season Sæbø and Mortensen (1995) found that leaf length in *L. perenne* and *Phleum pratense* was reduced and the stubble dry matter was increased at elevated $p_{\text{CO}_2}$.

In Experiment I, the frequency of sampling allowed us to compare the rates of change of carbohydrate concentrations in leaves. A rapid decrease in carbohydrate concentrations after defoliation indicated a high demand for carbohydrates relative to the supply from current photosynthesis (Fig. 2-1a). The initial rate of carbohydrate loss after defoliation was lower at elevated $p_{\text{CO}_2}$ than at ambient $p_{\text{CO}_2}$ because of the higher availability of assimilates from current photosynthesis.

In grasses like *L. perenne*, C demand for growth and growth-related respiration may be limited by a number of factors, such as the supply of N (Wilman and Wright 1983). Indeed, in our FACE experiment the yield response of *L. perenne* strongly depended on N fertilizer (Hebeisen *et al.* 1997). Thus, the lower carbohydrate concentrations in the high N supply treatments during initial regrowth suggest a N dependence of carbohydrate utilisation during regrowth (Figs 2-1a & 2-3). Further, minimum morning carbohydrate concentrations in the leaves growing under elevated $p_{\text{CO}_2}$ with low N supply were about twice as high as in the other treatments (Figs 2-1a, days 5–9 & 2-3, day 4). Such differences suggest a N-dependent sink limitation under elevated $p_{\text{CO}_2}$ with low N supply even shortly after defoliation. A tendency towards a reduced harvestable yield was observed at elevated $p_{\text{CO}_2}$ with low, but not high N supply (Hebeisen 1997; Hebeisen *et al.* 1997), further supporting this interpretation. Indications of limited C-sink activities at elevated $p_{\text{CO}_2}$ have also been found in experiments in controlled environment (e.g. Arp 1991).

**Table 2-1.** Residual leaf dry matter after defoliation relative to the total above ground dry matter present before defoliation of *L. perenne* from the ETH-FACE in July, 1993 (Experiment I) and in August, 1994 (Experiment II). Residual above ground dry matter was determined within 3 d after defoliation. Values are the means of three replicates. Within each experiment, values with common letters are not significantly different at $p \leq 0.05$ (multiple t-tests based on standard errors of least-squares means).

<table>
<thead>
<tr>
<th>Proportion of leaf dry matter remaining after defoliation</th>
<th>High N supply</th>
<th>Low N supply</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ambient $p_{\text{CO}_2}$</td>
<td>Elevated $p_{\text{CO}_2}$</td>
<td>Ambient $p_{\text{CO}_2}$</td>
</tr>
<tr>
<td>Experiment I</td>
<td>4 %$^a$</td>
<td>25 %$^b$</td>
</tr>
<tr>
<td>Experiment II</td>
<td>48 %$^{ab}$</td>
<td>43 %$^a$</td>
</tr>
</tbody>
</table>
About one week after defoliation, increasing carbohydrate concentrations indicated that C assimilation exceeded the immediate needs of the plant (Fig. 2-1a). The lag period until the beginning of carbohydrate accumulation in the leaves was, however, unaffected by the $p_{\text{CO}_2}$ treatments. Therefore, elevated $p_{\text{CO}_2}$ did not appear to accelerate metabolic recovery from defoliation in spite of the obvious increase in carbohydrate availability. Instead, the enhanced carbohydrate availability at elevated $p_{\text{CO}_2}$ may have been matched by a higher sink demand – possibly as a consequence of the respiratory needs for the maintenance and function of a larger root system (Jongen et al. 1995; Hebeisen et al. 1997) as no evidence for an enhanced initial rate of regrowth was observed (Stadelmann 1993).

After initial regrowth, carbohydrate concentrations in growing leaves rose steadily to exclusively $p_{\text{CO}_2}$-dependent final levels (Fig. 2-1). This was likely due to a N-mediated sink limitation as leaf-N concentrations, as well as differences between N treatments, declined towards the end of regrowth (not shown). Based on diurnal measurements of photosynthesis (Phillip A. Davey, University of Essex, personal communication) and using representative values for specific leaf weight (SLW) obtained in the growth chamber (Part 3), the amount of carbohydrate remaining in the leaves at the end of the night in the treatment at elevated $p_{\text{CO}_2}$ and low N supply was estimated to represent between 1.9 (day 0) and 1.1 (day 30) times the daily assimilation on a moderately sunny day.

2.5.2 Carbohydrate export from source leaves

The rates of apparent night-time export from the proximal sections of growing leaves increased after defoliation (Fig. 2-2a). This increase reflected a rising contribution of current assimilates in satisfying the plant’s C demand. At elevated $p_{\text{CO}_2}$, apparent night-time export temporarily exceeded that at ambient $p_{\text{CO}_2}$ around day 5 after defoliation. The effects of $p_{\text{CO}_2}$ on apparent night-time export might be related to limitations on daytime carbohydrate utilisation and export. However, an initial stoppage of translocation after defoliation as suggested by Richards (1993) would last for no more than 2 d; moreover, translocation capacity has been shown to respond both to leaf area and light conditions (Wardlaw 1990; Wimmers and Turgeon 1991) suggesting that translocation capacity adjusts to assimilate availability; similarly, recent results suggest that carbohydrate accumulation at elevated $p_{\text{CO}_2}$ in barley leaves was not caused by a limiting capacity for carbon export (Hibberd et al. 1996). In the absence of such limitations, the partitioning of assimilates in leaves between export and storage appears to be under endogenous control (Gordon 1986). This control is such as to maintain uninterrupted day-time export rates during fluctuations of photosynthesis in the short term and to retain sufficient carbohydrate to cover the needs anticipated for export during the night in the long term. Thus, the enhanced night-time export at elevated $p_{\text{CO}_2}$ likely reflected an increase in sink demand. As discussed above, an increase in sink demand might explain why the apparent recovery from defoliation was not accelerated at elevated $p_{\text{CO}_2}$ in spite of the higher carbohydrate availability (Fig. 2-1a). Nevertheless, assimilate demand at elevated
$p_{\text{CO}_2}$ and low N supply was insufficient to reduce the leaf carbohydrate concentration after defoliation to the level of the other treatments.

Towards the end of regrowth, decreasing apparent night-time export from growing leaves (Fig. 2-2) suggests that there was an increasing supply of carbohydrate from older leaves extending into the upper canopy. This effect was even more pronounced at elevated $p_{\text{CO}_2}$. Apparent night-time export from proximal leaf sections at elevated $p_{\text{CO}_2}$ decreased to near zero (Fig. 2-2a); nevertheless, a significant apparent night-time export persisted from distal leaf sections (Fig. 2-2b). Presumably, increased availability of carbohydrate at elevated $p_{\text{CO}_2}$ from older leaves, as well as from distal leaf sections, reduced the sink demand on proximal leaf sections.

#### 2.5.3 Comparison of sources: leaves and pseudo-stems

Carbohydrate concentrations in different leaf age classes (Fig. 2-3) and different leaf sections (Fig. 2-1), as well as their response to elevated $p_{\text{CO}_2}$, were generally quite similar. Carbohydrate concentrations at elevated $p_{\text{CO}_2}$ with low N supply were consistently higher than in the other three treatments; this corroborates the suggestion of a N-dependent sink limitation in plants subjected to that treatment. Plants grown at low N supply had a lower above ground dry matter yield (Hebeisen et al. 1997) and their root mass was larger (Jongen et al. 1995; Hebeisen et al. 1997); these symptoms strongly suggest nutrient limited growth and, as a consequence, a possible change in root:shoot allometry. Such a N-dependent sink limitation was most pronounced in first fully grown leaves. Differences in the effects of elevated $p_{\text{CO}_2}$ on carbohydrate availability between growing and fully grown leaves in grasses may be related to differences in their vascular links to sinks (Forde 1965; Davidson and Milthorpe 1966). However, recent work suggests the presence of a single pool of assimilates (de Visser et al. 1997).

The pseudo-stems from the treatment at elevated $p_{\text{CO}_2}$ with low N supply contained the highest carbohydrate concentrations (Fig. 2-4). Rates of net carbohydrate mobilisation from the pseudo-stems after defoliation suggest that the carbohydrate demand in excess of current assimilation was highest at ambient $p_{\text{CO}_2}$ and high N supply and lowest at elevated $p_{\text{CO}_2}$ and low N supply. While a part of this trend can be explained by the increased proportion of leaf dry matter remaining after defoliation at elevated $p_{\text{CO}_2}$ with low N supply (Tab. 2-1, Experiment II), it nevertheless supports our previous conclusion that the increase in carbohydrate availability exceeded any stimulation of sink demand due to elevated $p_{\text{CO}_2}$ (e.g. in the root system). Such an imbalance could be exacerbated by a potential $p_{\text{CO}_2}$-dependent reduction of respiration (González-Meler et al. 1996b and references therein).

#### 2.5.4 Structural C/N-ratios

High water-soluble carbohydrate concentrations in *L. perenne* were significantly correlated to an increased structural C/N-ratio (Fig. 2-5). This general correlation demonstrates that leaf water-soluble carbohydrate concentrations largely reflected the avail-
ability of C relative to that of N for incorporation into structural biomass. As growth, and thus C utilisation by sinks, in *L. perenne* was greatly influenced by N availability, this relation also supports the concept of carbohydrate concentrations reflecting the balance between C assimilation and utilisation.

While N availability obviously increased after fertilization 3 d after defoliation in Experiment I, the decrease in the water-soluble carbohydrate concentrations (Fig. 2-1a) and structural C/N-ratio in leaves had already started beforehand. Thus, the structural C/N-ratio reflected mainly reduced C availability and not the changing N availability. A strong decrease in the C/N ratio of newly formed leaf material after defoliation has recently been shown by de Visser et al. (1997) for *L. perenne* growing in hydroponic culture with constant N availability. It was interpreted as an adaptive response for maintaining the rate of leaf extension in spite of a reduced C availability.

The differences in the range of carbohydrate concentrations in growing leaves between Experiments I and II (Figs 2-1a & 2-3a) might suggest long-term adjustments of *L. perenne* to elevated $p_{\text{CO}_2}$ in the field. However, the relationship between water-soluble carbohydrate concentrations and structural C/N-ratios differed little between the two years (Fig. 2-5). These different ranges of carbohydrate concentrations possibly reflected an increased N availability in Experiment II as a consequence of N accumulation in the soil from continued fertilization (Zanetti *et al.* 1996). Consequently, there was no substantial change in the response of the carbohydrate source in *L. perenne* to elevated $p_{\text{CO}_2}$ during two growing seasons.

### 2.5.5 Conclusions

Assimilate concentration in shoots of *L. perenne* grown in repeatedly defoliated swards in the field was clearly increased at elevated $p_{\text{CO}_2}$, reflecting a change in the balance between C assimilation and utilisation. This was due to a persistent, direct stimulation of C assimilation and also to indirect effects such as an increased proportion of leaf dry matter remaining after defoliation. Elevated $p_{\text{CO}_2}$ reduced the impact of defoliation on carbohydrate concentrations. Assimilate demand after defoliation - as estimated from rates of apparent night-time assimilate export - appeared to be temporarily increased at elevated $p_{\text{CO}_2}$; however, recovery as detected from the incipient restoration of carbohydrate reserves was not accelerated. Instead, a persistent C sink limitation was evident at elevated $p_{\text{CO}_2}$ with low N supply.

Published as:

3. PHYSIOLOGICAL AND MORPHOLOGICAL ADJUSTMENTS TO ELEVATED $p_{CO_2}$ IN LOLIUM PERENNE L. IN THE ABSENCE OF A STIMULATION OF REGROWTH AFTER DEFOLIATION

3.1 Abstract

The regrowth of single, spaced plants of *Lolium perenne* L. (perennial ryegrass) after defoliation and their response to contrasting levels of the atmospheric partial pressure of CO$_2$ ($p_{CO_2}$) and nitrogen (N) supply was studied in controlled environments. The hypothesis was tested that an enhanced carbohydrate availability at elevated $p_{CO_2}$ would lead to a faster recovery from defoliation and an accelerated replenishment of carbohydrate reserves. Indeed, *L. perenne* produced more structural and/or non-structural dry matter per tiller, indicating an enhanced availability of carbon (C) building blocks for growth. In fact, this increase in dry mass per growing point (+33 % after apparent recovery) was the principal growth response to elevated $p_{CO_2}$. However, neither recovery from defoliation nor subsequent growth were significantly enhanced by elevated $p_{CO_2}$ at either generous or limiting N supply. The relative increase in tiller size at elevated $p_{CO_2}$ remained similar throughout and thus initial regrowth appeared to depend more directly on the vigour of meristems present at the time of defoliation than on C availability. It appears that enough carbohydrate reserves (1.5–2.5 mol kg$^{-1}$ as hexose equivalents in pseudo-stems) and/or leaf area remained after defoliation such that the regrowth of leaves was not limited by carbohydrate availability. A positive response to elevated $p_{CO_2}$ of total plant dry matter accumulation during the recovery phase may have been prevented by the presence of larger respiratory sinks (particularly roots). However, even during later regrowth and after recovery, there was no stimulation of tiller production or relative growth rate (RGR). Therefore, the higher leaf carbohydrate concentrations and the enhanced accumulation of carbohydrate reserves towards the end of regrowth (2.2–3.1 mol kg$^{-1}$ in first fully grown leaves and pseudo-stems) reflected an apparent sink limitation of the growth response to elevated $p_{CO_2}$. A reduced photosynthetic capacity per unit leaf area and a 10 % lower leaf area ratio (LAR) apparently decreased net carbon assimilation in plants growing at elevated $p_{CO_2}$ relative to plants growing at ambient $p_{CO_2}$. Possible underlying causes for this response to elevated $p_{CO_2}$ are discussed. It is concluded that in *L. perenne*, the reduction of the source:sink ratio by defoliation did not reverse the loss of responsiveness of growth to elevated $p_{CO_2}$ of the plants which is frequently found with increasing plant maturity in long-term studies on the effect of elevated $p_{CO_2}$ and which was also apparent in the present study.

3.2 Introduction

Rising atmospheric partial pressures of CO$_2$ ($p_{CO_2}$) lead to an increased efficiency of photosynthesis in C$_3$ plants (Long *et al.* 1995). A reduction of respiration rates has also been proposed (e.g. Bunce and Caulfield 1991; Amthor *et al.* 1992), but possible un-
underlying biochemical mechanisms are only just beginning to be elucidated (Gonzàlez-Meler et al. 1996b). A higher availability of assimilated carbon (C) at elevated $p_{CO_2}$ generally leads to enhanced growth (Baker and Enoch 1983). However, if environmental or developmental constraints restrict the growth response, various adjustment strategies can be observed. All of these appear to act in bringing C assimilation and demand back into balance: Photosynthetic acclimation may reduce C assimilation (Sage 1994). Alterations of plant morphology such as an increased specific leaf weight (SLW, the ratio of leaf dry mass to leaf area) and a reduced leaf area ratio (LAR, the ratio of leaf area to total plant dry mass) also reduce the C gain per plant (Poorter et al. 1996).

Defoliation of grassland plants by cutting or grazing greatly reduces their assimilatory surface. A stimulation of photosynthesis at elevated $p_{CO_2}$ might thus reduce the detrimental effects of defoliation on the plants’ C balance. Regrowth of leaves or at least the replenishment of carbohydrate stores may be accelerated.

Nevertheless, regularly defoliated swards of *Lolium perenne* L. (perennial ryegrass) exposed to elevated $p_{CO_2}$ in the field showed only a low responsiveness of aboveground yield to elevated $p_{CO_2}$; this responsiveness was dependent on nitrogen (N) fertilisation (Hebeisen et al. 1997). Measurements of carbohydrate concentrations during regrowth of *L. perenne* in the field suggested a persistent sink limitation at low N supply even shortly after defoliation (Part 2). In a previous study on the regrowth of *L. perenne* swards, it was concluded from a growth analysis of only aboveground plant material that treatment differences were established during the first days after cutting (Nijs et al. 1988b). However, no data on the residual biomass after defoliation were given.

Our objective was to characterise the effect of elevated $p_{CO_2}$ on the regrowth of *L. perenne* after defoliation and to identify causes that led to the limited growth response observed in the field.

An experiment in controlled environment chambers was designed for that purpose using single, spaced plants. As each individual plant can expand in three dimensions, a more pronounced growth response to elevated $p_{CO_2}$ is possible than in closed canopies (Lüscher et al. 1996). The use of single plants further allows the application of growth analysis techniques to entire plants. The approach chosen thus offers the opportunity to gain insight into adjustment processes which may govern the response of *L. perenne* to elevated $p_{CO_2}$. Two levels of N supply were employed with the aim to modify the potential growth response to elevated $p_{CO_2}$.

The hypothesis was tested that elevated $p_{CO_2}$ would enhance the growth of seedlings as well as the regrowth of defoliated plants of *L. perenne*, both of which have relatively low rates of C assimilation compared to C demand. Changes in source-sink relations due to defoliation and during regrowth should be reflected in the effect of elevated $p_{CO_2}$ on leaf carbohydrate concentrations (as a measure of carbohydrate availability) and on pseudo-stem carbohydrate concentrations (reflecting carbohydrate reserves). Further, it was expected that any adjustments to growth at elevated $p_{CO_2}$ (e.g. photosynthetic accli-
mation as in Rogers et al. 1995) would disappear temporarily after defoliation during regrowth in response to the reduced C assimilation, particularly at high N supply.

3.3 Materials and Methods

3.3.1 Plant material and growth conditions

Plants of *Lolium perenne* L. cv. Bastion were grown from seed in boxes, filled 5 cm deep with quartz sand (particle size 0.7–1.2 mm) and placed in Conviron PGV 36 (Conviron, Winnipeg, Manitoba, Canada) controlled environment chambers set to either 35 or 60 Pa partial pressure of CO\(_2\) (\(p_{\text{CO}_2}\)). \(p_{\text{CO}_2}\) was monitored using WMA-2 infrared gas analysers (PP-Systems, Hitchin, Herts, U.K.); excess CO\(_2\) was removed by forcing air through soda lime (Roth, Karlsruhe, Germany). Daylength was set to 16 h with 12 h at maximum PPFD (500 µmol m\(^{-2}\) s\(^{-1}\) produced from 5.2 kW of fluorescent tubes using Sylvania 215W very high output cool white and 2.5 kW of incandescent bulbs using Sylvania 100 W/277 V, each consuming approx. 75 W at 230 V; all Osram Sylvania Inc., St. Marys, PA, U.S.A.) and 2 h each of stepwise increasing and decreasing PPFD at the beginning and at the end of the day, respectively. Relative humidity was kept at 80 % and air temperature was set to 18/13 °C day/night. Plants were watered twice daily using a nutrient solution containing 7.5 mM NO\(_3\) according to Hammer et al. (1978), using 0.124 mM EDTA ammonium iron(III)salt (Fluka, Buchs, Switzerland) as a source of iron and without adjustment of pH. Plants and \(p_{\text{CO}_2}\) treatments were rotated between growth chambers at weekly intervals.

After 17 d, vigorous plants with one tiller and 2–3 leaves were selected in each \(p_{\text{CO}_2}\) treatment, trimmed to 5.5 cm of shoot and 2.5–3 cm of roots and transplanted singly into cylindrical pots of 7 cm diameter × 25 cm height, filled with 1.5 kg of quartz sand as above. The level of elevated \(p_{\text{CO}_2}\) was increased to 70 Pa. Nutrient solution was applied at a rate of 30 ml per pot twice daily.

Nitrogen treatments were started 39 d after sowing. Within each replication, plants were randomly allocated to either a low (LN) or a high (HN) nitrogen supply and to harvest dates. Pots were generously flushed with deionized water prior to the first application of new nutrient solutions at 100 ml per pot twice daily. To ensure non-limiting water supply with increasing plant size, the amount of nutrient solution applied was increased to 150 ml per pot twice daily 63 days after sowing. Nutrient solutions were prepared according to Hammer et al. (1978) without adjustment of pH, using 0.124 mM EDTA ammonium iron(III)salt (Fluka, Buchs, Switzerland) as a source of iron and contained the following additional modifications: For the LN solution, KNO\(_3\) and Ca(NO\(_3\))\(_2\) as in the original recipe were replaced by 0.5 mM KCl, 1 mM K\(_2\)SO\(_4\), 0.5 mM Ca(NO\(_3\))\(_2\) and 2 mM CaSO\(_4\) (final concentration of NO\(_3\)\(^–\): 1 mM). In the HN solution, the concentration of Ca(NO\(_3\))\(_2\) was changed from the original recipe to 6.25 mM (final concentration of NO\(_3\)\(^–\): 15 mM).
3.3.2 Sequential harvests during regrowth after defoliation

At 45 d after sowing (28 d after transplanting into pots), plants were defoliated to approx. 45 mm residual tiller height (measured from the point of attachment of roots; i.e. approx. 40 mm above ground). Regrowth was monitored using a total of seven harvests at 0, 2, 4, 7, 11, 17 and 28 d after defoliation. For each harvest, one set of plants, consisting of three replications, was sampled at the end of a photoperiod and another set at the beginning of the following photoperiod. Duplicate sets (twice three replications) were sampled for days 0, 4 and 17.

At each harvest, sand was carefully removed, then plants were separated into roots, pseudo-stems (stubble; essentially leaf sheaths and enclosed parts of the growing leaf), exposed parts of blades of growing leaves (no part of the sheath visible yet), blades of first fully grown leaves (the next older leaf on each tiller), blades of older leaves and necrotic tissue. Old leaves were classified as necrotic when more than two thirds of their area had senesced and were not included in the present analysis. Tiller number was determined by counting every shoot on which a leaf blade had appeared. The area of all leaf blade fractions was determined using a LI-3000A leaf area meter fitted with a belt conveyor (Lambda Instruments Corporation, Lincoln, Nebraska, U.S.A.). Leaves removed at defoliation were oven-dried (90 minutes at 105 °C, followed by 48 h at 65 °C); all other tissues were frozen in liquid N₂ within 30 minutes after processing and stored at -20 °C until lyophilisation. Dry material was weighed and finely ground for chemical analyses.

3.3.3 Water-soluble carbohydrate (WSC) analysis

WSC were extracted from 10 mg of sample material and analysed using an anthrone protocol optimised for the simultaneous determination of glucose, fructose, as well as other carbohydrates composed thereof, including fructan (Part 2). Previous measurements have demonstrated that starch in the morning is negligible in L. perenne (Part 2).

3.3.4 Elemental analysis

At least 20 mg of leaf material were analysed for nitrogen (N) and for carbon (C) by measuring thermal conductivity and infrared absorption, respectively, of combustion gases using an elemental analyser (LECO CHN-1000, LECO Corp., St. Joseph, MI, U.S.A.) calibrated against an acetanilide standard for elementary analysis (Merck, Darmstadt, Germany).

3.3.5 Analysis for Nitrate and Ammonium

Approximately 10 mg of finely ground leaf material were carefully suspended in 30 mm³ of ethanol and extracted in 1 cm³ of water for 30 min. The supernatant was analysed for nitrate and ammonium using an Evolution II autoanalyser (Alliance Instruments GmbH, Friedrichsdorf, Germany). Nitrate was reduced to nitrite using hydrazine sulphate and copper sulphate. Extinction at 540 nm was measured after a diazo coupling
of nitrite to sulphanilamide and α-naphthylethylenediamine. Ammonium was determined from the extinction at 660 nm after conversion to an indophenol dye according to DIN 38406 part 23 (ISO/DIS 11732-1994).

### 3.3.6 Photosynthesis measurements

Estimates of the maximum carboxylation velocity ($V_{c_{\text{max}}}$) of first fully grown leaves were obtained about one week after defoliation and at the end of the experiment. Response curves of assimilation rate ($A$) versus internal leaf CO$_2$ concentration ($C_i$) at saturating PPFD (1120 µmol m$^{-2}$ s$^{-1}$) were determined using the standard broad-leaf Parkinson leaf chamber connected to a CIRAS combined infra-red gas analyser system (both PP-Systems, Hitchin, Herts, U.K.). $V_{c_{\text{max}}}$, temperature corrected to 25 °C, was estimated as the slope of a linear regression of $A$ versus a function $f$ of $C_i$ and leaf temperature ($T$), derived from the model proposed by Farquhar and von Caemmerer (1982) and extended by the approximation for the temperature dependence of $V_{c_{\text{max}}}$ given in Kirschbaum and Farquhar (1984), with $R_d$ (day respiration) as the intercept:

$$A = V_{c_{\text{max}}}f - R_d$$

(3-1)

with

$$f = \frac{C_i - \Gamma_*}{C_i + K_c \left(1 + O_i/K_o\right)} \left[1 + 0.0505(T - 25) - 2.48 \cdot 10^{-4}(T - 25)^2 - 8.09 \cdot 10^{-5}(T - 25)^3\right]$$

(3-2)

and $A$, $V_{c_{\text{max}}}$ and $R_d$ in µmol m$^{-2}$ s$^{-1}$, $T$ in °C, $C_i$, $K_c$ (Michaelis constant of RuBisCO to CO$_2$) and $\Gamma_*$ (CO$_2$ compensation point in the absence of day respiration) in µmol mol$^{-1}$, $O_i$ (internal leaf O$_2$ concentration) and $K_o$ (Michaelis constant of RuBisCO to O$_2$) in mmol mol$^{-1}$ and using the following approximations: $\Gamma_* = 1.7$ °C (Farquhar 1988), $K_c = 39.05 \exp(0.086 T)$ and $K_o = 2.412 O_i \exp(0.086 T)/T$ (McMurtrie and Wang 1993).

### 3.3.7 Statistical analysis

Values of extensive quantities (dry mass, etc.) and ratios thereof were converted to their natural logarithms prior to statistical analysis. Means shown were obtained by back-transformation. Intensive quantities (concentrations, $V_{c_{\text{max}}}$) were estimated using non-transformed values. Statistical analyses were carried out using the GLM (general linear model) procedure of the SAS statistical analysis package (SAS Institute Inc., Cary, NC, USA). All tests were carried out at the 95% confidence level. Standard errors of means were calculated from the residual mean square error for each harvest.
3.4 Results

*Lolium perenne* plants grown for 45 d under elevated $p_{\text{CO}_2}$ were significantly heavier than those grown under ambient $p_{\text{CO}_2}$ (Fig. 3-1a–d, before defoliation). This size difference was evident in the leaf and pseudo-stem fractions; however, it was largest in the root fraction. At the same time, however, no significant effect of the N supply (6 d after the initiation of fertilisation treatments) was evident on total dry mass.

3.4.2 Relative growth rate

Total plant dry mass kept rising after defoliation (Fig. 3-1a). Relative growth rate (RGR, the slope of the natural logarithm of dry mass vs. time in Fig. 3-1) was assessed according to the method of Poorter (1986); it was always significantly greater than zero (Time effect significant, Tab. 3-1) and was unaffected by the $p_{\text{CO}_2}$ treatments (Time $\times$ $p_{\text{CO}_2}$ n.s., Tab. 3-1). Thus, the relative size advantage of the plants growing under elevated $p_{\text{CO}_2}$ over their counterparts at ambient $p_{\text{CO}_2}$ remained unchanged throughout the experiment.

While leaf dry mass and leaf area increased rapidly (Fig. 3-1b,e), pseudo-stems and roots ceased growing during the first 4 d after defoliation (Fig. 3-1c,d). Again, elevated $p_{\text{CO}_2}$ did not increase the relative growth rate of these plant fractions: the effect of $p_{\text{CO}_2}$ on

| Source of variation | Days 0-4 | | | Days 4-28 | | | |
|---------------------|----------|--------------------------|--------------------------|----------|--------------------------|--------------------------|
|                     | DF | Total DM | Tiller number | DF | Total DM | Tiller number |
| $p_{\text{CO}_2}$   | 1  | 4.873 *** | 0.431 *** | 1  | 6.086 *** | 0.498 *** |
| N                   | 1  | 0.163    | 1.161 ***  | 1  | 9.272 *** | 11.671 *** |
| $p_{\text{CO}_2} \times$ N | 1  | 0.121    | 0.017      | 1  | 0.051    | 0.029      |
| Time                | 2  | 1.114 *** | 0.753 ***  | 4  | 34.411 ***| 9.389 ***  |
| Time $\times$ $p_{\text{CO}_2}$ | 2  | 0.039    | 0.002      | 4  | 0.029    | 0.006      |
| Time $\times$ N     | 2  | 0.002    | 0.040      | 4  | 1.135 *** | 0.512 ***  |
| Time $\times$ $p_{\text{CO}_2} \times$ N | 2  | 0.019    | 0.015      | 4  | 0.083    | 0.007      |
| Replication        | 2  | 4.160 *** | 3.468 ***  | 2  | 3.773 *** | 3.935 ***  |
| Error              | 106| 0.050    | 0.024      | 146| 0.050    | 0.039      |

Table 3-1. Statistical analysis of total plant dry mass (DM) and tiller number and their development over time. The “Time” treatment tests whether the mean relative growth rate for a given parameter was different from zero over the time period indicated. Treatment effects on the relative growth rates appear as interactions with the “Time” treatment. Significant effects are indicated as follows: ***, $p \leq 0.001$. 
relative size differences remained unchanged (pseudo-stems) or even decreased (roots). A stimulation of initial leaf growth at elevated $p_{\text{CO}_2}$ (Fig. 3-1b,e; days 0–2) was no longer apparent when leaf growth was expressed relative to plant size (not shown). Relative differences between $p_{\text{CO}_2}$ treatments in both leaf dry mass and leaf area remained unchanged from day 2 throughout to the end of the experiment (Fig. 3-1b,e).

3.4.3 Morphology

3.4.3.1 Tillers

Tiller numbers were increased at elevated $p_{\text{CO}_2}$ relative to ambient $p_{\text{CO}_2}$ from the beginning of the experiment (Fig. 3-1f; $p_{\text{CO}_2}$ effect significant, Tab. 3-1). However, relative tillering rate was no longer affected by $p_{\text{CO}_2}$ and relative differences in tiller number remained therefore similar throughout the experiment (Time $\times$ $p_{\text{CO}_2}$ n.s., Tab. 3-1). Tillering proceeded rapidly (in the range of 0.1 d$^{-1}$ at high N supply) during the first 11 d after defoliation and only a transient depression in the rate of tiller production was apparent.

Table 3-2. Tiller size and structure averaged over days 11–28. Values with common letters are not significantly different at $p \leq 0.05$ (multiple t-tests based on standard errors of least-squares means).

<table>
<thead>
<tr>
<th>n</th>
<th>Quantity per tiller</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>High N supply</td>
<td></td>
<td>Low N supply</td>
</tr>
<tr>
<td></td>
<td>Ambient $p_{\text{CO}_2}$</td>
<td>Elevated $p_{\text{CO}_2}$</td>
<td>Ambient $p_{\text{CO}_2}$</td>
</tr>
<tr>
<td>Shoot DM (mg)</td>
<td>24</td>
<td>74.1$^b$</td>
<td>109.0$^c$</td>
</tr>
<tr>
<td>Root DM (mg)</td>
<td>24</td>
<td>20.4$^a$</td>
<td>27.7$^b$</td>
</tr>
<tr>
<td>Blades of growing leaves; DM, corrected for WSC (mg)</td>
<td>18</td>
<td>9.5$^a$</td>
<td>11.7$^b$</td>
</tr>
<tr>
<td>Blades of first fully grown leaves; DM, corrected for WSC (mg)</td>
<td>18</td>
<td>14.5$^b$</td>
<td>18.2$^c$</td>
</tr>
<tr>
<td>Pseudo-stem DM, corrected for WSC (mg)</td>
<td>12</td>
<td>16.6$^a$</td>
<td>24.6$^b$</td>
</tr>
<tr>
<td>Leaf area (cm$^2$)</td>
<td>24</td>
<td>5.87$^b$</td>
<td>7.12$^c$</td>
</tr>
</tbody>
</table>

relative size differences remained unchanged (pseudo-stems) or even decreased (roots). A stimulation of initial leaf growth at elevated $p_{\text{CO}_2}$ (Fig. 3-1b,e; days 0–2) was no longer apparent when leaf growth was expressed relative to plant size (not shown). Relative differences between $p_{\text{CO}_2}$ treatments in both leaf dry mass and leaf area remained unchanged from day 2 throughout to the end of the experiment (Fig. 3-1b,e).

Figure 3-1 (overleaf). Development of (a) total plant, (b) total leaf blade, (c) pseudo-stem and (d) root dry mass as well as (e) total leaf area and (f) tiller number in L. perenne. Plants were exposed to two $p_{\text{CO}_2}$ treatments ($\triangle\square$: ambient $p_{\text{CO}_2}$; $\blacktriangle\blacksquare$: elevated $p_{\text{CO}_2}$) and two N treatments ($\triangle\blacktriangle$: low N supply; $\square\blacksquare$: high N supply). Error bars indicate the standard error of means for each harvest with n=6 (n=12 for days 0, 4 and 17). For total plant and total leaf dry mass, values for intact plants before defoliation (b. d.) are shown (n=6).
Days after defoliation

**Total DM (g)**

- **Leaf DM (g)**
- **Pseudo-stem DM (g)**

*SE*
between days 2 and 4 after defoliation (Fig. 3-1f). Afterwards, rates of tiller production declined.

An effect of N supply on the number of tillers per plant was already apparent from the beginning (Fig. 3-1f; N effect significant, Tab. 3-1) and increased further during regrowth (Time × N significant for days 4–28, Tab. 3-1). Early effects of N supply were evident also for leaf and pseudo-stem dry mass. In contrast, roots showed an initial tendency towards a negative response to N supply; thus effects of N supply on total plant dry mass did not become apparent until more than a week after defoliation (Fig. 3-1a,d).

The larger size of plants at elevated $p_{\text{CO}_2}$ was mostly due to a higher dry mass per tiller (much greater relative effect of elevated $p_{\text{CO}_2}$ on total dry mass in Fig. 3-1a than on tiller number in Fig. 3-1f). While dry mass per tiller kept increasing with time in all treatments (faster increase with time of total plant dry mass in Fig. 3-1a than of tiller number in Fig. 3-1f), the relative effects of N and $p_{\text{CO}_2}$ treatments did not differ significantly before defoliation and during later regrowth. Averaged over days 11 to 28, dry mass per tiller was 33 % higher at elevated $p_{\text{CO}_2}$ (Tab. 3-2). This effect of elevated $p_{\text{CO}_2}$ was evident for both shoot and roots. It was not solely a consequence of WSC accumulation since a significant difference remained after correction for WSC, as shown for growing and first fully grown leaves as well as for pseudo-stems (Tab. 3-2). An increase in leaf area per tiller was apparent only at high N supply.

### 3.4.3.2 Dry matter partitioning

The root:shoot ratio was initially larger at elevated than at ambient $p_{\text{CO}_2}$ (on average 0.41 vs. 0.27, Fig. 3-2a). Due to a uniform cutting height of 4.5 cm and the greater size of plants at elevated $p_{\text{CO}_2}$, a larger fraction of the leaf dry matter was removed at elevated (89 %) than at ambient (75 %) $p_{\text{CO}_2}$. Thus, the $p_{\text{CO}_2}$-dependent difference in root:shoot ratio was increased by defoliation (Fig. 3-2a). However, the effect of $p_{\text{CO}_2}$ on root:shoot ratio disappeared rapidly within 4 d (HN) to 11 d (LN) after defoliation. Thereafter, the relative size of the root system was no longer influenced by the $p_{\text{CO}_2}$ treatments, both when compared at a given time or at a given shoot dry mass (Fig. 3-2a,d). Allometric differences were mainly due to N supply; they were established soon after defoliation and kept increasing with plant size.

The leaf weight ratio (LWR, the ratio of leaf blade to total plant dry mass) was greatly reduced by defoliation. Again, this reduction was more pronounced at elevated $p_{\text{CO}_2}$ than at ambient $p_{\text{CO}_2}$ due to the removal of a larger proportion of leaf tissue. Consequently,

**Figure 3-2 (right page).** Time course of the ratio of (a) root to shoot dry mass (R:S), (b) total leaf blade to total plant dry mass (LWR) and (c) total leaf area to total plant dry mass (LAR) in *L. perenne*. Allometric plots show (d) root vs. shoot dry mass for days 11-28 and (e) total leaf area vs. total plant dry mass for days 11-28. Plants were exposed to two $p_{\text{CO}_2}$ treatments (□: ambient $p_{\text{CO}_2}$; ■: elevated $p_{\text{CO}_2}$) and two N treatments (▲: low N supply; Δ: high N supply). Error bars indicate the standard error of means for each harvest with n=6 (n=12 for days 0, 4 and 17). For LWR and R:S, values for intact plants before defoliation (b. d.) are shown (n=6).
Shoot DM (g) 0.2 0.5 2 5 1
Root DM (g) 0.5 2 5 1
Leaf weight ratio 0.2 0.5 0.1
Days after defoliation 0 2 4 7 11 17 28
Leaf area ratio (cm$^2$ g$^{-1}$) 20 50 10 100
Root to shoot ratio 0.2 0.5 2 1
Total DM (g) 25 2 0 11 0
Leaf area (cm$^2$) 50 200 500 1000

(a) Root to shoot ratio
(b) Leaf weight ratio
(c) Leaf area ratio (cm$^2$ g$^{-1}$)
(d) Root DM (g) vs. Shoot DM (g)
(e) Leaf area (cm$^2$) vs. Total DM (g)
LWR was initially lower in plants growing under elevated $p_{CO_2}$ (Fig. 3-2b). LWR increased steadily during approximately 11 d after defoliation and approached the initial values at high but not at low N supply. These defoliation-induced differences were balanced after 4 (HN) to 11 (LN) days of regrowth when root:shoot ratios became similar between $p_{CO_2}$ treatments.

In contrast, after defoliation, LAR was always significantly lower in plants growing under elevated $p_{CO_2}$ when compared to plants growing under ambient $p_{CO_2}$ (Fig. 3-2c). LAR increased much faster than LWR and near maximal values were reached about 4 to 7 d after defoliation in all treatments. Subsequently, LAR decreased steadily in all treatments. Much higher values were reached and maintained at high N supply than at low N supply. It might be possible that the difference in LAR between plants of the same age exposed to contrasting $p_{CO_2}$ treatments was due to ontogenetic drift: Plants growing under elevated $p_{CO_2}$ were larger than their counterparts growing under ambient $p_{CO_2}$ (Fig. 3-

Figure 3-3. Specific leaf weight (SLW) of first fully grown leaves of *L. perenne* harvested in the morning (total height of bar) and its constituents: Water-soluble carbohydrates (WSC; open) and structure (SLW corrected for WSC; hatched). For days 7-28, structure is further divided into crude protein (N concentration, corrected for nitrate and ammonium and multiplied by 6.25; dense hatching) and others (light hatching). Plants were exposed to two $p_{CO_2}$ treatments (△ □: ambient $p_{CO_2}$; ■ ■: elevated $p_{CO_2}$) and two N treatments (△ ▲: low N supply; □ ■: high N supply). For each harvest, standard errors of means SLW (n=6), for WSC (n=3) and for leaf structure (n=3) are indicated at the top; standard errors of means for crude protein (n=3) are indicated to the right of each set of bars where appropriate.
LAR decreased during regrowth and thus with increasing plant size (Fig. 3-2c). However, an allometric plot of leaf area versus total plant dry mass for days 11–28 (Fig. 3-2e) demonstrates that for a given plant dry mass, plants growing under elevated $p_{\text{CO}_2}$ had about 10% less leaf area than plants growing under ambient $p_{\text{CO}_2}$, irrespective of N supply.

### 3.4.4 Components of specific leaf weight and maximum carboxylation velocity

Differences between the responses of LWR and LAR to elevated $p_{\text{CO}_2}$ suggest an alteration of leaf composition. Losses of WSC after defoliation and accumulation after apparent recovery (about one week after defoliation) accounted for most of the changes over time in the specific leaf weight (SLW, leaf dry mass per leaf area) of first fully grown leaves (Fig. 3-3) and growing leaves (not shown). In contrast, changes in the amount of structural leaf material per unit leaf area were small and only a transient decrease was observed after defoliation. Treatment effects on SLW were also largely due to differences in the amount of WSC per unit leaf area. Only at the end of the experiment was there a significantly higher area density of structural leaf material at low N supply. However, the composition of structural leaf dry matter changed significantly: plants growing at low N supply had a significantly lower estimated protein content per unit leaf area than those at high supply (Fig. 3-3). Further, a significantly lower estimated protein content per unit area in plants growing under elevated relative to ambient $p_{\text{CO}_2}$ was evident on days 11 and 28 after defoliation.

Leaf N content is closely linked to leaf photosynthetic capacity (Field and Mooney 1986). Indeed, $V_{\text{c max}}$ was lower at low than at high N supply both one and four weeks after defoliation (N effect in Tab. 3-3, Time × N n.s.). In contrast, photosynthetic acclimation to elevated $p_{\text{CO}_2}$ was not yet evident one week after defoliation, but became manifest during later regrowth ($p_{\text{CO}_2}$ effect, Time × $p_{\text{CO}_2}$ in Tab. 3-3). By the end of the experiment, photosynthetic capacity in all treatments was considerably lower than after defoliation.

<table>
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<th>Source</th>
<th>DF</th>
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<tbody>
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<td>$p_{\text{CO}_2}$</td>
<td>1</td>
<td>3102</td>
<td>*</td>
</tr>
<tr>
<td>N</td>
<td>1</td>
<td>1953</td>
<td>*</td>
</tr>
<tr>
<td>$p_{\text{CO}_2} \times N$</td>
<td>1</td>
<td>33</td>
<td></td>
</tr>
<tr>
<td>Time</td>
<td>1</td>
<td>8204</td>
<td>***</td>
</tr>
<tr>
<td>Time × $p_{\text{CO}_2}$</td>
<td>1</td>
<td>1635</td>
<td>*</td>
</tr>
<tr>
<td>Time × N</td>
<td>1</td>
<td>57</td>
<td></td>
</tr>
<tr>
<td>Time × $p_{\text{CO}_2} \times N$</td>
<td>1</td>
<td>85</td>
<td></td>
</tr>
<tr>
<td>Error</td>
<td>11</td>
<td>303</td>
<td></td>
</tr>
</tbody>
</table>

**Table 3-3.** Analysis of variance of the maximum carboxylation velocity ($V_{\text{c max}}$) of first fully grown leaves of *L. perenne*. Significant effects are indicated as follows: *, $p \leq 0.05$; ***, $p \leq 0.001$. 
the first week of regrowth (Time effect in Tab. 3-3). While reliable estimates of the maximal rate of electron transport (J_{\text{max}}), could not be obtained, plots of A vs. C suggested that assimilation rates at high C were also reduced in plants growing at elevated p_{\text{co2}} relative to plants growing at ambient p_{\text{co2}} (not shown).

3.4.5 Water-soluble carbohydrate (WSC) concentrations

Initial WSC concentrations measured in the morning in both leaves and pseudo-stems were higher at low than at high N supply (Fig. 3-4). They also tended to be lower at elevated than at ambient p_{\text{co2}}, but this effect was significant only for pseudo-stems (Fig. 3-4c). Leaf and pseudo-stem WSC concentrations decreased rapidly after defoliation. There were no significant treatment effects on the initial rate of WSC loss. The total decrease in WSC concentration was similar in three of the four treatments and a minimum in the range of 0.5 to 1.5 mol kg\(^{-1}\) was reached about 4 d after defoliation. In contrast, the total decrease was smaller and the minimum was reached earlier in the one treatment at elevated p_{\text{co2}} with high N supply. However, this treatment also had the lowest leaf WSC concentrations before defoliation.

Less than one week after defoliation, WSC concentrations began to rise again. WSC concentrations were significantly higher at elevated than at ambient p_{\text{co2}} from day 11 and 17 after defoliation in leaves and pseudo-stems, respectively (Fig. 3-4). Higher WSC concentrations at low than at high N supply were evident almost throughout the experiment both in leaves and in pseudo-stems. In first fully grown leaves, this N effect was most pronounced at ambient p_{\text{co2}} at the last two harvests with a similar tendency being apparent from day 4 after defoliation (Fig. 3-4b). Such a CO\(_2\) × N interaction was absent in growing leaves and pseudo-stems.

3.5 Discussion

For the first time, carbohydrate and growth dynamics have been followed simultaneously during regrowth after defoliation at contrasting p_{\text{co2}}. There was a pronounced relative growth response to elevated p_{\text{co2}} during early development in L. perenne; however, regrowth of plants defoliated 45 d after sowing was not enhanced by elevated p_{\text{co2}}. Both RGR and relative tillering rate were unaffected by p_{\text{co2}}. Moreover, growth under elevated p_{\text{co2}} reduced LAR, N concentration per unit leaf area as well as Vc_{\max} when compared to ambient p_{\text{co2}}; plants at elevated p_{\text{co2}} accumulated higher concentrations of WSC after recovery from defoliation.

Figure 3-4 (right page). Time course of morning water-soluble carbohydrate (WSC) concentrations in blades of (a) growing and (b) first fully grown leaves and in (c) pseudo-stems of L. perenne. Plants were exposed to two p_{\text{co2}} treatments (△□: ambient p_{\text{co2}}; ▲■: elevated p_{\text{co2}}) and two N treatments (△▲: low N supply; □■: high N supply). WSC concentrations are expressed as moles of hexose equivalents per unit dry weight. Error bars indicate the standard error of means for each harvest with n=3 (n=6 for pseudo-stems at harvests 0, 4 and 17).
Carbohydrate concentration as hexose equivalents (mol kg\(^{-1}\))

Days after defoliation

Carbohydrate concentration as hexose equivalents per unit structural DM (mol kg\(^{-1}\))

Pseudo-stems

First fully grown leaves

Growing leaves

Growing leaves

Growing leaves

Growing leaves
3.5.1 Stimulation of seedling growth by elevated $p_{\text{CO}_2}$ in L. perenne

Large differences in plant size were evident after 45 d of growth under elevated compared to ambient $p_{\text{CO}_2}$ (Fig. 3-1). Loehle (1995) proposed that a temporary stimulation of growth at the beginning of experiments might be due largely to the accumulation of non-structural carbohydrate. However, in the present study, initial carbohydrate concentrations tended to be lower at elevated $p_{\text{CO}_2}$ (Fig. 3-4) and therefore a true acceleration of seedling growth must be postulated. While leaf carbohydrate concentrations are typically increased at elevated $p_{\text{CO}_2}$ (Farrar and Williams 1991), a pronounced decrease has also been observed in seedlings of Agrostis capillaris (Baxter et al. 1995) together with a strong relative growth response to elevated $p_{\text{CO}_2}$ (Baxter et al. 1994). A positive growth response to elevated $p_{\text{CO}_2}$ has also been observed in young, uncut plants of L. perenne grown either singly (Marks and Clay 1990; Ryle et al. 1992a; Roumet et al. 1996) or in swards (Schenk et al. 1995; Schenk et al. 1996). I concluded that there exists a potential for a growth response to elevated $p_{\text{CO}_2}$ in L. perenne and that growth was apparently $C$ limited during early development. Therefore, I expected a similar stimulation of growth during an initial period after defoliation when $C$ availability would be low due to the loss of most leaf material.

3.5.2 Initial regrowth after defoliation not accelerated at elevated $p_{\text{CO}_2}$

Defoliation was performed at a uniform height above ground, as it is typical for agricultural practice. However, a greater proportion of leaf dry matter was removed in the initially taller plants growing under elevated $p_{\text{CO}_2}$ leading to a smaller residual leaf area than in the shorter plants at ambient $p_{\text{CO}_2}$; the pre-defoliation differences in root:shoot ratio were therefore further enhanced. Hence, the conditions immediately after defoliation were somewhat different from those expected in a field situation: swards have been found to adjust the length of their leaf sheaths to the prevailing cutting height (Davies 1988). However, unlike an established sward, single young plants are amenable to a complete growth analysis including allometric relationships. Further, the chosen approach of exposing plants to the treatments prior to defoliation has the advantage of avoiding the confounding of the response to defoliation with the acclimation to new treatments.

By comparison of the present results to those from a field experiment using free air $\text{CO}_2$ enrichment (FACE) technology (Part 2), the relevance of the conclusions drawn from this experiment may be assessed. Basic carbohydrate patterns and effects of $p_{\text{CO}_2}$ were similar in this study as in the field: While both leaf carbohydrate concentrations and their decrease after defoliation (Fig. 3-4) were roughly similar to Experiment I of the field study (Fig. 2-1a), the decrease in the concentrations both in leaves and pseudostems was more pronounced than in Experiment II of the field study (Figs 2-3 & 2-4). However, the effect of defoliation on carbohydrate reserves in pseudo-stems was quali-
tatively similar to the response observed in other controlled environment chamber and greenhouse experiments (e.g. Sullivan and Sprague 1943; Davies 1965; Gonzales et al. 1989; Prud'homme et al. 1992). Further, carbohydrate concentrations in pseudo-stems and treatment effects similar to those found during later regrowth in this experiment (Fig. 3-4c) were also found in the field (Fig. 2-4).

Carbon availability was severely reduced during initial regrowth (Fig. 3-4, days 0–7 after defoliation) and dry matter partitioning was apparently shifted to favour leaf growth (Fig. 3-1b,c,d). In spite of the removal of a large majority of all leaf material, however, the plants kept increasing in total dry weight (Fig. 3-1a; Time effect significant for days 0–4 in Tab. 3-1). Thus, they had a positive net C balance at all times. A contribution to current C assimilation by green leaf sheaths (Davies et al. 1983) may have led to a smaller impact of defoliation on whole plant photosynthesis than on leaf dry mass.

Apparently, leaf growth proceeded in proportion to plant size, and a larger leaf area per plant was attained at elevated $p_{\text{CO}_2}$ within 2 d after defoliation. Further, rates of photosynthesis per unit leaf area are generally increased at elevated $p_{\text{CO}_2}$ (Long et al. 1995; and as observed e.g. in Nijs et al. 1989; Ryle et al. 1992). Thus, assimilate supply in the plants growing under elevated $p_{\text{CO}_2}$ likely exceeded that under ambient $p_{\text{CO}_2}$ shortly after defoliation. Nevertheless, there was no stimulation of leaf growth in relation to plant size at elevated $p_{\text{CO}_2}$ (similar relative growth rate after day 2, Fig. 3-1b,e), and the growth of pseudo-stems and roots resumed after a similar lag in both $p_{\text{CO}_2}$ treatments (Fig. 3-1c,d). Could it be that regrowth was not carbon limited?

While the remobilisation of nitrogenous compounds in addition to carbohydrates after defoliation has been firmly established, little is known about the effect of N reserves on the rates of regrowth (Volenc et al. 1996). Nitrate uptake in defoliated L. perenne has been shown to depend on the nutrient status of the plants (Macduff et al. 1989) and nitrate reductase activity in leaves was found to increase after defoliation (Boucaud and Bigot 1989). Further, the expression of nitrate reductase has been shown to be inducible by carbohydrate (see Koch 1996). Thus, N reduction should not be limiting growth as long as mineral N and energy in the form of carbohydrate are available. Nevertheless, it cannot be entirely excluded that regrowth was limited by a shortage of reduced N, not even at high N supply.

Even though carbohydrate concentrations in pseudo-stems were lower at elevated than at ambient $p_{\text{CO}_2}$, all concentrations were well above the value of approx. 15% (corresponding to about 0.9 mol kg$^{-1}$) judged as non-limiting for the production of new regrowth in L. perenne (Davies et al. 1972; Davies 1988). The halt of net growth of lower priority sinks like pseudo-stems and roots (Fig. 3-1c,d) does not necessarily imply that C availability was limiting for leaf growth, as leaves are supplied with high priority after defoliation (Richards 1993). Further, it has been proposed that leaf area expansion can often be regarded as “sink limited”, as it is to some extent independent of leaf growth in terms of dry matter accumulation (van Loo 1993). In fact, recent evidence suggests that the C requirements for leaf area growth can be greatly reduced after defo-
iliation in *L. perenne*, allowing for an efficient restoration of assimilatory surface even if C availability is very low (de Visser *et al.* 1997). A transient reduction of the amount of structural dry matter invested per unit leaf area was also apparent in the present study (Fig. 3-3, day 2). Therefore, the regrowth of leaf area – and possibly also leaf dry matter – was probably not carbon limited in this experiment.

However, an explanation is still needed why the accumulation of dry matter in pseudo-stems and roots was not stimulated at elevated $p_{CO_2}$. Leaf carbohydrate concentrations initially decreased at similar rates (Fig. 3-4a,b) in both $p_{CO_2}$ treatments. Such similarities suggest that the decrease in the ratio of C supply and demand was alike both in extent and in duration between $P_{CO_2}$ treatments, in spite of the greater leaf area at elevated $P_{CO_2}$ from day 2 after defoliation. What could be the reason for such a response?

Differences between $P_{CO_2}$ treatments in LWR persisted beyond day 2 after defoliation (Fig. 3-2b). They were mainly due to a larger root system in the plants growing at elevated $p_{CO_2}$ (Fig. 3-1d), the carbohydrate requirements of which may have matched the increase in C assimilation at elevated $p_{CO_2}$. This could explain why, initially, the carbohydrate availability at elevated $p_{CO_2}$ apparently remained below that at ambient $p_{CO_2}$ (Fig. 3-4) thus preventing a faster accumulation of dry matter during early regrowth.

Recovery from defoliation was indicated by the resumption of pseudo-stem and root growth as well as by the attainment of maximal values of LAR around days 4 to 7 in each treatment (Fig. 3-2c). At the same time, the beginning of carbohydrate accumulation in pseudo-stems (Fig. 3-4) also suggested that carbohydrate availability started to exceed the immediate needs of the plant. Thus, in spite of a high percentage of leaf area removed, plants recovered very rapidly. Similarly, *L. perenne* in hydroponic culture was found to obtain the majority of the C incorporated into newly formed leaf tissue from current photosynthesis 3 d after defoliation (de Visser *et al.* 1997).

3.5.3 Similar relative growth rates after recovery from defoliation

3.5.3.1 Differences in plant size dominated by early effects of elevated $p_{CO_2}$

While recovery from defoliation was apparently not accelerated in the present experiment, further growth might still have been enhanced at elevated $p_{CO_2}$ – particularly, as the plants used were relatively small and grew singly in spaced pots so that competition for light and self-shading were minimal and growth after recovery was almost exponential (Fig. 3-1a). The growth response to elevated $p_{CO_2}$ has been suggested to be much more pronounced in expanding than in steady-state systems in a comparison of single plants in artificial gaps to plants in closed canopies in the field (Lüscher *et al.* 1996). Nevertheless, whole plant RGR was similar between $p_{CO_2}$ treatments throughout the experiment (Tab. 3-1, $Time \times P_{CO_2}$ n.s.; Fig. 3-1a). The response of RGR to elevated $p_{CO_2}$ has often been limited to seedling development (e.g. Ryle *et al.* 1992a), a developmental
stage during which plants may be generally more responsive to any type of resource enrichment (Baker and Enoch 1983; Loehle 1995), or to the initial phase of experiments (e.g. Hardacre (1979) cited in Newton 1991; den Hertog et al. 1996; and as reviewed in Poorter 1993; Poorter et al. 1996). From the definition of RGR it follows that a relative difference in plant biomass established during early development at elevated $p_{\text{CO}_2}$ will persist throughout further growth unless RGR decreases below that at ambient $p_{\text{CO}_2}$. Such a response has also been observed in *L. perenne* (Ryle et al. 1992a). In fact, an enhanced yield after regrowth under elevated $p_{\text{CO}_2}$ in *L. perenne* as reported in several studies (Hardacre et al. 1986; Nijs et al. 1988b; Schenk et al. 1996) may be the consequence of an initial size advantage due to a larger residual biomass (as also observed in Sæbø and Mortensen 1995; Schenk et al. 1996) without any true growth stimulation after defoliation, as demonstrated in the present study.

### 3.5.3.2 Greater availability of carbohydrates for storage

Even though there was no relative growth response to elevated $p_{\text{CO}_2}$, a more pronounced accumulation of non-structural carbohydrate both in source and storage tissues (Fig. 3-4) suggests that the availability of carbohydrate was greatly increased at elevated $p_{\text{CO}_2}$ during later regrowth. Carbon supply may have exceeded the demand for structural growth and the needs for respiration, and thus more carbohydrate was partitioned to storage. The higher concentration of carbohydrate reserves in plants exposed to elevated $p_{\text{CO}_2}$ may allow for an enhanced tolerance to subsequent defoliation or similar stresses.

As C export from leaves does not appear to be limited by the capacity for translocation (Wardlaw 1990; Hibberd et al. 1996), the accumulation of carbohydrate in the source presumably reflected an imbalance between assimilate supply and demand. This interpretation is supported by the effect of low N supply, which also led to reduced growth and increased carbohydrate accumulation. However, the stronger effect of $p_{\text{CO}_2}$ on carbohydrate accumulation in first fully grown leaves at high N supply when compared to that at low N supply does not fit this interpretation. At high N supply, a more positive relative growth response to elevated $p_{\text{CO}_2}$ (Schenk et al., 1996; and as reviewed by Conroy and Hocking, 1993; Poorter et al., 1996) and thus a higher increase in demand for carbohydrate is expected than at low N supply (Rogers et al. 1996). This should lead to a more pronounced carbohydrate accumulation in response to elevated $p_{\text{CO}_2}$ at low rather than at high N supply. However, it is conceivable that near maximal carbohydrate concentrations were already reached at low N supply combined with ambient $p_{\text{CO}_2}$ and no significant further increase was possible at elevated $p_{\text{CO}_2}$.

Which factors could have been responsible for the observed lack of a relative growth response in *L. perenne* and the apparent sink limitation of carbohydrate utilisation?
3.5.3.3 No stimulation of tiller production in spite of higher carbohydrate availability

Total plant RGR is the sum of relative tillering rate and the relative growth rate of mean tiller mass (van Loo 1992). While the average dry mass per tiller in *L. perenne* kept increasing until the end of the experiment, the relative effect of elevated $p_{\text{CO}_2}$ on the average tiller dry mass was similar before defoliation and at the end of the experiment (compare Fig. 3-1a,f). Thus, a higher relative tillering rate would have been required for an enhanced RGR at elevated $p_{\text{CO}_2}$. While elevated $p_{\text{CO}_2}$ had apparently stimulated tillering during early plant development, an increased relative tillering rate was no longer sustained by elevated $P_{\text{CO}_2}$ after defoliation (Fig. 3-1f; Tab. 3-1). Instead, not only the concentration of non-structural carbohydrate but also the structural dry mass per tiller was increased at elevated $P_{\text{CO}_2}$ (Tab. 3-2). Since the size distribution of tillers was not determined in this experiment, it is not possible to distinguish whether these effects were due to an increase in the size of fully grown tillers, or whether the fraction of small, newly formed, tillers was smaller. However, for both cases it can be concluded that *L. perenne* failed to invest any additionally available C at elevated $P_{\text{CO}_2}$ into the production of additional growing points during regrowth. This is quite surprising because the rate of tiller production appeared transiently depressed after defoliation (Fig. 3-1f, days 2–4), suggesting that assimilates may have been limiting. Indeed, the size of the carbohydrate pool has been successfully included as a determinant of tiller production in modelling the regrowth of spaced *L. perenne* plants after defoliation (van Loo 1993) and a higher tiller number per plant was found in seedlings grown at elevated $P_{\text{CO}_2}$ for 45 days in the present study. A stimulation of tiller production at elevated $P_{\text{CO}_2}$ has also been shown e.g. in single seedlings of wheat (Balaguer *et al.* 1995; Christ and Körner 1995) and rice (Seneweera *et al.* 1994), supporting that tiller production in grasses can be responsive to assimilate availability. However, no stimulation of tiller production at elevated $P_{\text{CO}_2}$ was observed in a study on single, young plants of *L. perenne* (Ryle *et al.* 1992a).

It is well known that tillering is reduced in *L. perenne* if low ratios of red to far-red light indicate the presence of neighbouring plants (Deregibus *et al.* 1983). However, at least during the first few weeks of regrowth in this experiment, such an effect was probably not very important as plants were grown singly in pots with the distance between pots increasing with plant size (initial density 85 pots m$^{-2}$; final density 15 pots m$^{-2}$) and as the light in the growth chamber had a much higher ratio of red to far-red light (2.1 as determined by Lötscher 1994) than sunlight (1.2 according to Casal *et al.* 1990). Therefore, it appears that the rate of tiller production was determined by nutrient availability rather than by C supply, since there was a significant stimulation of tillering rate in the high N treatment compared to the low N treatment. At high N supply, rates of tiller production up to day 11 in both $P_{\text{CO}_2}$ treatments approached the value of 0.1 d$^{-1}$ considered as maximal for *L. perenne* by van Loo (1993) based on a leaf appearance rate of 0.14 d$^{-1}$. Unfortunately, leaf appearance rate was not determined in this study. However, leaf appearance rates as high as 0.19 d$^{-1}$ (allowing for a maximal relative tillering rate of
0.13 d\(^{-1}\) according to Neuteboom and Lantinga (1989) have been reported for some genotypes of *L. perenne* during regrowth in summer in the greenhouse (Davies 1974).

In some, but not all, studies with *L. perenne* grown in swards, a higher tiller density was found at elevated \(p_{\text{CO}_2}\), sometimes depending on season and/or temperature treatments (Soussana *et al.* 1994; Sebø and Mortensen 1995) or N supply (Soussana *et al.* 1994; Schenk *et al.* 1996). However, effects of \(p_{\text{CO}_2}\) on tiller density in swards cannot be directly compared to effects on the relative rate of tiller production as it is the result of the equilibrium between tiller production and tiller death. Further, tiller production in swards is strongly influenced by neighbouring plants through light quality as discussed above.

Only at high N supply was the higher dry matter accumulation per tiller at elevated \(p_{\text{CO}_2}\) reflected in a higher leaf area per tiller (Tab. 3-2). Together with the higher photosynthetic capacity in the high N treatment, this greater difference in leaf area per tiller may have contributed to the larger effect of elevated \(p_{\text{CO}_2}\) on carbohydrate concentrations in first fully grown leaves at high compared to low N supply (Fig. 3-4b).

### 3.5.4 Maintaining balanced source and sink activities at elevated \(p_{\text{CO}_2}\)

#### 3.5.4.1 Unchanged dry matter partitioning to root and shoot

How did the plants balance their C budget given that the initiation of new sinks was not accelerated at elevated \(p_{\text{CO}_2}\) in this experiment? It has often been proposed that the partitioning of biomass between shoot and root is such as to maintain a functional balance between shoot and root activities, i.e. carbon and nutrient uptake (Brouwer 1962; Davidson 1969). At elevated \(p_{\text{CO}_2}\), photosynthesis and thus the productivity of the shoot is increased. As discussed in Hilbert and Reynolds (1991), a stable plant N concentration can only be maintained during growth if a functional balance of root and shoot is restored. The plant can (i) increase its rate of nutrient uptake which requires an increase in the dry matter partitioning towards the root unless the specific absorption rate for N can be increased. Alternatively (ii), the amount of N needed in the shoot can be reduced at the expense of the photosynthetic efficiency of the shoot biomass, either by a down-regulation of photosynthesis per unit leaf area or by a reduction of nitrogen-costly assimilatory surface (i.e. a reduction of LAR). In case (i), whole plant RGR is likely to increase in response to elevated \(p_{\text{CO}_2}\); however, in case (ii) growth will not be stimulated if a reduction of shoot photosynthetic surface and capacity fully counterbalances the stimulatory effect of elevated \(p_{\text{CO}_2}\) on photosynthesis (den Hertog *et al.* 1996).

A \(p_{\text{CO}_2}\)-independent allometric relationship for root vs. shoot dry mass (Fig. 3-2d) demonstrates that dry matter partitioning between roots and shoot was not altered by elevated \(p_{\text{CO}_2}\) in *L. perenne* after plants had recovered from defoliation. Similar observations of mostly unchanged dry matter partitioning to shoot and roots have been made in seedlings of different grass species (Bowler and Press 1993; Baxter *et al.* 1994; Hunt *et
al. 1995) even though RGR was enhanced in several of these experiments. In fact, even an increased partitioning of new growth to the shoot was observed in young, hydroponically grown plants of *Dactylis glomerata* exposed to elevated $p_{CO_2}$ (Gunn *et al.* 1996). In contrast, a higher proportion of new growth was found to be allocated to roots at elevated $p_{CO_2}$ in uncut plants of *L. perenne* grown in soil at ambient temperature, though no such difference was found at an elevated temperature (Nijs and Impens 1997). Contrasting observations even within the same species emphasise that different strategies may be employed to balance root and shoot activity, possibly depending on environmental conditions and the history of a plant. It has been postulated that elevated $p_{CO_2}$ only induces a change in dry matter partitioning between roots and shoot if growth is limited by nutrient supply (Stulen and Den Hertog 1993). However, higher relative rates of nutrient uptake and thus an enhanced partitioning of new growth to roots may not be required if the stimulation of photosynthesis is offset by a down-regulation of photosynthesis or a reduction of LAR. Further, it has been suggested that partitioning to roots versus the shoot responds primarily to the availability of N to the roots rather than to the C:N ratio in some part of the plant (Fichtner *et al.* 1993; Beck 1994).

### 3.5.4.2 Adjustments at the leaf level to both elevated $p_{CO_2}$ and low N supply

As discussed above, instead of increasing the rate of C utilisation by sinks, an alternative strategy to maintain balanced source sink relations at elevated $p_{CO_2}$ is to reduce the production of assimilates by the source. Differences in LWR between $p_{CO_2}$ treatments declined rapidly after defoliation and subsequently a similar proportion of total plant dry mass was partitioned to leaves in both $p_{CO_2}$ treatments (Fig. 3-2b). However, at elevated $p_{CO_2}$, specific leaf weight was significantly higher (Fig. 3-3). Increases in specific leaf weight have been attributed both to changes in leaf anatomy and to an accumulation of non-structural carbohydrate (Acock and Pasternak 1986; Vu *et al.* 1989). Major alterations of *L. perenne* leaf morphology due to elevated $p_{CO_2}$ are unlikely since almost all of the difference in SLW was accounted for by a massive accumulation of WSC (Fig. 3-3). Thus, a greater proportion of the dry matter partitioned to leaves accumulated as WSC (Fig. 3-3) and less as structural material. As a consequence, LAR was significantly reduced at elevated $p_{CO_2}$ (Fig. 3-2c). A reduced LAR in spite of a similar allocation of dry matter to leaves is a typical response to elevated $p_{CO_2}$ which has also been observed in many other studies with different species (Poorter *et al.* 1996). Further, N area density was reduced at elevated relative to ambient $p_{CO_2}$ (Fig. 3-3), possibly indicating an allocation of N resources away from leaves. Similar reductions in the N concentration per unit leaf area in *L. perenne* have also been observed in other studies (Nijs *et al.* 1995; Soussana *et al.* 1996). Such a response to elevated $p_{CO_2}$ is to be expected according to resource optimisation models when the relative availability of N to that of C is reduced (Hilbert *et al.* 1991; Woodrow 1994). Since a large fraction of leaf N is typically bound in ribulose-1,5-bisphosphate carboxylase oxygenase (RuBisCO), the primary enzyme of photosynthesis, a reduced N allocation to leaves typically in-
volves a noticeable withdrawal of N from RuBisCO, although other components of the photosynthetic apparatus can also be reduced (Long et al. 1995; Nijs et al. 1995). This phenomenon is known as photosynthetic acclimation. Indeed, a reduced $V_{\text{c, max}}$ was apparent at low N availability throughout and also became evident at elevated $p_{\text{CO}_2}$ towards the end of the experiment (Tab. 3-3). In addition, reduced assimilation rates were apparent in plants grown at elevated $p_{\text{CO}_2}$ even for high values of $C_r$. The reduction in $V_{\text{c, max}}$ is in accordance with measurements of $V_{\text{c, max}}$ and RuBisCO antigen in *L. perenne* grown in the field at low N supply (Rogers et al. 1995). While some allocation of N away from RuBisCO may be expected even at non-limiting N availability due to an increased photosynthetic N use efficiency at elevated $p_{\text{CO}_2}$, such a response was only observed in a minority of species (Sage 1994). Instead, in most cases, photosynthetic acclimation was related to a limitation of growth by either pot size or nutrient supply (Sage 1994; Long et al. 1996). Photosynthetic acclimation in this experiment was not an effect of root growth being limited by pot size: Acclimation was more pronounced at low than at high N supply even though the root system in plants grown at high N supply was significantly larger than in plants grown at low N supply. Thus a limiting availability of nutrients may have been ultimately responsible for the acclimation processes observed. However, Woodrow (1994) suggested that even at excess nutrient supply photosynthetic acclimation may occur if sink capacity cannot be increased.

From these arguments it can be concluded that the amount of C assimilated per unit of shoot dry mass at elevated $p_{\text{CO}_2}$ was reduced both as a consequence of an accumulation of a greater fraction of leaf dry matter as WSC and of a reduced photosynthetic capacity per unit leaf area. This reduction of the efficiency of the shoot appears to have counteracted the stimulatory effect of elevated $p_{\text{CO}_2}$ on photosynthesis leading to balanced root and shoot activities at elevated $p_{\text{CO}_2}$ in the absence of a stimulation of RGR.

### 3.5.5 Sink limited growth response to elevated $p_{\text{CO}_2}$ in *L. perenne*

A stimulation of growth by elevated $p_{\text{CO}_2}$ was prevented by the failure of *L. perenne* to activate new sinks (tiller buds) in response to an increased C availability. As indicated by the accumulation of carbohydrates and the down-regulation of photosynthesis, growth after recovery from defoliation was apparently sink limited. It cannot be excluded that a stimulation of growth by elevated $p_{\text{CO}_2}$ would have been possible at even higher rates of nutrient supply. Possibly, a very high (and continuous) nutrient supply may be required for a sustained stimulation of RGR at elevated $p_{\text{CO}_2}$ (e.g. by the use of hydroponic culture, as in Roumet et al. 1996). However, in spite of an overall tendency towards a higher responsiveness of growth to elevated $p_{\text{CO}_2}$ at high relative to low N supply, some studies have also found a pronounced stimulation of growth at low N supply (Conroy 1992; Poorter et al. 1996). Since a stimulation of growth at elevated $p_{\text{CO}_2}$ did occur during seedling development at moderately high N supply (most if not all of this effect was already apparent at after 39 d of growth at 7.5 mM $\text{NO}_3^{-}$; not shown), it is
suggested that N supply in the high N treatment did not limit the growth response to elevated $p_{CO_2}$.

While *L. perenne* is known to be very responsive in its growth to N supply (Wilman and Wright 1983), it appears that this species is not well adapted to take prolonged advantage of an increased $p_{CO_2}$. The growth response to stimuli such as N (Beck 1994) and light (Baraldi *et al*. 1995; Kraepiel *et al*. 1995) likely involves specific reactions mediated by phytohormones while such a specific response to $p_{CO_2}$ may not have evolved as $p_{CO_2}$ is relatively constant in most environments. While adaptations to changes in $p_{CO_2}$ are apparent in plants growing at different altitudes, there are also important changes in other parameters (Mott 1990); further, $p_{CO_2}$ remains in a constant ratio to $p_{O_2}$ and thus only the saturation of RuBisCO, but not the proportion of assimilates lost to photorespiration will change with elevation. It has been suggested that short-term responses of C partitioning to environmental changes are mediated by their effects on sugar concentration gradients which control transport in the phloem (Minchin *et al*. 1994a; Minchin *et al*. 1994b). Further, it appears likely that the feed-back inhibition of photosynthesis in response to an insufficient sink for carbohydrates is mediated by the accumulation of sugars in the leaf (Sheen 1994). However, the extent to which the availability of C substrate controls sink growth as proposed by Farrar and Williams (1991) and Farrar (1992) appears to vary between plant species and tissues (Stitt and Schulze 1994). While effects of carbohydrate availability on the expression of genes related to carbohydrate metabolism, storage, respiration and other functions in sinks have been demonstrated, this response appears to be often modulated by plant hormones or light (Koch 1996). Very little is known so far about the contribution of carbohydrate availability to the regulation of developmental processes such as the activation of dormant buds or the formation of vascular bundles (Pollock and Farrar 1996). However, it is conceivable that a limited responsiveness of some developmental processes to carbohydrate availability restricts the growth response of sinks under elevated $p_{CO_2}$. Thus, it has been suggested that the relative growth response to elevated $p_{CO_2}$ is highest in potentially fast-growing wild species and crop species (Poorter *et al*. 1996) which have been selected for maximal growth under favourable conditions and in which growth may be largely source limited.

In a field study using FACE technology (Hebeisen *et al*. 1997), an increased SLW in *L. perenne* grown in monocultures (Stadelmann 1993) in combination with increased WSC concentrations (Part 2) and photosynthetic acclimation (Rogers *et al*. 1995) at elevated $p_{CO_2}$ suggested that largely similar adjustment strategies operated at the leaf level as in the present study. However, in the field, the relative yield increase due to elevated $p_{CO_2}$ was higher at high N supply. Further, root:shoot partitioning at elevated $p_{CO_2}$ was apparently altered in favour of roots (Jongen *et al*. 1995; Hebeisen *et al*. 1997). Thus, morphological adjustments were different, possibly related to the fact that in the present study, *L. perenne* was grown as single plants and not in swards. However, it is also conceivable that the increased dry matter partitioning to roots at elevated $p_{CO_2}$ was due to a reduced availability of N from the soil at elevated $p_{CO_2}$ in the field. A reduction of N availability from the soil at elevated $p_{CO_2}$ has been proposed based on the observation
that the proportion of N derived from symbiotic N\(_2\) fixation was increased in \textit{T. repens} when grown in a parallel experiment in the field but not when grown in sand in the growth chamber (Zanetti 1997).

### 3.5.6 Conclusions

Plants of \textit{L. perenne} exposed to elevated \(p_{\text{CO}_2}\) in the growth chamber produced more structural and/or non-structural dry matter per tiller, indicating an enhanced availability of C building blocks for growth. In fact, this increase in dry mass per growing point was the principal growth response to elevated \(p_{\text{CO}_2}\). Even though the higher C availability at elevated \(p_{\text{CO}_2}\) might be expected to alleviate the impact of defoliation and thus to promote regrowth under favourable conditions, no such effect could be observed. Initial regrowth appeared to depend more directly on the vigour of meristems present at the time of defoliation than on C availability, as relative differences in tiller size were maintained. It appears that enough carbohydrate reserves and/or leaf area remained after defoliation such that the regrowth of leaves was not limited by carbohydrate availability. A positive response to elevated \(p_{\text{CO}_2}\) of total plant dry matter accumulation during the recovery phase may have been prevented by the presence of larger respiratory sinks (particularly roots). However, even during later regrowth and after recovery, there was no stimulation of tiller production or RGR. Therefore, the higher leaf carbohydrate concentrations and the enhanced accumulation of carbohydrate reserves towards the end of regrowth reflected an apparent sink limitation of the growth response to elevated \(p_{\text{CO}_2}\). It is concluded that in \textit{L. perenne}, the reduction of the source:sink ratio by defoliation did not reverse the loss of responsiveness of growth to elevated \(p_{\text{CO}_2}\) of the plants which is frequently found with increasing plant maturity in long-term studies on the effect of elevated \(p_{\text{CO}_2}\) and which was also apparent in the present study.
4. CARBOHYDRATES IN TRIFOLIUM REPENS L. IN RELATION TO REGROWTH AFTER DEFOLIATION AT ELEVATED $P_{\text{CO}_2}$

4.1 Abstract

An increased efficiency of photosynthesis at an elevated atmospheric partial pressure of CO$_2$ ($p_{\text{CO}_2}$) may compensate for the loss of leaf area at defoliation. The following hypothesis was tested in controlled environment chambers and under field conditions: After a severe defoliation, an enhanced carbon (C) availability at elevated $p_{\text{CO}_2}$ would lead to a faster recovery and regrowth of *Trifolium repens* L. (white clover) and an accelerated replenishment of carbohydrate reserves, irrespective of nitrogen (N) supply. Indeed, WSC concentrations in leaves and stolons were consistently increased at elevated $p_{\text{CO}_2}$ throughout all experiments and starch concentrations at elevated $p_{\text{CO}_2}$ were frequently at least twice as high as concentrations at ambient $p_{\text{CO}_2}$ (leaf starch concentrations at elevated $p_{\text{CO}_2}$ were typically in the range of 0.4–0.8 mol kg$^-1$ after apparent recovery). Together with a significantly accelerated replenishment of stolon starch reserves, this indicated that carbohydrate availability was persistently enhanced in *T. repens* growing at elevated $p_{\text{CO}_2}$. Both in the field and in the growth chamber, carbohydrate mobilisation after defoliation continued beyond the apparent recovery of the plant carbohydrate status at elevated $p_{\text{CO}_2}$, suggesting that control mechanisms were unable to respond to the enhanced rates of photosynthesis per unit leaf area at elevated $p_{\text{CO}_2}$. Likewise, the time needed until plants had recovered from defoliation was similar under elevated and ambient $p_{\text{CO}_2}$; this was assessed from incipient carbohydrate accumulation, from the resumption of growth in roots and stolons or from the restoration of characteristic morphological ratios. Indirect evidence obtained in the growth chamber suggested that, in part, this lack of an accelerating effect of elevated $p_{\text{CO}_2}$ may have been due to a longer duration of leaf expansion. A transient stimulation of relative growth rate (RGR) at elevated $p_{\text{CO}_2}$ in the growth chamber was only observed once a sufficient leaf area had been restored. This stimulation of RGR was similar in both N treatments and was not the consequence of carbohydrate accumulation. However, the growth stimulation (18 %) remained small in comparison to the persistent effect of elevated $p_{\text{CO}_2}$ on the dry mass accumulated per growing point (28 %). An enhanced accumulation of non-structural carbohydrates and an increased production of structural tissue components at the expense of additional leaf area led to a decrease in leaf area ratio (LAR, -12 % after recovery) and appeared to aid in maintaining balanced source–sink relations at elevated $p_{\text{CO}_2}$. However, there was no down-regulation of photosynthesis per unit leaf area. Only in the growth chamber, an accumulation of starch at elevated $p_{\text{CO}_2}$ in combination with low N supply suggested that symbiotic N$_2$ fixation did not fully compensate for the differences in N supply from fertilization. Nevertheless, similar values of RGR in all treatments during the last week of the experiment suggest that the plants were able to adjust to the relative availability of C and N. It is concluded that the long-term growth response of *T.*
repens to elevated $p_{\text{CO}_2}$ was restricted by internal or external factors other than N supply and that this restriction was only briefly released after defoliation.

4.2 Introduction

At an elevated atmospheric partial pressure of CO$_2$ ($p_{\text{CO}_2}$), an increased efficiency of photosynthesis (Long et al. 1995) in C$_3$ plants as well as possibly reduced rates of respiration (e.g. Bunce and Caulfield 1991; Amthor et al. 1992) generally lead to enhanced growth (Baker and Enoch 1983). However, in many species, a stimulation of the relative growth rate (RGR, the rate of dry mass increase relative to current dry mass) is most pronounced during an initial phase of exposure to elevated $p_{\text{CO}_2}$ (Poorter 1993; Poorter et al. 1996). In long-term experiments, alterations of plant morphology – particularly a reduction of the leaf area ratio (LAR, the ratio of leaf area to total plant dry mass) and of the root:shoot ratio (Poorter 1993) – and physiological acclimation – mainly a reduction in photosynthetic capacity (Sage 1994) – often greatly reduce the stimulation of growth in response to elevated $p_{\text{CO}_2}$. Both an alteration of the root:shoot ratio and a down-regulation of photosynthesis have been associated mainly with a limiting nutrient supply (Stulen and Den Hertog 1993; Sage 1994) and thus suggest that the growth response to elevated $p_{\text{CO}_2}$ is often limited by sink development.

Defoliation of grassland plants by cutting or grazing greatly reduces their assimilatory surface. Carbohydrate reserves are depleted and may even become limiting for initial regrowth after a severe defoliation (Richards 1993). Thus, the rate of growth after defoliation as well as the replenishment of carbohydrate reserves may depend strongly on assimilate supply. A stimulation of photosynthesis at elevated $p_{\text{CO}_2}$ may thus reduce the detrimental effects of defoliation on the plant’s carbon (C) balance and accelerate recovery.

However, as discussed in the previous chapter, differences between $p_{\text{CO}_2}$ treatments in the morphology of the plants after defoliation and physiological acclimation appeared to completely balance the gain in photosynthetic efficiency under elevated $p_{\text{CO}_2}$ during regrowth of Lolium perenne. It was suggested that similar mechanisms may have been responsible for the lack of an increase in agronomic yield (5 cm cutting height) in L. perenne growing in the ETH FACE (Free Air Carbon Dioxide Enrichment) experiment (Hebeisen et al. 1997). At least in the field, nitrogen (N) availability may have limited the responsiveness of L. perenne to elevated $p_{\text{CO}_2}$. In contrast, a pronounced increase in harvestable yield was observed in Trifolium repens L. (white clover). T. repens is largely independent of mineral soil N due to its ability to obtain its N from a symbiosis with Rhizobium bacteria. Therefore, acclimation responses may be less likely to occur in this species and a stimulation of growth may be expected at least after defoliation.

Ryle et al. (1992) concluded that a persistent growth response to elevated $p_{\text{CO}_2}$ could be obtained by maintaining ramets of T. repens at constant mass by continuous removal of new expanded leaves. However, their growth response depended largely on the production of larger stolon systems at elevated $p_{\text{CO}_2}$ during an initial phase of acclimation to the
treatments. It would therefore be of interest to examine if a stimulation of regrowth in *T. repens* could also be observed in plants already acclimatised to elevated $p_{\text{CO}_2}$ and under a less stringent defoliation regime. Such a response has been suggested by Nijs *et al.* (1988a) based on results from swards of *T. repens* exposed to elevated $p_{\text{CO}_2}$ in the greenhouse. However, no data were collected before defoliation or during the first few days of regrowth to further support this suggestion. In a study with single ramets of *T. repens*, Scheidegger and Nösberger (1983) did not find a stimulation of the production of new growing points either during acclimation to elevated $p_{\text{CO}_2}$ or during regrowth after only moderate defoliation.

A study was therefore undertaken to identify and characterise the mechanisms governing the response of *T. repens* to elevated $p_{\text{CO}_2}$ during regrowth after severe defoliation and to relate them to the effect of elevated $p_{\text{CO}_2}$ on harvestable yield observed in the field.

An experiment was set up in controlled environment chambers using spaced ramets of *T. repens* in sand. As each individual plant can expand in three dimensions, a more pronounced growth response to elevated $p_{\text{CO}_2}$ is possible than in closed canopies (cf. Lüscher *et al.* 1996). The use of single plants further allowed the application of growth analysis techniques to entire plants. The approach chosen thus offers the opportunity to gain insight into acclimation processes which may govern the response of *T. repens* to elevated $p_{\text{CO}_2}$. Two levels of N supply were employed to modify the demand for carbohydrate by modulating growth rates and/or the dependence on symbiotic N$_2$ fixation.

The hypothesis was tested that elevated $p_{\text{CO}_2}$ would enhance the recovery of defoliated stolons of *T. repens*, as they have relatively low rates of C assimilation compared to C demand. Changes in source:sink relations due to defoliation and during regrowth should be reflected in the effect of elevated $p_{\text{CO}_2}$ on leaf carbohydrate concentrations (as a measure of carbohydrate availability in relation to sink demand) and on stolon carbohydrate concentrations (reflecting carbohydrate reserves). An increase in sink demand relative to the plant’s leaf area would increase apparent night-time export. It was expected that growth would be more responsive to elevated $p_{\text{CO}_2}$ than in *L. perenne* and thus morphological adjustments and photosynthetic acclimation would be of lesser importance in *T. repens*.

**4.3 Materials and Methods**

**4.3.1 Growth chamber experiment**

**4.3.1.1 Plant material and growth conditions**

Two clones of *Trifolium repens* L. cv. Milkanova were established from stolons sampled in fumigated plots of the FACE field experiment and were propagated in Conviron PGV 36 (Conviron, Winnipeg, Manitoba, Canada) controlled environment chambers set to either 35 or 70 Pa partial pressure of CO$_2$ ($p_{\text{CO}_2}$). $p_{\text{CO}_2}$ was monitored using WMA-2
infrared gas analysers (PP-Systems, Hitchin, Herts, U.K.); excess CO₂ was removed by forcing air through soda lime (Roth, Karlsruhe, Germany). Air temperature was set to 18/13 °C day/night. Daylength was set to 16 h with 12 h at maximum PPFD (500 µmol m⁻² s⁻¹ produced from 5.2 kW of fluorescent tubes using Sylvania 215W very high output cool white and 2.5 kW of incandescent bulbs using Sylvania 100 W/277 V, each consuming approx. 75 W at 230 V; all Osram Sylvania Inc., St. Marys, PA, U.S.A.) and 2 h each of stepwise increasing and decreasing PPFD at the beginning and at the end of the day, respectively.

Cuttings (3–4 nodes; with leaves larger than stage 0.6–0.7 according to Carlson 1966a removed) were rooted in boxes filled 5 cm deep with sand (particle size 0.7–1.2 mm). PPFD was increased from 310 µmol m⁻² s⁻¹ to 500 µmol m⁻² s⁻¹ in three steps over 22 d. Relative humidity was set to 99 % initially and decreased to 80 % in two steps over 14 d. Cuttings were initially kept moist by spraying de-ionised water. After 6 d, they were watered twice daily with half-strength nutrient solution and, after 14 d, full-strength nutrient solution was applied. The nutrient solution was modified from Hammer et al. (1978) as follows: Ca(NO₃)₂ as in the original solution was replaced by 2.5 mM CaSO₄ (final concentration of NO₃⁻: 2.5 mM), and 0.124 mM EDTA ammonium iron(III)salt (Fluka, Buchs, Switzerland) was used as a source of iron; no adjustment of the final pH was done. At weekly intervals, plants and pCO₂ treatments were rotated between growth chambers and the sand was inoculated with a suspension of Rhizobium leguminosarum bv. trifolii strain RBL 5020 (kindly provided by Dr. H. P. Spaink, Leiden, NL) to facilitate nodulation.

After 18 d of growth, cuttings with three to four fully expanded leaves were selected and transferred into plastic boxes 150 mm wide × 365 mm long × mm 140 deep filled with quartz sand as above and with the root space separated longitudinally using a plastic board. Above and below ground competition was minimised by arranging four cuttings of the same pCO₂ treatment per box such that on both sides of the separation one cutting was growing towards the centre of the box from each end. Roots were trimmed to a length of four to five cm where necessary to facilitate planting. Any secondary stolons growing from the basal node were removed. Flower buds, which were assumed to have been induced before the preparation of cuttings, were excised throughout the first five weeks of growth. Regularly, extending stolons were pinned down onto the substrate by means of U-shaped pieces of plastic-coated wire to facilitate the outgrowth of root buds. Nutrient solution was applied by sprinkling with 400 ml per box twice daily to provide a non-limiting water supply.

After 25 d of growth, boxes were generously flushed with deionized water and then randomly allocated to either a low (1 mM NO₃⁻) or a high N (15 mM NO₃⁻) treatment. Nutrient solutions were modified from Hammer et al. (1978) as described in Part 3.
4.3.1.2 Sequential harvests during regrowth after defoliation

After 35 d of growth (17 d after transplanting into boxes), plants were defoliated. All leaves that had reached or exceeded stage 0.4 of development according to Carlson (1966a) were excised close to the base of the petiole or at the sand surface level. Regrowth was monitored using a total of seven harvests at 0, 2, 3, 4, 7, 11, and 22/23 d after defoliation (the last harvest had to be distributed over two days due to the large size of the plants). For each harvest, one set of plants, consisting of four replicates, was sampled at the end of a photoperiod and another set at the beginning of the following photoperiod. A duplicate set of plants was sampled for day 0.

Sand was carefully removed. Active growing points (all buds which had produced at least one leaf with a visible petiole) were counted. In addition, growing points which had produced more than three fully expanded leaves and at least one elongated internode were counted and classified as secondary stolons. Plants were separated into blades of growing leaves, blades of first (youngest) fully expanded leaves (stage 0.9–1.0 of development according to Carlson 1966a), blades of older leaves, petioles, new stolon parts (distal to the node of attachment of the 2nd youngest leaf removed at defoliation at each growing point), old stolon (extended proximal internodes on the main stolon, mostly stunted proximal internodes on secondary stolons), roots and necrotic tissue (less than one third green). The area of all leaf blade fractions was determined using a LI-3000A leaf area meter fitted with a belt conveyor (Lambda Instruments Corporation, Lincoln, Nebraska, U.S.A.). Tissue removed at defoliation was oven-dried for 1 h at 105 °C followed by 48 h at 65 °C; all other tissues were frozen in liquid N₂ within 30 minutes after processing and stored at -20 °C until lyophilisation. Dry material was weighed and finely ground for chemical analyses.

4.3.2 Field experiment

Monocultures of *Trifolium repens* L. cv. Milkanova were grown in the field at Eschikon near Zürich, Switzerland as described for the frequent defoliation treatment in Zanetti (1996). Briefly: Swards were established at ambient $p_{\text{CO}_2}$ in August 1992. From the end of May 1993, a FACE system (Free Air Carbon Dioxide Enrichment; Lewin et al. 1994) was used to increase $p_{\text{CO}_2}$ from ambient (35 Pa) to 60 Pa. The swards were defoliated at approximately 5 cm above ground at regular intervals of four to six weeks. N fertilizer was applied after each cut as ammonium nitrate solution. N doses for the regrowth period under investigation in 1993 were 1.5 g m⁻² and 6 g m⁻², applied three days after the cut in the low and high N treatment, respectively (10 g m⁻² yr⁻¹ and 42 g m⁻² yr⁻¹); these were increased to 1.7 g m⁻² and 7 g m⁻², applied on the day after the cut in 1994 (14 g m⁻² yr⁻¹ and 56 g m⁻² yr⁻¹). The experiments were set up as a split plot design with three blocks (replications) on the basis of the crops grown in previous years and with $p_{\text{CO}_2}$ as the main plot factor and N supply as sub-plot factor (for details see Hebeisen et al. 1997).
Field experiment I. In 1993, from mid-July to mid-August, first (youngest) fully expanded leaves (stage 0.9–1.0 according to Carlson 1966a) were sampled at intervals throughout regrowth, starting immediately before defoliation. On each occasion, one set of approximately 10 leaves per plot was sampled on the previous evening (17h–21h) and another set on the morning (6h–10h) of the day indicated in the figures.

Field experiment II. In 1994, from the beginning of August to the beginning of September, main stolons (as defined in Thomas 1987) were sampled at intervals throughout regrowth, starting immediately before defoliation. Each time, 8 main stolons bearing at least one fully developed leaf were carefully detached from their roots and excised. Stolons were divided into an ‘apical section’ distal to the node of attachment of the first leaf that would be (petiole longer than 5 cm) or was removed at defoliation and a proximal section of 5 internodes, designated as ‘old stolon’. First (youngest) and second fully expanded leaves (where available) were excised and all other leaves including growing leaves which had completely emerged from their stipule as well as all petioles were discarded. In both experiments, the samples were frozen in liquid N₂ immediately after processing, stored at -20 °C, lyophilised and finely ground.

Temperature at 2 m above ground and global irradiance (300–2500 nm) were obtained from meteorological stations in the vicinity of the FACE rings. Daily mean temperatures ranged from 12 to 22 °C for both experiments (Fig. 4-1). The mean daily radiant exposure was 17.5 MJ m⁻² for Experiment I and 15.1 MJ m⁻² for Experiment II. During both experimental periods, there was sufficient water supply from rainfall (Experiment I: 135 mm rainfall vs. 112 mm potential evaporation; Experiment II: 145 mm rainfall vs. 70 mm potential evaporation). Experiment II started at the end of a drought period, but some rainfall (5, 8 and 6 mm, respectively) had occurred on each of the two days before defoliation and on the day of defoliation itself (for details see Hebeisen 1997). Thus, it is assumed that regrowth was not seriously restricted by drought.

4.3.3 Chemical analysis

Approximately 10 mg of sample material were extracted twice with 1 cm³ of 80 % (v/v) ethanol in water at 80 °C for 30 min. The combined supernatants, as well as the pellets, were stored at -20 °C. Carbohydrates in the extract (i.e. highly soluble carbohydrates, comprising hexoses, sucrose and maltose) were designated as ‘water-soluble carbohydrate’ (WSC) and analysed using an anthrone protocol optimised for the simultaneous determination of glucose, fructose, and other carbohydrates composed thereof, e.g. sucrose (Part 2).

Figure 4-1 (right page). Time course of temperature at 2 m above ground (upper line, right axis) and solar irradiance (lower line, left axis) near the ETH FACE array for the regrowth periods (a) from July 14, 1993 (0 mark at 12 h noon) to August 13, 1993 and from (b) August 8, 1994 to September 2, 1994. Symbols (right axis) indicate the average ± SD radiant exposure on the harvest date up to the time when samples were collected in the morning and during the day (●) or at the end of the day (○).
Global irradiance (W m\(^{-2}\))

Temperature (°C) / radiant exposure (MJ m\(^{-2}\))

Days after defoliation
The starch in the pellets remaining after the extraction of WSC was determined as previously described (Part 2). In brief, glucose released by enzymatic hydrolysis (Schweizer 1986) was determined enzymatically using hexokinase and glucose-6-phosphate dehydrogenase as modified from Farrar (1993).

All carbohydrate concentrations were expressed as moles of glucose equivalents per unit of carbohydrate-free (structural) dry mass. For the calculation of carbohydrate-free dry mass, it was assumed that WSC consisted of two thirds sucrose and one third hexoses with an average mass of 174.15 g per mole of hexose equivalents. Starch was assumed to consist of poly-glucose with an average mass of 162.14 g per mole of hexose equivalents. While the true composition of water-soluble carbohydrates may vary between almost pure sucrose and about one half hexoses, depending on tissue, time of day and developmental stage of the plant (Gordon et al. 1987; Lüscher 1989; Baur-Höch et al. 1990), the error introduced by these deviations remains well below 1 % for the range of WSC concentrations found in the present study.

At least 20 mg of leaf material were analysed for nitrogen (N) and for carbon (C) by measuring thermal conductivity and infrared absorption, respectively, of combustion gases using an elemental analyser (LECO CHN-1000, LECO Corp., St. Joseph, MI, U.S.A.) calibrated against an acetonilide standard for elementary analysis (Merck, Darmstadt, Germany).

Nitrate and ammonium concentrations were determined in approximately 10 mg of finely ground leaf material as described in Part 3.

4.3.4 Photosynthesis measurements

Estimates of the maximum carboxylation velocity (Vc\textsubscript{max}) of first fully expanded leaves were obtained in the growth chamber experiment between 12 and 22 d after defoliation. Measurements were made on single leaflets and analysed as described in Part 3.

4.3.5 Statistical analysis

Values of extensive quantities (dry mass, etc.) and ratios thereof were converted to their natural logarithms prior to statistical analysis. Means shown were obtained by back-transformation. Intensive quantities (concentrations, Vc\textsubscript{max}) were estimated using non-transformed values. The FACE experiment was analysed as a split-plot design (Gomez and Gomez 1984) with \( p_{\text{CO}_2} \) as main plot factor and N supply as sub-plot factor and three replications. The growth chamber experiment was analysed as a randomised complete block design. To minimise effects of variation in the initial plant size, the deviation from treatment means in the amount of leaf area removed at defoliation was used as a covariate in the analysis of all extensive quantities. Statistical analyses were carried out using the GLM (general linear model) procedure of the SAS statistical analysis package (SAS Institute Inc., Cary, NC, USA). All tests were carried out at the 95 % confidence level. Unless indicated otherwise, standard errors of means were calculated from the residual mean square error for each harvest.
4.4 Results

4.4.1 Growth chamber experiment

4.4.1.1 Growth and morphology

A. Initial conditions and effect of defoliation

Plants grown at elevated $p_{\text{CO}_2}$ had significantly more dry mass than plants at ambient $p_{\text{CO}_2}$ (Fig. 4-2a, before defoliation). However, there was no effect of $p_{\text{CO}_2}$ on the total number of growing points (Fig. 4-3b, before defoliation). Thus all effects of $p_{\text{CO}_2}$ on plant size were due to the production of more dry matter per growing point. In addition, plants grown at high N supply had significantly more dry mass than plants grown at low N supply (Fig. 4-2a, before defoliation).

While care was taken to ensure a uniform removal of all leaves at or beyond developmental stage 0.4 according to Carlson (1966a), a greater proportion of leaf dry matter remained in the treatment at low N supply and ambient $p_{\text{CO}_2}$ (3.4 %) than in the other three treatments (2.3 %, cf. Fig. 4-2b). However, this was probably the consequence of differences in plant morphology rather than of experimental errors, as similar deviations for the root:shoot ratio (Fig. 4-4a) and the leaf weight ratio (LWR, the ratio of leaf blade to total plant dry mass, Fig. 4-4b) were evident already before defoliation.

B. Time course of regrowth

Total plant dry mass remained approximately unchanged during the first 3 d at high N supply and during the first 4 d at low N supply (Fig. 4-2a). While there was no clear effect of $p_{\text{CO}_2}$ treatments on dry matter development during early regrowth, growth proceeded significantly faster at elevated than at ambient $p_{\text{CO}_2}$ between days 4 and 7 after defoliation. Also, the effect of the N treatments increased significantly during the first week after defoliation. However, there were no more treatment effects on total plant growth rates beyond day 7. Still, the mean whole plant RGR between days 0 and 11 (see Section C for the selection of the endpoint of regrowth) was higher both at elevated $p_{\text{CO}_2}$ relative to ambient $p_{\text{CO}_2}$ and at high N supply relative to low N supply (Time $\times$ $p_{\text{CO}_2}$ and Time $\times$ N effect, respectively, in Tab. 4-1). A similar effect was also evident when plants at day 11 were compared to undefoliated plants.

Leaf growth proceeded rapidly and without any substantial lag after defoliation (Fig. 4-2b). Small initial differences in leaf dry mass between treatments had disappeared by day 3 after defoliation. While growth rates at ambient $p_{\text{CO}_2}$ declined after day 4, such a decline was only apparent after day 7 at elevated $p_{\text{CO}_2}$. As a consequence, plants growing at elevated $p_{\text{CO}_2}$ had a significantly higher leaf dry mass from day 7 after defoliation. An essentially similar development over time was also evident for petioles (Fig. 4-2e). Total plant leaf area was even transiently lower at elevated than at ambient $p_{\text{CO}_2}$ (Fig. 4-3a).
Figure 4-2. Development of (a) total plant, (b) total leaf blade, (c) total stolon, (d) root and (e) petiole dry mass (DM) in T. repens. Plants were grown in the growth chamber and exposed to two $p_{CO_2}$ treatments ($\triangle$ square: ambient $p_{CO_2}$; $\blacksquare$: elevated $p_{CO_2}$) and two N treatments ($\Delta \blacksquare$: low N supply; $\square \blacksquare$: high N supply). Error bars indicate the average standard error of means for each harvest as derived from the error mean square of an ANOVA over all harvests with the deviation from treatment means in the amount of leaf area removed at defoliation as a covariate. Time point b. d. indicates values before defoliation.
Leaf area (cm²)

Number of growing points

Number of secondary stolons

Days after defoliation
Figure 4-3 (left page). Development of (a) total leaf area, (b) total number of growing points and (c) number of secondary stolons in *T. repens*. Plants were grown in the growth chamber and exposed to two *p*$_{\text{CO}_2}$ treatments (△: ambient *p*$_{\text{CO}_2}$; ▲: elevated *p*$_{\text{CO}_2}$) and two N treatments (△: low N supply; □: high N supply). Error bars indicate the average standard error of means for each harvest as derived from the error mean square of an ANOVA over all harvests with the deviation from treatment means in the amount of leaf area removed at defoliation as a covariate. Time point b. d. indicates values before defoliation.

This condition persisted from day 3 after defoliation until leaf area growth rates started to decline at ambient *p*$_{\text{CO}_2}$ after day 4. Plants growing at elevated *p*$_{\text{CO}_2}$ had a smaller number of fully developed leaves on days 3 and 4 after defoliation (Fig. 4-5b). However, the average area of fully expanded leaves was always larger at elevated *p*$_{\text{CO}_2}$ (Fig. 4-5a). Thus, the total leaf area of plants growing at elevated *p*$_{\text{CO}_2}$ exceeded that of plants growing at ambient *p*$_{\text{CO}_2}$ as soon as the number of fully expanded leaves became similar.

Table 4-1. Statistical analysis of total plant dry mass (DM), total leaf blade dry mass, total stolon dry mass, root dry mass, total leaf area and number of growing points and their development during regrowth after defoliation of *T. repens* in controlled environment chambers. To avoid any difficulties in the interpretation due to apparent changes in partitioning at the end of the experiment, only data for days 0 and 11 have been used. The “Time” treatment tests whether the mean relative growth rate for a given parameter was different from zero between these two days. Treatment effects on the relative growth rates appear as interactions with the “Time” treatment. The covariate accounts for deviations from treatment means in the amount of leaf area removed at defoliation. Significant effects are indicated as follows: *, p ≤ 0.05; **, p ≤ 0.01; ***, p ≤ 0.001.

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>DF</th>
<th>Total DM</th>
<th>Leaf DM</th>
<th>Stolon DM</th>
<th>Root DM</th>
<th>Leaf area</th>
<th>Grow. pts.</th>
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<tr>
<td></td>
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<td>p</td>
<td>MS</td>
<td>p</td>
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<td>1.49 ***</td>
<td>1.48 ***</td>
<td>1.77 ***</td>
<td>0.73 ***</td>
<td>0.45 *</td>
<td>0.04</td>
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<td>6.37 ***</td>
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<td>0.27</td>
<td>0.45 ***</td>
<td>0.00</td>
<td>0.63 *</td>
<td>0.12 *</td>
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<tr>
<td>Time × <em>p</em>$_{\text{CO}_2}$ × N</td>
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<td>0.34 ***</td>
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<tr>
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<td>0.03</td>
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<td>0.09</td>
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<td>1.37 ***</td>
<td>1.40 ***</td>
<td>1.37 ***</td>
<td>1.24 ***</td>
<td>0.62 ***</td>
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<td>0.09</td>
<td>0.01</td>
<td>0.02</td>
<td>0.10</td>
<td>0.03</td>
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</tbody>
</table>
in both $p_{CO_2}$ treatments about 7 d after defoliation. Between days 0 and 11, only the relative growth rate of leaf dry mass but not of leaf area was higher at elevated than at ambient $p_{CO_2}$ (Time × $p_{CO_2}$ effect in Tab. 4-1).

**Figure 4-4 (left page).** Time course of the ratio of (a) root to shoot dry mass (R:S), (b) total leaf blade to total plant dry mass (LWR) and (c) total leaf area to total plant dry mass (LAR) in *T. repens*. Plants were grown in the growth chamber and exposed to two $p_{CO_2}$ treatments ($\triangle \square$: ambient $p_{CO_2}$: $\blacktriangle \blacksquare$: elevated $p_{CO_2}$) and two N treatments ($\triangle \blacktriangle$: low N supply; $\square \blacksquare$: high N supply). Error bars indicate the standard error of means for each harvest with n=8 (n=16 for day 0, n=4 for day 4). For LWR and R:S, values for intact plants before defoliation (b. d.) are shown (n=6).

**Figure 4-5.** Time course of (a) leaf area per fully expanded leaf and (b) number of fully expanded leaves per plant in *T. repens*. Plants were grown in the growth chamber and exposed to two $p_{CO_2}$ treatments ($\triangle \square$: ambient $p_{CO_2}$; $\blacktriangle \blacksquare$: elevated $p_{CO_2}$) and two N treatments ($\triangle \blacktriangle$: low N supply; $\square \blacksquare$: high N supply). Error bars indicate in (a) the standard error of means for each harvest with n=8 and in (b) the average standard error of means for each harvest as derived from the error mean square of an ANOVA over all harvests with the deviation from treatment means in the amount of leaf area removed at defoliation as a covariate.
Net growth of stolons ceased during the first 4 to 7 d after defoliation (Fig. 4-2c). Particularly at low N supply, there appeared to be even a transient loss of total stolon dry matter. However, there was no significant effect of $p_{CO_2}$ on either the duration of the lag phase or on the overall stolon relative growth rate (no Time × $p_{CO_2}$ effect in Tab. 4-1). In contrast, stolon relative growth rate was significantly lower at low than at high N supply throughout the experiment (Time × N effect in Tab. 4-1). Net growth of roots also ceased during the first week after defoliation (Fig. 4-2d). Over the whole experiment, root relative growth rate was significantly higher at elevated than at ambient $p_{CO_2}$ (Time × $p_{CO_2}$ effect in Tab. 4-1) and this effect appeared to originate mostly from the period between days 4 and 7.

The production of new growing points (Fig. 4-3b) did not match the time course of dry matter development (Fig. 4-2a). The number of growing points per plant continued to rise during the first 3 d after defoliation before stagnating briefly. Notably, this transient stop in the production of new growing points was similar in all treatments. Even though the number of growing points per plant was significantly higher at elevated than at ambient $p_{CO_2}$ at day 11 after defoliation, an apparent stimulation of the production rate of new growing points was significant only at the $p \leq 0.1$ level (Fig. 4-3b, Time × $p_{CO_2}$ effect in Tab. 4-1). An effect of N treatments on the relative growth rate of the number of growing points was significant but seemed to disappear towards the end of the experiment. Relative differences in the number of growing points were small when compared to differences in plant dry mass. However, the number of growing points classified as secondary stolons was transiently higher at elevated than at ambient $p_{CO_2}$ between days 7 and 11 (Fig. 4-3c).

C. Morphology and dry matter partitioning

Differences between $p_{CO_2}$ treatments in total plant dry mass in the absence of or with only small differences in the number of growing points suggest that the most prominent growth response to elevated $p_{CO_2}$ was the production of more dry mass per growing point. Such a response was confirmed for all major tissue fractions (Tab. 4-2). A comparison of the structural dry mass per growing point demonstrates that this response included a stimulation of structural growth. Both in stolons and in first fully expanded leaves, there was a significant effect of $p_{CO_2}$ on the structural dry mass per growing point. While there were consistent differences in the dry mass per growing point, an increase in the effect of elevated $p_{CO_2}$ from days 0 to 11 was only significant for the leaf dry mass per growing point but not for leaf area or total dry mass (Tab. 4-2). When the amount of dry mass per growing point after recovery at day 11 was compared with that of undefoliated plants, the effect of $p_{CO_2}$ did not change significantly over time for any parameter.

Root:shoot ratio was significantly increased as a consequence of defoliation (Fig. 4-4a) and declined during regrowth as new shoot material was produced. Values similar to
those observed before defoliation were reached about 11 d after defoliation. However, the decrease over time continued in most treatments until the end of the experiment. Treatment differences in root:shoot ratio between treatments were only slightly altered by defoliation. Throughout regrowth, root:shoot ratio was higher at low than at high N supply (significant overall and for days 2, 3, 7, 11, 22/23). In addition, root:shoot ratio was mostly lowered in response to elevated $p_{\text{CO}_2}$ at low N supply; at high N supply, this effect was less pronounced or even reversed ($p_{\text{CO}_2}$ effect significant overall and for days 0, 7, 11, 22/23; $p_{\text{CO}_2} \times N$ interaction significant overall and for days 7, 22/23).

As discussed above, not all the treatments were equally affected by defoliation. This was also reflected in LWR (Fig. 4–4b). Due to the apparently faster initial leaf development at ambient $p_{\text{CO}_2}$, LWR was transiently higher at ambient than at elevated $p_{\text{CO}_2}$ after 3 d of regrowth (significant for day 4) until treatment effects similar to those before defoliation were restored by day 7. From day 7, there was no more effect of $p_{\text{CO}_2}$ on LWR at high N supply. In contrast, LWR was significantly lower than in the other three treatments at ambient $p_{\text{CO}_2}$ with low N supply ($p_{\text{CO}_2}$ effect, N effect and $p_{\text{CO}_2} \times N$ interaction all significant for days 7–22/23). After 11 d of regrowth, LWR had reached near original values and subsequently did not rise much further.

### Table 4-2
Partitioning of total plant dry mass (DM) per growing point at 0 and 11 d after defoliation of *T. repens* grown in controlled environment chambers. Values with common letters are not significantly different at $p \leq 0.05$ (multiple t-tests based on standard errors of least-squares means).

<table>
<thead>
<tr>
<th></th>
<th>Day 0</th>
<th>Day 11</th>
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<tbody>
<tr>
<td></td>
<td>n Quantity per growing point</td>
<td>n Quantity per growing point</td>
</tr>
<tr>
<td></td>
<td>High N</td>
<td>Low N</td>
</tr>
<tr>
<td>Total plant DM (mg)</td>
<td>16</td>
<td>30.5</td>
</tr>
<tr>
<td>Total leaf blade DM (mg)</td>
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<td>0.95</td>
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<tr>
<td>Root (mg)</td>
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<tr>
<td>Total stolon DM,</td>
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<td>12.3</td>
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<tr>
<td>corrected for WSC (mg)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blades of first fully</td>
<td>8</td>
<td>3.9</td>
</tr>
<tr>
<td>expanded leaves; DM,</td>
<td></td>
<td></td>
</tr>
<tr>
<td>corrected for WSC (mg)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total leaf area (cm^2)</td>
<td>16</td>
<td>0.10</td>
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</table>
Values for LAR (Fig. 4-4c) were consistently lower at elevated than at ambient $p_{\text{CO}_2}$ (significant overall and for days 2, 3, 4, 11, 22/23). This effect was most pronounced during early regrowth, when the plants at ambient $p_{\text{CO}_2}$ had more fully expanded leaves, but remained evident in both N treatments throughout. There was a tendency towards a reduced LAR at low N supply relative to high N supply; however, this effect was only significant at day 11 after defoliation. Maximal values of LAR had been reached 11 d after defoliation with only small changes occurring thereafter.

From day 11, three of the four treatments showed a similar trend with time for each of root:shoot ratio, LWR, and LAR. However, the response of the treatment at elevated $p_{\text{CO}_2}$ with high N supply was different. Compared to what could be expected from the trend in the other treatments, root:shoot ratio at day 22/23 was too high (Fig. 4-4a) and both LAR and LWR were too low (Fig. 4-4b,c). This indicates a relative change in partitioning towards the roots. As the plants at elevated $p_{\text{CO}_2}$ with high N supply had the highest root dry mass of all treatments, this response might reflect an attempt to over-

![Figure 4-6](image_url)

**Figure 4-6.** Specific leaf weight (SLW) of first fully grown leaves of *T. repens* harvested in the morning (total height of bar) and its constituents: Total non-structural carbohydrate (TNC; open) and structure (SLW corrected for TNC; hatched). For days 11-22/23, structure is further divided into crude protein (N concentration, corrected for nitrate and ammonium and multiplied by 6.25; dense hatching) and others (light hatching). Plants were grown in the growth chamber and exposed to two $p_{\text{CO}_2}$ treatments (△: ambient $p_{\text{CO}_2}$; ■: elevated $p_{\text{CO}_2}$) and two N treatments (△: low N supply; □: high N supply). For each harvest, standard errors of means SLW (n=8), for TNC (n=4) and for leaf structure (n=4) are indicated at the top; standard errors of means for crude protein (n=4) are indicated to the right of each set of bars where appropriate.
come a limitation encountered in the root space (e.g. full exploitation of the root space, excessive temporary depletion of N concentration between additions of nutrient solution). However, by day 22/23, many plants at elevated $p_{\text{CO}_2}$ with high N supply had also started to grow flower buds. In contrast, the number of developing flower buds remained small in the other treatments. Thus, the changes in partitioning may also have been related to a transition from vegetative to generative growth. To avoid any complication of the interpretation of the growth response to treatments, it was therefore decided to use day 11 as the endpoint of the experiment for all relative growth rate calculations in Tab. 4-1.

D. Components of specific leaf weight and maximum carboxylation velocity

Differences in the treatment effects on LWR and LAR suggest that there may have been alterations of leaf structure. Compared to the large effect of elevated $p_{\text{CO}_2}$ on specific leaf weight (SLW), the increase in structural dry matter per unit leaf area at elevated $p_{\text{CO}_2}$ was small (Fig. 4-6) and only consistently apparent at high N supply ($p_{\text{CO}_2} \times N$ interaction significant for day 22/23). In addition, there was significantly less structural dry matter per unit leaf area at low than at high N supply throughout the experiment. Thus, most of the treatment effects on SLW and most of the increase over time were due to an accumulation of nonstructural carbohydrates, particularly at low N supply. Organic leaf N content per unit area was significantly lower at low than at high N supply (Fig. 4-6, shown as estimated crude protein). In contrast, there was no consistent effect of elevated $p_{\text{CO}_2}$ on the content of N per unit leaf area (increased at high N supply but decreased at low N supply). Thus, treatment effects were similar to those on leaf structure. Leaf photosynthetic capacity is frequently correlated to leaf N content per unit area. While there was a tendency towards a reduced $V_{\text{cmax}}$ in first fully expanded leaves (Tab. 4-3), paralleling the lower N content per unit area, this effect was not statistically significant. No tendency towards an effect of elevated $p_{\text{CO}_2}$ on $V_{\text{cmax}}$ was apparent.

4.4.1.2 Dynamics of carbohydrate concentrations

Starch is the main storage carbohydrate in _T. repens_, both in the short and in the long term (Smith 1973; Gordon 1986; Gordon _et al_. 1987). Sucrose serves as the main trans-

<table>
<thead>
<tr>
<th>Maximum carboxylation velocity (µmol m$^{-2}$ s$^{-1}$)</th>
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<tr>
<td>High N supply</td>
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<tr>
<td>----------------</td>
</tr>
<tr>
<td>Ambient $p_{\text{CO}_2}$</td>
</tr>
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<td>93</td>
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</table>
port sugar. Hexoses and their phosphorylated forms as well as maltose mainly act as intermediates and do not normally accumulate to a significant extent (Gordon et al. 1987; Lüscher 1989; Baur-Höch et al. 1990). Therefore, WSC concentrations in *T. repens* are interpreted as a measure for the availability of carbohydrate in a transport-related pool. While *T. repens* has been shown to accumulate considerable quantities of pinitol (Davis and Nordin 1983), there is no evidence for a physiological role of pinitol as a reserve compound during regrowth after defoliation (Baur-Höch et al. 1990). Therefore, no effort was made to include pinitol in the present study.

**A. Leaves**

As changes in leaf structural composition were small compared to changes in leaf carbohydrate content (Fig. 4-6), qualitatively similar time courses and diurnal differences were obtained for leaf carbohydrate concentrations expressed per unit of structural dry mass as for concentrations expressed per unit leaf area. Therefore, only concentrations based on structural dry mass are shown.

Fully expanded leaves were not available until 4 d after defoliation. Therefore, carbohydrate concentrations in leaf blades removed at defoliation were included in Fig. 4-7a,b for comparison. Morning starch concentrations in first fully expanded leaves were significantly higher in plants grown at elevated $p_{\text{CO}_2}$ than in plants grown at ambient $p_{\text{CO}_2}$ ($p_{\text{CO}_2}$ effect significant overall and for days 7, 11, 22/23; Fig. 4-7a). This difference was much more pronounced in plants grown at low than in plants grown at high N supply ($p_{\text{CO}_2} \times N$ effect significant overall and for days 7, 11). Further, leaf starch concentrations were also significantly higher in plants grown at low N supply than in plants grown at high N supply (N effect significant overall and for days 7, 11, 22/23). Four days after defoliation, morning starch concentrations were relatively low in all treatments. While there was little change over time in plants grown at ambient $p_{\text{CO}_2}$ with high N supply, leaf starch concentrations rose significantly at elevated $p_{\text{CO}_2}$ as well as at low N supply, and there was a dramatic increase in the combined treatment. Starch concentrations in first fully grown leaves at the end of the experiment were similar to those observed in the pooled fraction of leaf blades removed at defoliation with similar treatment effects. At least part of the remaining difference is likely explained by the fact that samples were taken at different times of the day as evening starch concentrations were even more similar (not shown). Treatment effects on morning leaf WSC concentrations were much smaller than those on starch concentrations (Fig. 4-7b). Nevertheless, WSC concentrations in first fully expanded leaves were significantly higher at elevated than at ambient $p_{\text{CO}_2}$ throughout. Further, WSC concentrations were higher at low N supply than at high N supply (significant overall and for days 7, 11), but no interaction between N and $p_{\text{CO}_2}$ treatments was found. There was a significant increase in leaf WSC concentrations over time (days 4–23), which was more pronounced at elevated than at ambient $p_{\text{CO}_2}$ between days 4–7. Final concentrations were only slightly lower than those observed at the beginning of the experiment.
Figure 4-7. Time course of morning concentrations of (a, c, e) starch and (b, d, f) water-soluble carbohydrate (WSC) measured in (a, b) blades of first (youngest) fully expanded leaves, (c, d) old stolon sections and (e, f) apical stolon sections of *T. repens* grown in controlled environment chambers. For day 0 after defoliation, carbohydrate concentrations in a combined fraction of leaf blades removed at defoliation are shown in (a, b) as no fully expanded leaves were left on the plants. Plants were exposed to two $p_{\text{CO}_2}$ treatments ($\triangle \square$: ambient $p_{\text{CO}_2}$; ■■: elevated $p_{\text{CO}_2}$) and two N treatments ($\triangle \blacktriangle$: low N supply; ■■■: high N supply). Carbohydrate concentrations are expressed as moles of hexose equivalents per unit of structural dry matter. Error bars indicate the standard error of means (n=4) for each harvest.
**B. Stolons**

Starch concentrations in old stolon sections were significantly higher at elevated $p_{\text{CO}_2}$ than at ambient $p_{\text{CO}_2}$ (significant overall and for days 3, 4, 22/23; Fig. 4-7c). They were also higher at low than at high N supply (significant overall and for days 3, 4, 7). During the first week after defoliation, the effect of elevated $p_{\text{CO}_2}$ on starch concentrations was consistently more pronounced at low than at high N supply. Even though this interaction was no longer apparent at later harvests, there were, however, no significant differences between treatments in the rate of starch mobilisation from old stolon sections. The replenishment of starch reserves between days 11 and 22/23 after defoliation was significantly faster at elevated than at ambient $p_{\text{CO}_2}$ (Time $\times p_{\text{CO}_2}$ interaction significant for days 11–22/23). WSC concentrations in old stolon sections were consistently higher at elevated $p_{\text{CO}_2}$ (significant overall and for days 0, 3, 7, 11, 23; Fig. 4-7d). In contrast, there was no consistent effect of N supply. After defoliation, there was a small but distinct decrease in old stolon WSC concentrations with a minimum reached between days 4 and 7. Subsequently, concentrations rose to and beyond the initial values. This time course did not differ between treatments.

Starch concentrations in apical stolon sections were consistently higher at elevated than at ambient $p_{\text{CO}_2}$ (significant overall and for days 3, 4, 7, 11, 22/23; Fig. 4-7e) and at low N supply (significant overall and for days 3, 4, 7, 11, 22/23). Similar to old stolon sections, the effect of elevated $p_{\text{CO}_2}$ on starch concentrations was consistently more pronounced at low than at high N supply during the first week after defoliation. Starch concentrations in apical stolon sections dropped immediately after defoliation and stayed at a very low level for several days. An accumulation of starch started after day 4 but be-
came more pronounced after day 7, particularly so at elevated $p_{\text{CO}_2}$ and at low N supply (significant $\text{Time} \times p_{\text{CO}_2}$ and $\text{Time} \times \text{N}$ interactions for days 7–22/23). Concentrations of WSC in apical stolon sections were mostly higher at elevated $p_{\text{CO}_2}$ (significant overall and for days 2, 23; Fig. 4-7f), and tended to be higher at high N supply except at the last harvest (significant for day 0). They remained largely unchanged during the first week after defoliation and subsequently increased to a similar extent as in old stolon sections. As in old stolon sections, this time course did not differ between treatments.

C. **Diurnal changes in carbohydrate concentrations**

Diurnal differences in carbohydrate concentrations were determined for the last three harvests (Fig. 4-8). While WSC concentrations did not differ significantly between leaves harvested in the evening and leaves harvested in the morning (Fig. 4-8b), there were pronounced diurnal differences in starch concentrations (Fig. 4-8a). As discussed in Part 2, diurnal accumulation of carbohydrate in leaves reflects transient storage to support night-time export and leaf respiration and was thus designated as ‘apparent night-time export’. Apparent night-time export was of a similar magnitude at all three harvest dates. While values were greater at elevated $p_{\text{CO}_2}$ (significant overall and for day 7; Fig. 4-8a), there was no significant effect of the N treatments.

4.4.2 Field experiments

In the field, there was no significant effect of the N fertilisation treatments on either starch or WSC concentrations. Therefore, only means over both N treatments are shown.

4.4.2.1 Experiment I

A. **Morning carbohydrate concentrations**

Throughout the regrowth period from July to August 1993, concentrations of both WSC ($p_{\text{CO}_2}$ effect significant for days 0, 20; Fig. 4-9b) and starch ($p_{\text{CO}_2}$ effect significant overall and for days 0, 9; Fig. 4-9a) in first fully expanded leaves of *T. repens* were higher in plants grown at elevated $p_{\text{CO}_2}$ than at ambient $p_{\text{CO}_2}$. After defoliation, there was a significant decrease in the concentration of both WSC and starch. Carbohydrate concentrations recovered between days 5 and 9 but tended to decline towards the end of the experiment. For starch, the initial decrease as well as the subsequent recovery was more pronounced at elevated $p_{\text{CO}_2}$ than at ambient $p_{\text{CO}_2}$ (Time $\times p_{\text{CO}_2}$ interaction significant for days 0–3 and for days 5–9). In contrast, the effect of $p_{\text{CO}_2}$ on WSC concentrations did not change over time.

B. **Diurnal changes in carbohydrate concentrations**

As in the growth-chamber experiment, pronounced diurnal differences in leaf starch concentrations (Fig. 4-10a) but no significant diurnal changes in leaf WSC concentra-
tions (Fig. 4-10b) contributed to the apparent night-time export. Apparent night-time export showed a consistent tendency towards greater values at elevated than at ambient $p_{\text{CO}_2}$ (significant overall and for days 3, 5, 30, Fig. 4-10a). Apparent night-time export

![Figure 4-9](image)

*Figure 4-9.* Time course of morning concentrations of (a) starch and (b) water-soluble carbohydrate (WSC) were measured in blades of first (youngest) fully expanded leaves of *T. repens* from monocultures in the ETH-FACE array (Field experiment I; July-August, 1993). Experimental plots were subjected to two $p_{\text{CO}_2}$ treatments (O: ambient $p_{\text{CO}_2}$; ●: elevated $p_{\text{CO}_2}$) and two N treatments; however, because of the lack of significant N effects, averaged results over the N treatments are shown. Carbohydrate concentrations are expressed as moles of hexose equivalents per unit of structural dry mass. Error bars indicate the standard error of means (n=6) for each harvest.

![Figure 4-10](image)

*Figure 4-10.* Time course of apparent night-time export of carbohydrate as estimated from the differences in (a) starch and (b) water-soluble carbohydrate (WSC) concentrations measured in the evening and the following morning in blades of first (youngest) fully expanded leaves of *T. repens* from monocultures in the ETH-FACE array (Field experiment I; July-August, 1993). Experimental plots were subjected to two $p_{\text{CO}_2}$ treatments (O: ambient $p_{\text{CO}_2}$; ●: elevated $p_{\text{CO}_2}$) and two N treatments; however, because of the lack of significant N effects, averaged results over the N treatments are shown. Carbohydrate concentrations are expressed as moles of hexose equivalents per unit of structural dry matter. Error bars indicate the standard error of means (n=6) for each harvest.
rose from day 0 to day 5 after defoliation. This increase was significantly more pronounced at elevated than at ambient $p_{\text{CO}_2}$. Subsequently, values remained similar at ambient $p_{\text{CO}_2}$, while a transient decrease was observed at elevated $p_{\text{CO}_2}$.

### 4.4.2.2 Experiment II

#### A. Leaves

During the regrowth period from August to September 1994, starch concentrations in leaves of *T. repens* were consistently higher at elevated than at ambient $p_{\text{CO}_2}$ (Fig. 4-11a,c). This effect was significant overall and for day 8 after defoliation in first (youngest) fully expanded leaves (Fig. 4-11a). In second fully expanded leaves, the effect of elevated $p_{\text{CO}_2}$ was significant overall and for days 0 and 8 after defoliation (Fig. 4-11c). In contrast, WSC concentrations were not significantly increased at elevated $p_{\text{CO}_2}$ even though a tendency towards higher concentrations was apparent at most harvests (Fig. 4-11b,d). Leaf starch concentrations were low at the beginning of the experiment and rose to a maximum between one and two weeks after defoliation before declining again. This increase and the subsequent decrease were more pronounced at elevated than at ambient $p_{\text{CO}_2}$ (Fig. 4-11a,c). Concentrations of WSC rose up to day 8 (starting from day 4 in first fully expanded leaves) and remained constant thereafter. There was no effect of elevated $p_{\text{CO}_2}$ on the time course of WSC concentrations (Fig. 4-11b,d).

#### B. Stolons

In old stolon sections, there was a consistent tendency towards higher starch concentrations at elevated $p_{\text{CO}_2}$ (Fig. 4-11e). WSC concentrations were only significantly increased by elevated $p_{\text{CO}_2}$ before defoliation (Fig. 4-11f, day 0). In contrast, in apical stolon sections, higher concentrations at elevated $p_{\text{CO}_2}$ than at ambient $p_{\text{CO}_2}$ were observed both for starch (significant overall and for day 14; Fig. 4-11g) and for WSC (significant overall and for day 0; Fig. 4-11h). After defoliation, starch concentrations in old stolon sections markedly decreased up to day 8 after defoliation (Fig. 4-11e). Subsequently, there was a pronounced accumulation of starch and initial starch concentrations were exceeded at the end of the experiment. There was no significant effect of elevated $p_{\text{CO}_2}$ on either the remobilisation or the accumulation of starch. WSC concentrations showed only a slight decrease after defoliation but increased to concentrations higher than the initial level before day 8 after defoliation (Fig. 4-11f). Again, there was no effect of elevated $p_{\text{CO}_2}$ on the time course of WSC concentrations.

In apical stolon sections, there was a significant decrease in both starch (Fig. 4-11g) and WSC (Fig. 4-11h) concentrations during the first 4 d after defoliation which was significantly more pronounced at elevated $p_{\text{CO}_2}$ for WSC. As a consequence, starch concentrations on days 4 and 8 after defoliation no longer differed between $p_{\text{CO}_2}$ treatments. Both WSC and starch concentrations started to recover before day 8 after defoliation. The accumulation of starch was more pronounced at elevated than at ambient $p_{\text{CO}_2}$ (Fig. 4-11g).
For the first time, the effect of elevated $p_{CO_2}$ on the shoot carbohydrate concentration of a forage legume has been studied during regrowth. Similar patterns of carbohydrate concentrations in stolons of *T. repens* in the field and in the growth chamber suggest a broad similarity of the responses to elevated $p_{CO_2}$ in both systems. In spite of strong evidence for an increased availability of carbohydrates at elevated $p_{CO_2}$, recovery from defoliation did not appear to be significantly accelerated at elevated $p_{CO_2}$. The time lag until an increase in stolon and root dry mass could be observed was largely similar in both $p_{CO_2}$ treatments and only the replenishment of reserves proceeded faster at elevated $p_{CO_2}$.

### 4.5 Discussion

For the first time, the effect of elevated $p_{CO_2}$ on the shoot carbohydrate concentration of a forage legume has been studied during regrowth. Similar patterns of carbohydrate concentrations in stolons of *T. repens* in the field and in the growth chamber suggest a broad similarity of the responses to elevated $p_{CO_2}$ in both systems. In spite of strong evidence for an increased availability of carbohydrates at elevated $p_{CO_2}$, recovery from defoliation did not appear to be significantly accelerated at elevated $p_{CO_2}$. The time lag until an increase in stolon and root dry mass could be observed was largely similar in both $p_{CO_2}$ treatments and only the replenishment of reserves proceeded faster at elevated $p_{CO_2}$.

#### 4.5.1 Analysis of response mechanisms in controlled environment chambers

**4.5.1.1 Enhanced photosynthesis and carbohydrate availability at elevated $p_{CO_2}$**

Rates of photosynthesis per unit leaf area are generally increased at elevated $p_{CO_2}$ (Long *et al.* 1995). In accordance with previous reports of increased leaf photosynthesis in *T. repens* (e.g. Scheidegger and Nösberger 1984), measurements at the end of regrowth suggested a 51% stimulation of the light saturated rate of photosynthesis per unit leaf area at elevated relative to ambient $p_{CO_2}$ (results not shown). Indeed, WSC concentrations suggested that carbohydrate availability was higher in plants growing at elevated than in plants growing at ambient $p_{CO_2}$ throughout the regrowth period (Fig. 4-7b,d,f).

The removal of all fully developed leaves of *T. repens* has been shown to cause reduced or retarded growth of many tissues: Newly produced leaves had a smaller area (Carlson 1966b; King *et al.* 1978; Baur-Höch 1988) and both the rate of leaf emergence (Carlson 1966b) and the number of nodes produced on lateral stolons (King *et al.* 1978) were reduced. Hence, an attractive interpretation would be that these responses were caused by the reduction in the amount of assimilates available from current photosynthesis. Consequently, the reduction in leaf size was interpreted as evidence for a lack of assimilates by Baur-Höch (1988). I therefore expected that the enhanced availability of carbohydrates observed at elevated $p_{CO_2}$ would compensate at least partly for the effects of defo-
lilation and lead to a growth advantage for plants exposed to elevated $p_{\text{CO}_2}$ during early regrowth.

4.5.1.2 No acceleration of recovery from defoliation at elevated $p_{\text{CO}_2}$

Growth of total plant dry matter ceased completely during the first 3 d after defoliation at high N supply and during the first 4 d after defoliation at low N supply (Fig. 4-2a). Initially, the production of leaf dry matter was balanced by a temporary loss of dry matter from stolons and roots (Fig. 4-2c,d), largely due to the mobilisation of carbohydrate reserves (Fig. 4-7c,d). Thus, the plants were just barely able to maintain a balanced C budget after defoliation. Further, rates of stolon starch mobilisation were similar in all treatments (Fig. 4-7c) and appeared to be even somewhat higher at elevated $p_{\text{CO}_2}$ and low N supply than in the other treatments. These findings suggest that the demand for carbohydrates not covered by current photosynthesis after defoliation was similar relative to the amount of stolon tissue present in the plants in both $p_{\text{CO}_2}$ treatments.

Even though the rate of reserve mobilisation was not reduced, one might still expect the recovery from defoliation to be accelerated as a consequence of the enhanced efficiency of photosynthesis at elevated $p_{\text{CO}_2}$. However, net growth of stolons and roots did not resume until between 4 to 7 d after defoliation (Fig. 4-2c,d). There was no distinguishable effect of $p_{\text{CO}_2}$ on the duration of this lag. At about the same time, a rise in leaf and stolon carbohydrate concentrations indicated that carbohydrate availability started to exceed the immediate needs of the plant, particularly at elevated $p_{\text{CO}_2}$ (Fig. 4-7a,b).

Nevertheless, reserve mobilisation appeared to continue in all treatments until stolon starch concentrations had been reduced to low values. Replenishment of stolon starch reserves did not start until about 7 to 11 d after defoliation (Fig. 4-7c,e). This was only just before recovery was complete at about 11 d after defoliation, as assessed from the restoration of near initial and/or stable values of root:shoot ratio, LWR and LAR (Fig. 4-4). Again, there was no evidence for an acceleration of recovery under elevated $p_{\text{CO}_2}$.

4.5.1.3 Which processes were important in determining the demand for carbohydrates after defoliation?

The time course of the number of fully expanded leaves per plant (Fig. 4-5b) suggests that the plants grown at elevated $p_{\text{CO}_2}$ took longer to expand new leaves, since the number of growing points was similar in both $p_{\text{CO}_2}$ treatments (Fig. 4-3b). As a consequence, plants grown at ambient $p_{\text{CO}_2}$ transiently had a greater leaf area per plant than their counterparts at elevated $p_{\text{CO}_2}$ until day 7 after defoliation (Fig. 4-3a) when the fraction and total number of fully expanded leaves became similar in both $p_{\text{CO}_2}$ treatments (Fig. 4-5b). During that period, the difference in LAR between $p_{\text{CO}_2}$ treatments was even more pronounced than during later regrowth (Fig. 4-4c). The plants growing at elevated $p_{\text{CO}_2}$ produced leaves with a greater area than the plants growing at ambient $p_{\text{CO}_2}$ (Fig. 4-5a) as previously observed (Scheidegger and Nösberger 1984; Ryle and Powell 1992).
Therefore, one might speculate that these leaves took longer to expand because of their larger area. Carlson (1966b) reported that less time was required for the full expansion of new leaves after defoliation or shading of *T. repens* cv. ladino. While his interpretation was based on a postulated action of growth substances, it might also be possible that the duration of leaf expansion depended on final leaf size (which was reduced in both of his treatments) or on C availability. However, another study found no effect of defoliation on the duration of leaf expansion (King *et al.* 1978).

The finding of an apparent retardation of leaf development at elevated $p_{CO_2}$ was quite unexpected. In a study with ramets of *T. repens* which were pre-grown at 20 vs. 100 Pa $p_{CO_2}$, defoliated and regrown at ambient $p_{CO_2}$, Baur-Höch (1988) found that after 5 d, the plants pre-grown at high $p_{CO_2}$ had produced a greater amount of leaf dry matter per plant than the plants pre-grown at low $p_{CO_2}$. Unfortunately, leaf area was not determined in that experiment. Since the Carlson scale of leaf development was not re-validated for the two $p_{CO_2}$ treatments used in the present study, it cannot be entirely excluded that there were systematic differences in the stage of development of the oldest leaves left on the plants at defoliation. However, it appears highly unlikely that such systematic errors caused the apparent lag in leaf development as there was no consistent effect of $p_{CO_2}$ on the proportion of leaf dry matter remaining on the plants (cf. Fig. 4-2b). One possible explanation for the absence of a stimulatory effect of $p_{CO_2}$ on the regrowth of leaves after defoliation would be that carbohydrates never limited leaf growth in any treatment because, generally, the carbohydrate demand of the apex in defoliated *T. repens* is met with relatively high priority (Chapman and Robson 1988; Chapman *et al.* 1991). How does the level of reserves available before defoliation in the present experiment compare to other experiments?

In an attempt to elucidate the effect of different levels of carbohydrate reserves on regrowth, Baur-Höch (1990) grew rooted cuttings of *T. repens* at 20 and 100 Pa $p_{CO_2}$. After a severe defoliation, similar to that imposed in the growth chamber experiment of this study, regrowth was enhanced at elevated $p_{CO_2}$ during the first 5 days after defoliation. It was concluded that this difference was due to the higher stolon total non-structural carbohydrate (TNC) content of 22.4 % in the plants pre-grown at 100 Pa $p_{CO_2}$ compared to a content of 14.3 % in the plants pre-grown at 20 Pa $p_{CO_2}$. However, as $p_{CO_2}$ is known to increase also leaf size (Scheidegger and Nösberger 1984; this study) which is largely determined during early growth (discussed in Chapman 1989), this initial growth advantage may represent a carry-over effect from the pre-defoliation growth conditions rather than a consequence of differing stolon TNC reserve concentrations.

In an experiment involving repeated pre-cutting and shading treatments to reduce stolon carbohydrate content, Scheidegger and Nösberger (1983) were able to produce plants with a TNC concentration in their stolons of between 4.7 and 21.9 %. However, after a defoliation leaving only leaves at stage 0.5 according to Carlson (1966a), they found no consistent relation between initial RGR and stolon carbohydrate reserves. In fact, the
highest initial relative growth rate for total plant and leaf dry mass was observed in the plants with only 9% TNC in their stolons.

Thus, it appears that even stolon TNC concentrations as low as 9% may already be non-limiting for the regrowth of leaves after defoliation. In the present experiment, stolon TNC concentrations before defoliation were in the range of 14.6% (ambient $p_{\text{CO}_2}$ and high N supply) to 31.0% (elevated $p_{\text{CO}_2}$ and low N supply). The lack of a stimulation of initial leaf growth suggests that carbohydrate reserves in combination with current assimilation were sufficient to support maximal rates of leaf growth in all treatments. Thus, it is conceivable that effects of defoliation on leaf size as discussed above were not under the direct control of carbohydrate availability but might have depended on other factors, e.g. on changes in light quality at the apices and stolons (cf. Robin et al. 1992), on changes in the microclimate at the apex or on alterations in the hormone status of the plant (as postulated by Carlson 1966b; cf. Thomas 1987 for a discussion of hormonal effects on leaf size).

4.5.1.4 A model for carbohydrate relations during the recovery phase

Similarly, the mobilisation of starch may be, at least in part, under hormonal control. After a defoliation of $T. \text{repens}$ ramets with a simultaneous excision of the root system, a pronounced accumulation of sucrose and hexose indicated that starch was being mobilised well in excess of the demand for regrowth (Baur-Höch et al. 1990). In fact, the mobilisation of starch proceeded at rates similar to those observed in defoliated plants with intact roots. It was postulated that starch degradation after defoliation was controlled by gibberellins exported from stolon tips since starch degradation was significantly reduced in plants whose stolon tips were removed at the time of defoliation. Recent results of Satya Pasumarty (Massey University, personal communication) support the hypothesis that the mobilisation of starch is controlled by the balance of the stimulatory effect of gibberellins released from the apex (Cohen and Dovrat 1976) and the inhibitory effect of abscisic acid (ABA) released from leaves.

The operation of such a regulatory mechanism may explain why there was no effect of $p_{\text{CO}_2}$ on the rate and duration of starch mobilisation even though WSC concentrations suggested a higher carbohydrate availability at elevated than at ambient $p_{\text{CO}_2}$ throughout the experiment (note the rise in leaf WSC concentrations already apparent between days 4–7, particularly at elevated $p_{\text{CO}_2}$): If the phase of starch mobilisation in old stolons continues until newly grown leaves produce sufficient ABA to stop further starch mobilisation, then the accumulation of new reserves can only start after a sufficient number of new leaves has been produced; therefore, reserve accumulation in stolons is little affected by the photosynthetic efficiency of these leaves and its influence on carbohydrate availability. Such a model would also explain the observation made by Baur-Höch (1990) that the replenishment of reserves appeared to depend on the residual leaf area rather than on the amount of reserves present at the time of defoliation. Similar control
mechanisms may have been responsible for the transient halt in the production of new growing points between days 3 and 4 (Fig. 4-3b) which also appeared independent of carbohydrate availability.

In contrast to the lack of an effect of $p_{\text{CO}_2}$ on the mobilisation phase, there was a distinct stimulation of the rate of reserve starch accumulation in stolons by elevated $p_{\text{CO}_2}$ (Fig. 4-7c,e). As a consequence, plants growing at elevated $p_{\text{CO}_2}$ accumulated significantly more starch in their stolons during the last two weeks of the experimental period than the plants growing at ambient $P_{\text{CO}_2}$. The final concentration of total nonstructural carbohydrate at high N supply showed a similar effect of $p_{\text{CO}_2}$ as that found by Scheidegger and Nösberger (1984) using much more extreme levels of $P_{\text{CO}_2}$ (20 Pa vs. 100 Pa).

Does such an enhanced accumulation of reserves confer an advantage to plants exposed to frequent, severe defoliation when grown at elevated $p_{\text{CO}_2}$? Depending on the critical concentration of stolon carbohydrate reserves necessary to support maximum rates of leaf growth after defoliation, there may be a short period of time during which this level of reserves has already been reached at elevated $p_{\text{CO}_2}$ but not yet at ambient $p_{\text{CO}_2}$. However, during the accumulation phase, the difference in time when a given TNC concentration in total stolon tissue is reached by plants in contrasting $p_{\text{CO}_2}$ treatments never appears to exceed 10 d. Further, a level of 10 % TNC in the dry matter is reached no later than about 17 d after defoliation in all treatments. Thus, for most defoliation intervals, the advantage that is conferred by the higher rate of starch accumulation at elevated $p_{\text{CO}_2}$ will be small or non-existent.

### 4.5.1.5 Stimulation of growth during the recovery phase

In spite of the similar duration of the recovery from defoliation, there was a stimulation of RGR during the first 11 d of regrowth. This stimulation was evident for total dry mass, leaf dry mass and root dry mass (significant Time $\times$ $p_{\text{CO}_2}$ effects in Tab. 4-1), but not for stolon dry mass or leaf area. A similar effect of elevated $p_{\text{CO}_2}$ was also evident when plants at day 11 were compared to uncut plants before defoliation (not shown). Thus, in spite of the apparent initial lag in leaf development, the plants growing at elevated $p_{\text{CO}_2}$ were able to further increase their size advantage during regrowth.

In fact, most of the stimulation of both leaf and root growth at elevated $p_{\text{CO}_2}$ appears to have originated from the period around days 4 to 7 when the relative growth rate of leaf area declined at ambient but not at elevated $p_{\text{CO}_2}$ (Fig. 4-3a) and plants at elevated $p_{\text{CO}_2}$ started to have a greater leaf area than plants at ambient $p_{\text{CO}_2}$. A stimulation of growth at elevated $p_{\text{CO}_2}$ was no longer apparent after day 7. These findings contrast with the conclusion drawn by Nijs et al. (1988a) that a difference between $P_{\text{CO}_2}$ treatments in the dry matter accumulated above cutting height originated during the first days of regrowth of swards of T. repens (differences were already evident at the first harvest three days after defoliation).

Which are the underlying processes that led to such a growth response?
A. A tendency towards an increased relative growth rate of the number of growing points

Total plant RGR can be partitioned as the sum of the relative growth rate of the number of growing points and the relative growth rate of the average dry mass per growing point (van Loo 1992). However, attempts to assign this growth stimulation to either a faster increase in the number of growing points or to a faster increase in the dry mass per growing point yielded somewhat inconclusive results: There was a tendency towards an increased relative growth rate of the number of growing points at elevated $p_{\text{CO}_2}$ (Fig. 4-3b). However, this effect was only significant at $p \leq 0.1$, even though a significant difference in the number of growing points per plant was observed at day 11 after defoliation. At the same time, significantly more growing points were classified as secondary stolons (Fig. 4-3c) suggesting that growing points may have been more vigorous at elevated than at ambient $p_{\text{CO}_2}$.

In contrast, there was no change with time in the relative effect of $p_{\text{CO}_2}$ on the average dry mass per growing point (Tab. 4-2). An effect of $p_{\text{CO}_2}$ on the relative growth rate of leaf dry mass per growing point was evident during regrowth of defoliated plants. However, no such effect was found in a comparison of plants at day 11 with plants before defoliation (not shown). Thus, the effect of $p_{\text{CO}_2}$ on the relative growth rate of leaf dry mass per growing point may have been the consequence of a greater relative effect of elevated $p_{\text{CO}_2}$ on leaf dry mass in fully expanded leaves (which were predominant after recovery) than in developing leaves (the only leaf category remaining after defoliation).

It therefore appears that the increase in total plant RGR (+18 %) observed at elevated relative to ambient $p_{\text{CO}_2}$ was related to a somewhat higher (+16 %) relative growth rate of the number of growing points per plant at elevated $p_{\text{CO}_2}$. While the effect of $p_{\text{CO}_2}$ on the total number of growing points became significant only by day 11 after defoliation, the effect on the number of growing points classified as secondary stolons was apparent (though not significant) already at day 7 and thus may have been related to the observed stimulation of whole plant RGR. However, a detailed analysis of the relationship between the production of growing points and dry matter production is not possible since no information has been recorded about the size distribution of leaves and internodes, on the presence of nodal roots or on the productivity of individual growing points. Similarly, it is not possible to determine whether the increased production of growing points at elevated $p_{\text{CO}_2}$ was due to a faster leaf appearance rate or due to an enhanced activation of buds.

Was this apparent stimulation of the rate of production of new growing points related to defoliation? Even though an effect of $p_{\text{CO}_2}$ was apparent following defoliation, there was no significant difference in the number of growing points counted after 35 d of establishment and growth at contrasting levels of $p_{\text{CO}_2}$ (starting with the preparation of cuttings with a similar number of nodes). Also, no further stimulation of whole plant RGR was apparent beyond day 7 after defoliation (Fig. 4-2a). These observations suggest that the response to elevated $p_{\text{CO}_2}$ may have been dependent on the defoliation. Yet, Scheid-
egger and Nösberger (1984) did not find an effect of even more distinct levels of $p_{\text{CO}_2}$ (100 Pa vs. 20 Pa) on the production of new growing points in $T. \text{repens}$ during either acclimation to elevated $p_{\text{CO}_2}$ or regrowth after defoliation. However, defoliation was much less severe in their study than in the present experiment: They removed only fully expanded leaves (stage 1.0 on the scale of Carlson 1966a). As demonstrated by Baur-Höch (1988), a similarly lenient defoliation treatment had a much smaller effect on the production of secondary stolons than a more severe defoliation treatment (which was comparable to that applied in the present experiment). In a study of Ryle and Powell (1992) with plants defoliated to approximately one fully expanded leaf per every two stolons every other day, elevated $p_{\text{CO}_2}$ stimulated the rate of stolon production exclusively during an initial acclimation phase. As there were also other pronounced effects of such a high defoliation frequency on plant morphology, their results are not directly comparable to the present experiment. However, the present results support the conclusion that elevated $p_{\text{CO}_2}$ can enhance the production of new growing points after severe or repeated defoliation. It therefore appears that either leaf appearance rate or the activation of axillary buds was C limited and thus responsive to elevated $p_{\text{CO}_2}$ during a brief phase after defoliation.

While elevated $p_{\text{CO}_2}$ stimulated the appearance of new growing points about one week after defoliation, a transient halt in the production of new growing points between days 3 and 4 after defoliation was apparent in all treatments and was not affected by differences in carbohydrate availability between $p_{\text{CO}_2}$ treatments (Fig. 4-3b). While this halt was clearly related to defoliation (a similar effect was observed after defoliation of $L. \text{perenne}$ in Part 3) the effect may have been mediated not through carbohydrate availability but through some other mechanism.

**B. More dry matter per growing point**

In spite of the enhanced production of new growing points at elevated $p_{\text{CO}_2}$, size differences were largely due to a greater dry mass per growing point throughout the experiment (Tab. 4-2): Averaged over both N treatments, a 24 % higher dry mass per plant at elevated $p_{\text{CO}_2}$ before the cut resulted from a 2 % lower number of growing points and a 26 % higher dry mass per growing point; 11 d after defoliation, a 42 % higher dry mass per plant at elevated $p_{\text{CO}_2}$ resulted from a 12 % higher number of growing points and a 28 % higher dry mass per growing point. Similarly, in a study on the regrowth of $T. \text{repens}$ at 20 Pa vs. 100 Pa $p_{\text{CO}_2}$, Scheidegger and Nösberger (1984) found a very pronounced effect of elevated $p_{\text{CO}_2}$ on the dry mass produced per growing point. At the same time, they observed that as much as 82 % of the difference in leaf dry mass observed between treatments after 18 d of regrowth was accounted for by carbohydrate accumulation; in stolons and roots, this contribution was still 30 % and 18 %, respectively. While carbohydrate accumulation also contributed significantly to the effect of elevated $p_{\text{CO}_2}$ on plant dry mass in the present experiment, a significant effect of elevated $p_{\text{CO}_2}$ on the structural dry mass produced per growing point remained in leaves and stolons (Tab. 4-2). The increase in leaf dry mass at elevated $p_{\text{CO}_2}$ was mainly the result of
both a higher leaf area per leaf and a greater SLW. Only the increase in SLW could be largely attributed to an accumulation of non-structural carbohydrates, particularly at low N supply (Fig. 4-6). There were no significant differences between $p_{\text{CO}_2}$ treatments in the number of fully expanded leaves per growing point after recovery (less than 2% at day 11, compare Fig. 4-5b with Fig. 4-3b).

### 4.5.1.6 Was the growth response to elevated $p_{\text{CO}_2}$ limited by N availability?

Pronounced effects on RGR were also observed in response to the N treatments (Fig. 4-2, Time × N effect in Tab. 4-1). Thus, differences which had developed during the first week of growth at contrasting levels of N supply were further increased for total dry mass (Fig. 4-2a), stolon dry mass (Fig. 4-2c), leaf area (Fig. 4-3a) and the number of growing points (Fig. 4-3b). It is known that high levels of NO$_3^-$ fertilisation can stimulate growth even relative to well-nodulated plants with efficient symbiotic N$_2$ fixation (Chapman 1989). However, the transient nature of this growth stimulation (no or only small effects of N treatments on growth after day 7) suggests that growth was only temporarily limited by N availability.

In fact, the transient stimulation of RGR at elevated $p_{\text{CO}_2}$ was observed in both N treatments during a period when RGR differed between N treatments but was no longer apparent at the end of regrowth when RGR was similar in both N treatments. This suggests that the lack of a persistent relative growth response to elevated $p_{\text{CO}_2}$ did not depend on N supply.

But could it be that the absence of a relative growth response to elevate $p_{\text{CO}_2}$ during the first four days of regrowth was due to a N limitation of leaf growth? It is known that defoliation reduces rates of symbiotic N$_2$ fixation in *T. repens* (Hart 1987). Marriott and Haystead (1992) found an increase in the (small) contribution of fertiliser N to the total N uptake after lenient defoliation. However, the decrease in N$_2$ fixation activity after defoliation was concluded to depend on a decrease in N demand due to reduced growth (Hartwig *et al.* 1994). Similarly, Seresinhe *et al.* (1994) found in a field study that the contribution of symbiotic N$_2$ fixation to the N supply of *T. repens* did not depend on the height of defoliation; they concluded that the rate of regrowth was not limited by the supply of N from symbiotic N$_2$ fixation. Further, it has been shown that N redistributed from stolons and roots makes up a large percentage of the N found in new regrowth in *T. repens*, and putative storage proteins have been identified which are degraded during regrowth and subsequently resynthesized (Corre *et al.* 1996). However, it is not yet known whether the availability of these storage proteins contributes to defoliation tolerance. At least the plants growing at high N supply should have had ample N reserves, even in the case that N assimilation after defoliation had been limited. Thus, it appears highly unlikely that the responsiveness of initial regrowth to elevated $p_{\text{CO}_2}$ was limited by N availability.
In either case, a more detailed analysis of partitioning during regrowth should allow to gain some insight into the strategies which *T. repens* uses to adjust to different levels of C and N availability during regrowth.

### 4.5.1.7 Maintaining balanced carbon and nitrogen relations at elevated $p_{\text{CO}_2}$

#### A. Changes in root:shoot ratio in response to nitrogen availability

In agreement with common findings, the root:shoot ratio of *T. repens* was increased at low N supply (Fig. 4-4a). At the same time, LWR was decreased (Fig. 4-4b). There was no effect of elevated $p_{\text{CO}_2}$ on the root:shoot ratio or on LWR at high N supply (Fig. 4-4a,b). Only at low N supply, the root:shoot ratio was decreased and LWR increased by elevated $p_{\text{CO}_2}$. A detailed analysis of the plants before defoliation suggests that both the effect of $p_{\text{CO}_2}$ on LWR and the interaction with N supply were due to the accumulation of carbohydrate in leaves (cf. Fig. 4-7). For LWR of the undefoliated plants calculated on the basis of TNC-corrected dry mass, only a reduction at low N supply was found (not shown). Similarly, no more effect of $p_{\text{CO}_2}$ was apparent at either level of N supply if the root:shoot ratio was calculated on the basis of TNC-corrected dry mass (not shown).

The effect of elevated $p_{\text{CO}_2}$ on the root:shoot ratio contrasts with typical findings for other plant species (Poorter 1993; Poorter et al. 1996). However, observations of a reduced root:shoot ratio at elevated $p_{\text{CO}_2}$ in *T. repens* have also been made in other growth chamber experiments with N-free nutrient solution (Ryle and Powell 1992; Zanetti 1997). Interestingly, Zanetti (1997) also found a reduction of the root:shoot ratio in response to elevated $p_{\text{CO}_2}$ in a high N supply treatment (7.5 mM N as NH$_4$NO$_3$). It appears that *T. repens* does not increase root growth in response to an increased availability of C relative to that of mineral N. In species relying on soil N for their N supply, an increase in dry matter partitioning to roots in response to elevated $p_{\text{CO}_2}$, which is frequently observed at a limiting availability of soil N, is interpreted as an adaptive strategy to balance the uptake of N and the assimilation of C (e.g. Stulen and Den Hertog 1993). As *T. repens* has been shown to be able to increase its rate of symbiotic N$_2$ fixation at elevated $p_{\text{CO}_2}$ in the growth chamber as well as in the field (Masterson and Sherwood 1978; Zanetti et al. 1996), there may be no need to increase the exploitation of the root space in search of N, even if growth rates are increased at elevated $p_{\text{CO}_2}$.

#### B. Adjustment to an increased carbon availability at elevated $p_{\text{CO}_2}$

The effect of elevated $p_{\text{CO}_2}$ on total plant dry mass was much greater than the effect on leaf area per plant (Figs 4-2a & 4-3a). Therefore, LAR was significantly lower at elevated than at ambient $p_{\text{CO}_2}$ throughout the experiment in spite of the greater leaf area per leaf (Fig. 4-4c). No consistent effect of N supply on LAR was observed.

As LAR was reduced but RGR was temporarily increased at elevated $p_{\text{CO}_2}$, there must have been a greater demand for carbohydrates per unit leaf area at elevated $p_{\text{CO}_2}$. This increase in demand was reflected in a consistent tendency towards a higher apparent
night-time carbohydrate export at elevated $p_{\text{CO}_2}$ over the period examined (Fig. 4-8). The effect of elevated $p_{\text{CO}_2}$ was most pronounced and statistically significant at day 7 after defoliation, around the time of maximal growth stimulation at elevated $p_{\text{CO}_2}$.

Diurnal differences in leaf carbohydrate concentrations were exclusively due to changes in the starch concentration (Fig. 4-8a). There was no significant diurnal accumulation of WSC (Fig. 4-8b). This contrasts with observations made by Scheidegger and Nösberger (1984) for $T. \text{repens}$ grown at a $p_{\text{CO}_2}$ of 100 Pa. They proposed that the apparent diurnal accumulation of ethanol-soluble carbohydrate indicated that the capacity of the leaves to accumulate starch had been exceeded. This was apparently not the case in the present experiment.

Clearly, the decrease in LAR (-12 % at day 11, averaged over both N treatments), balanced part of the stimulation of the rate of photosynthesis per unit leaf area at elevated $p_{\text{CO}_2}$. Such a response is typical for most plant species (Poorter 1993; Poorter et al. 1996; Part 3). However, the observed stimulation of RGR and the subsequently higher rates of reserve accumulation demonstrate that there remained a stimulation of photosynthesis in relation to standing plant biomass. But what caused the transient nature of the RGR stimulation? Carbohydrate availability kept increasing past the time of the RGR stimulation until the end of the experiment. Thus, other causes must be postulated. Even though care was taken to remove neighbouring plants with increasing plant size, photomorphogenetic responses to light quality (Robin et al. 1992; Lötscher 1994) cannot be excluded. Light quality perceived at the stolon tips has been shown to affect branching intensity by modulating the delay in branch appearance (Robin et al. 1994). Thus, a change in light quality at the stolon tips as more and more leaves unfolded may have prevented a further stimulation of branching at elevated $p_{\text{CO}_2}$. Since branching activity may also be influenced by phytohormones (Thomas 1987), the hormonal balance which was proposed to control the mobilisation of starch may also affect the responsiveness to elevated $p_{\text{CO}_2}$ of the production of new growing points. As in the present experiment, such or similar mechanisms may even lead to what appears as a priority of carbohydrate accumulation over a stimulation of growth in response to enhanced carbohydrate availability. In a speculative model, Acock (1990) proposed that a production of new growing points in response to elevated $p_{\text{CO}_2}$ occurs only after a plant has increased its dry matter density to a maximum. This model clearly does not apply in the present case because the stimulation of growth was maximal when carbohydrate reserves were lowest. More likely, reserve production, in the sense that carbohydrate is accumulated at the expense of potential growth (Stitt and Schulze 1994), may have evolved in $T. \text{repens}$ as an adaptation to frequent defoliation.

C. Effects of different levels of nitrogen supply

While N supply had strongly affected growth rates during a period of adjustment to the N treatments, and continued to do so during early regrowth, total plant RGR was apparently no longer affected by the N treatments from day 7 after defoliation. However, the
distribution of dry matter in the plant (in particular the root:shoot ratio, Fig. 4-4a) re-
ained different between N treatments. Apparently this shift in morphology allowed the
plants to maintain a similar RGR. A similar adjustment to different levels of N supply
which resulted in comparable growth was demonstrated in *Urtica dioica* (Beck 1994).

It appears that N supply also affected the reaction of leaf structural composition to ele-
vated $p_{\text{CO}_2}$ (Fig. 4-6). At low N supply, nearly all additional C accumulated as TNC. In
contrast, at high N supply, the structural dry mass per unit leaf area was also increased.
In fact, the continuous accumulation of starch in leaves of plants growing at elevated
$p_{\text{CO}_2}$ with low N supply (Fig. 4-7a) in spite of enhanced rates of apparent night-time ex-
port (Fig. 4-8a) suggests that not all assimilates could be exported during a complete
day/night cycle. There is ample evidence that the diurnal accumulation of starch is ad-
justed according to needs for a continued export of assimilates to sinks during the night
(Gordon 1986; Stitt and Schulze 1994). Therefore carbohydrate remaining in the leaves
at the end of the night may be seen as surplus to the plant’s demand for growth and re-
serve formation (Stitt and Schulze 1994). However, due to a feedback of sucrose con-
centration on the allocation of assimilate to starch (Stitt 1991), some increase in starch
production may also occur as a side effect of an increased sucrose concentration driving
higher rates of export. Such a mechanism may explain why local starch accumulation
occurred even when only small patches of leaves on large trees were exposed to ele-
vated $p_{\text{CO}_2}$ (Körner and Würth 1996) – a treatment judged by the authors not to have any
measurable influence on the source-sink relations in the whole plant. Thus, a moderate
increase in leaf starch content may be regarded as a standard effect of elevated $p_{\text{CO}_2}$.
However, the large effect of elevated $p_{\text{CO}_2}$ on leaf starch concentrations at low N supply
(also in comparison to effects observed in the field, Fig. 4-11a,c) suggests that the utili-
sation of the extra C assimilated was at least partly limited by low N supply. Interest-
ingly, starch concentrations in leaves mostly exceeded those in stolons, particularly at
low N supply. It is not known what mechanism prevented the accumulation of more
starch in the stolons in the present growth chamber experiment (as opposed to the field
experiment, Fig. 4-11e,g).

A limiting role of low N supply was also suggested by the lower organic leaf N content
per unit area (Fig. 4-6, shown as estimated crude protein). This low N concentration was
paralleled by a tendency towards a lower $V_{\text{c max}}$ at low N supply (Tab. 4-3). However,
there was no consistent effect of $p_{\text{CO}_2}$ on leaf N concentrations and no differences in
$V_{\text{c max}}$ were observed between $p_{\text{CO}_2}$ treatments. Similarly, Lewis (1994) found no signifi-
cant effect of elevated $p_{\text{CO}_2}$ on *T. repens* grown in FACE at high (4 weeks) defoliation
frequency and low N supply – a treatment which was also examined in the present
study; however, at low (8 weeks) defoliation frequency, a significant acclimation of
$V_{\text{c max}}$ was observed. An acclimation of photosynthetic rates in response to growth at
contrasting levels of $p_{\text{CO}_2}$ (100 Pa vs. 20 Pa) was also reported by Scheidegger and Nös-
berger (1984) and in response to elevated $p_{\text{CO}_2}$ (68 Pa vs. 34 Pa) with a concomitant 3 °C
increase in temperature by Ryle *et al.* (1992b). Thus, it cannot be excluded that the ap-
parent imbalance between photosynthesis and utilisation of assimilates for structural
growth eventually would have led to photosynthetic acclimation. However, during the regrowth period examined, a reduction of C assimilation at elevated $p_{\text{CO}_2}$ was only achieved through the reduction in LAR.

Since the fraction of N derived from symbiotic $\text{N}_2$ fixation was not determined, the extent to which an increase in symbiotic $\text{N}_2$ fixation may have helped to maintain a similar RGR in both N treatments cannot be assessed. Similarly, it can only be speculated, that a stimulation of symbiotic $\text{N}_2$ fixation as demonstrated by Zanetti et al. (1996) may have helped to cover the presumably higher N demand for the enhanced growth at elevated $p_{\text{CO}_2}$. In such a case, a higher rate of symbiotic $\text{N}_2$ fixation would also act as a sink for some of the additional C fixed at elevated $p_{\text{CO}_2}$. However, as discussed above, symbiotic $\text{N}_2$ fixation clearly did not fully compensate for the reduced N supply in the low N treatment.

### 4.5.2 Validity of these interpretations under field conditions

#### 4.5.2.1 Higher carbohydrate availability at elevated $p_{\text{CO}_2}$

Consistently higher concentrations of WSC (Figs. 4-9b & 4-11b,d,f,h) suggest that carbohydrate availability in *T. repens* exposed to elevated $p_{\text{CO}_2}$ in the field was increased in both experiments due to a persistent stimulation of photosynthesis at elevated $p_{\text{CO}_2}$ (Lewis 1994). Even though the extent of the effect of elevated $p_{\text{CO}_2}$ on WSC concentrations in first fully expanded leaves was more pronounced in Experiment I than in Experiment II, there were similar tendencies in both experiments which were consistent for nearly every time point. The interpretation that WSC concentrations are closely linked to the transport pool and, therefore, reflect carbohydrate availability is supported by the very similar effect of elevated $p_{\text{CO}_2}$ on WSC concentrations in every tissue examined as well as by the broad similarity of their time course in various tissues (Fig. 4-11b,d,f,h).

#### 4.5.2.2 No qualitative effect of $p_{\text{CO}_2}$ on the time course of stolon carbohydrate concentrations

While the higher carbohydrate availability at elevated $p_{\text{CO}_2}$ was reflected in consistently higher stolon starch concentrations, changes in stolon starch concentrations after defoliation followed a very similar pattern in both $p_{\text{CO}_2}$ treatments (Fig. 4-11e,g). Starch concentrations declined and started to rise again after about one week, thus following a time course essentially similar to that observed in the growth chamber experiment (Fig. 4-7c) or in published studies in the greenhouse (Moran et al. 1953) and in the growth chamber (Gordon et al. 1986; Baur-Höch et al. 1990; Kang and Brink 1995). In contrast to the initial hypotheses, the depletion of starch reserves was clearly not reduced at elevated $p_{\text{CO}_2}$. In fact, there was even a tendency towards a more pronounced mobilisation of starch reserves at elevated $p_{\text{CO}_2}$ (Fig. 4-11e,g). Similarly, a more pronounced drop in the WSC concentration of apical stolon sections was observed at elevated relative to ambient $p_{\text{CO}_2}$. 
In accordance with previous observations in the growth chamber, there was no evidence for an earlier start of the accumulation of new reserves. However, between days 8–14, rates of starch accumulation tended to be higher at elevated $p_{\text{CO}_2}$ in old stolons and were significantly enhanced in young stolon tissue (Fig. 4-11e,g). Subsequently, the effect of $p_{\text{CO}_2}$ on stolon starch concentrations remained unchanged. As in the growth chamber experiment, neither the rate nor the duration of starch mobilisation from stolons appeared to be controlled by carbohydrate availability. Can the very limited extent of this response to elevated $p_{\text{CO}_2}$ be explained by a model similar to that put forward for the growth chamber experiment?

### 4.5.2.3 Leaf carbohydrate concentrations suggest non-optimal regulation of reserve mobilisation

Leaf WSC concentrations in *T. repens* in undisturbed, fully developed plants were mostly low (Figs 4-9b, day 30 & 4-11b,d, days 0, 25). Significantly higher leaf WSC (and starch) concentrations at day 0 of Experiment I (Fig. 4-9) may be related to a series of comparatively cold days and nights just prior day 0 (Fig. 4-1). A pronounced accumulation of TNC in leaves of *T. repens* grown at low temperature as demonstrated by Boller and Nösberger (1983) may be related to the relatively high temperature optimum of this species (about 24 °C as estimated by Mitchell 1956b for growth at constant temperature in a controlled environment).

In spite of the relatively severe defoliation (removal of most leaves larger than Carlson stage 0.4–0.5, estimated from data given in Stadelmann 1993), there was only a short drop in stolon WSC concentrations and no decrease in leaf WSC in Experiment II (Fig. 4-11b,d,f,h). A rise in WSC concentrations in all tissues after day 4 indicated that carbohydrate availability recovered rapidly. At ambient $p_{\text{CO}_2}$, the time course of leaf WSC concentrations was mirrored by similar changes in starch concentrations. However, while the rise in leaf WSC concentrations at elevated $p_{\text{CO}_2}$ was similar to that at ambient $p_{\text{CO}_2}$, leaf starch concentrations at elevated $p_{\text{CO}_2}$ rose to a pronounced maximum around day 8 after defoliation, particularly in second fully expanded leaves (Figs 4-9 & 4-11a,b,c,d). As discussed above, a feedback from carbohydrate can lead to increased leaf starch concentrations if the carbohydrate availability in a plant is high relative to the demand, particularly at elevated $p_{\text{CO}_2}$. But what processes caused this strong increase in carbohydrate availability about one week after defoliation?

Differences in the physiological characteristics of leaves developing in full light (after defoliation) compared to leaves grown in the shade (before defoliation) were not likely: Unless young leaves of *T. repens* are prevented by a tall competitor from reaching the top of the canopy by the time they become flat (shortly before full expansion), their photosynthetic apparatus is able to adjust to the new light conditions (Dennis and Woledge 1982; Dennis and Woledge 1983).

More likely, carbohydrate availability at elevated $p_{\text{CO}_2}$ rose so strongly because the mobilisation of stolon starch reserves continued even though high rates of photosynthesis
of the developing canopy at elevated $p_{\text{CO}_2}$ would have been already sufficient to cover the plant’s needs for carbohydrate. As discussed above for the growth chamber experiment, it appears that the control of the duration and rate of starch mobilisation is such as to meet the needs for carbohydrate of a plant defoliated and regrowing at ambient $p_{\text{CO}_2}$, resulting in only small fluctuations in leaf WSC and starch concentrations (Figs 4-9 & 4-11a,b,c,d). At elevated $p_{\text{CO}_2}$, the plants were unable to take advantage of their better carbohydrate status and temporarily accumulated carbohydrate in their leaves instead of starting earlier with the accumulation of new reserves in stolons.

While there are several studies on the effect of defoliation on stolon carbohydrate reserves in *T. repens*, published information about the time course of leaf carbohydrate concentration is scarce. TNC concentrations averaged over three different pre-cutting treatments appear compatible with an earlier rise in leaf as opposed to stolon TNC concentrations and with a slight decrease in leaf TNC concentrations towards later regrowth (Kang and Brink 1995).

### 4.5.2.4 Higher demand for carbohydrates per unit leaf area

There was always a pronounced apparent night-time export (Fig. 4-10a), even though leaves retained some starch at the end of the night once that WSC concentrations had recovered (Fig. 4-9a,b, days 9–30). At elevated $p_{\text{CO}_2}$, the amount of carbohydrate lost from leaves during the night was nearly twice as high as that at ambient $p_{\text{CO}_2}$. It is likely that some of this difference in apparent night-time export rate was balanced by a lower LAR as demonstrated in the growth chamber experiment. However, if the decrease in LAR in the field was within a similar range as in the growth chamber, then this would indicate that carbohydrate utilisation by sinks was significantly enhanced at elevated $p_{\text{CO}_2}$ in *T. repens*. In part, this utilisation may have consisted of carbohydrate storage, but it appears likely that more carbohydrate was being used for growth processes. A consistently higher agronomic yield of *T. repens* at elevated $p_{\text{CO}_2}$ has been observed in the FACE experiment (Hebeisen et al. 1997). For the regrowth periods shown here, the average enhancement of yield (5 cm cutting height) at elevated $p_{\text{CO}_2}$ was 24% in Experiment I and 32% in Experiment II (Hebeisen 1997). Experiments with root ingrowth bags indicated a higher production of new roots during the summer of 1993 (Jongen et al. 1995). Thus, productivity per unit of ground area was increased at elevated $p_{\text{CO}_2}$. However, this increase in yield cannot be related directly to the productivity of growing points or biomass (RGR) since the relationship between total biomass and leaf area or the number of growing points is not known in that field experiment. Zanetti et al. (1996) demonstrated that *T. repens* grown at elevated $p_{\text{CO}_2}$ in FACE obtained a greater proportion of its N from symbiotic N$_2$ fixation than when grown at ambient $p_{\text{CO}_2}$. Thus, symbiotic N$_2$ fixation may represent another important sink for the extra C fixed at elevated $p_{\text{CO}_2}$. Unlike in the growth chamber experiment, symbiotic N$_2$ fixation in the field appeared to fully compensate for differences in N availability between N treatments, as there were no significant differences in carbohydrate accumulation or apparent night-
time export. Similarly, N treatments had no significant effect on the yield in the field (Hebeisen et al. 1997).

### 4.5.3 Conclusions

Both in the field and in a controlled environment, increased carbohydrate concentrations and a significantly accelerated replenishment of stolon starch reserves indicated that C availability was consistently enhanced in *T. repens* growing at elevated $p_{\text{CO}_2}$. Together with a stimulation of the production of structural tissue components, the carbohydrate accumulation resulted in a higher dry mass per growing point as observed in the growth chamber. In fact, this increase in dry mass per growing point was the principal growth response to elevated $p_{\text{CO}_2}$ in the growth chamber. Even though the higher C availability at elevated $p_{\text{CO}_2}$ might be expected to alleviate the impact of defoliation and thus to promote regrowth under favourable conditions, initial regrowth was not enhanced. During the first four days after defoliation, regrowth proceeded essentially in proportion to the existing dry mass and appeared to depend more directly on the vigour of meristems present at the time of defoliation than on either carbohydrate reserves or rates of current photosynthesis. Similarly, both in a controlled environment and under field conditions, the duration of carbohydrate mobilisation after defoliation was similar in both $p_{\text{CO}_2}$ treatments and continued beyond the apparent recovery of the plants’ carbohydrate status at elevated $p_{\text{CO}_2}$. Thus, control mechanisms were apparently unable to respond to the enhanced rates of photosynthesis per unit leaf area at elevated $p_{\text{CO}_2}$. Nevertheless, a brief phase of enhanced RGR at elevated $p_{\text{CO}_2}$ in combination with an increased production of new growing points was observed in the growth chamber about one week after defoliation. Thus, in agreement with the initial hypothesis, regrowth was temporarily limited by C availability after a severe defoliation, and the responsiveness of growth to elevated $p_{\text{CO}_2}$, as it is typically observed in seedlings, was transiently restored after defoliation. However, the increase in RGR remained small in comparison to the persistent effect of elevated $p_{\text{CO}_2}$ on the dry mass accumulated per growing point. Even though there was a pronounced accumulation of leaf starch at low N supply in the growth chamber, RGR was not persistently altered by N treatments. It is therefore concluded that the long-term response of *T. repens* to elevated $p_{\text{CO}_2}$ was restricted by internal or external factors other than N availability and that this restriction was only briefly released after defoliation. As patterns of carbohydrate concentrations were similar in the field and in the growth chamber, the higher leaf carbohydrate concentrations and the enhanced accumulation of carbohydrate reserves towards the end of regrowth reflected an apparent sink limitation of the growth response to elevated $p_{\text{CO}_2}$ in both systems.
5. GENERAL DISCUSSION: LIMITATIONS IN THE RESPONSE OF REGROWTH AND GROWTH TO ELEVATED $P_{\text{CO}_2}$ - A COMPARISON OF TWO SPECIES

In a combination of field and growth chamber experiments, the main hypothesis tested was that an enhanced C availability at elevated atmospheric $P_{\text{CO}_2}$ would alleviate the impact of defoliation on source–sink relations and thus stimulate regrowth and recovery of carbohydrate reserves after defoliation. Indeed, higher concentrations of non-structural carbohydrates were generally observed in both $L. \text{perenne}$ and $T. \text{repens}$. Also, a consistent increase in the amount of dry matter accumulated per growing point or per unit leaf area indicated an enhanced availability of C building blocks.

5.1 Limited or no relative growth response in controlled environments.

5.1.1 No stimulation of initial regrowth after defoliation

However, both in $L. \text{perenne}$ and $T. \text{repens}$, there was no evidence for a specific enhancement of leaf growth during the first few days of regrowth at elevated $P_{\text{CO}_2}$ in the growth chamber. Neither the higher carbohydrate availability in $T. \text{repens}$ nor the initially lower carbohydrate reserve concentrations in $L. \text{perenne}$ had a clear effect on initial regrowth. Instead, initial regrowth appeared to occur in proportion to the size of existing organs and the apparent vigour of shoot meristems. These results suggest that the availability of carbohydrate from reserves and current photosynthesis was non-limiting for the regrowth of leaves in both experiments. Differences in the time course of initial regrowth between $L. \text{perenne}$ and $T. \text{repens}$ may be related to their different patterns of leaf development and to differences in the severity of defoliation. While there was little residual leaf area in $T. \text{repens}$, photosynthesis of leaf sheaths and residual parts of leaf blades in $L. \text{perenne}$ may have contributed significantly to carbohydrate availability. Therefore, it cannot be excluded that the regrowth of new leaves in $L. \text{perenne}$ was limited by factors other than carbohydrate availability and a stimulation of regrowth at elevated $P_{\text{CO}_2}$ would have been observed after a more severe defoliation. However, the treatments employed in the growth chamber experiments were chosen such as to approximate the severity of defoliation in the field experiments, which represents typical agricultural practice for swards cut for hay. Further, even if a more severe defoliation regime were to cause a true C limitation of leaf regrowth, morphological adjustments may still counteract a stimulation of regrowth at elevated $P_{\text{CO}_2}$ (cf. Section 5.4).

5.1.2 Growth stimulation depended on an enhanced production of new growing points

Three distinct growth processes which might be affected by elevated $P_{\text{CO}_2}$ were identified in the general introduction (Part 1). Both structural and non-structural dry mass accu-
mulation per growing point were clearly enhanced at elevated $p_{\text{CO}_2}$ and this appeared to be the principal growth response to elevated $p_{\text{CO}_2}$ in both *L. perenne* (Part 3) and *T. repens* (Part 4). In both species, relative treatment effects on the dry mass accumulated per growing point were essentially similar before defoliation and during regrowth. Apparently, the relative effect of elevated $p_{\text{CO}_2}$ on dry matter accumulation per growing point remained unchanged once established and no longer contributed to differences in RGR once the plants had acclimatised to growth at elevated $p_{\text{CO}_2}$.

Rates of development of newly formed organs were not studied specifically, but as suggested by the observations made in *T. repens*, leaf expansion did not appear accelerated at elevated $p_{\text{CO}_2}$ (Part 4). Similarly, the rate of leaf growth at elevated $p_{\text{CO}_2}$ in *L. perenne* appeared to be proportional to the greater plant size rather than being specifically enhanced. However, further research would be necessary to clarify these points.

Finally, a transient increase in the relative growth rate of the number of growing points at elevated $p_{\text{CO}_2}$ was limited to *T. repens* and became evident only after the plants had regained a positive C balance. Even though there was a considerable delay, this effect was related to defoliation as no similar stimulation of the production of new growing points at elevated $p_{\text{CO}_2}$ was apparent in intact plants or during later regrowth. However, elevated $p_{\text{CO}_2}$ did not appear to influence the extent of a transient halt in the production of new growing points observed shortly after defoliation in both *T. repens* and *L. perenne*. As the extent of this halt appeared independent of all treatments, it appeared to be mediated by some consequence of defoliation other than carbohydrate availability.

### 5.1.3 Insufficient information available on growing point densities in the field

Single, spaced plants were used in the growth chamber experiments as they had been designed to explore the potential for a growth response to elevated $p_{\text{CO}_2}$ during regrowth after defoliation under favourable conditions. In a closed canopy in the field, the production of new growing points is limited by the presence of competitors. Thus, the actual area density of growing points is the result of the production of new growing points and the death of old growing points. In principle, both processes might be affected by elevated $p_{\text{CO}_2}$ either directly or through a feedback of effects on canopy structure.

While growing point density in the field was increased at elevated $p_{\text{CO}_2}$ in *L. perenne* (Stadelmann 1993; Hebeisen 1997; Herbert Blum, Institute of Plant Sciences, ETH Zürich, personal communication) there was no evidence for a significant change in *T. repens* (Stadelmann 1993). Thus, it appears that any yield increase in *T. repens* was probably due to an increased dry matter production per growing point; in contrast, above-ground dry matter production per growing point in *L. perenne* was likely similar or even slightly reduced at elevated compared to ambient $p_{\text{CO}_2}$. However, particularly for *T. repens*, these results are still very preliminary in character and more detailed morphological research will be necessary to characterise the growth response of these two species to elevated $p_{\text{CO}_2}$ in the field.
5.1.4 What factors may have limited the growth response to elevated $p_{\text{CO}_2}$ in L. perenne and T. repens?

Is it possible from the data available to further characterise the factors which control growth rates and likely prevent a higher responsiveness of growth to elevated $p_{\text{CO}_2}$?

The responsiveness of plant growth to elevated $p_{\text{CO}_2}$ is generally thought to decrease if other factors – such as nutrient supply – limit growth (Baker and Enoch 1983; Bazzaz 1990; Poorter et al. 1996). Nevertheless, in some studies a similar relative growth response to elevated $p_{\text{CO}_2}$ was observed both at limiting and at ample nitrogen (N) supply (Conroy 1992). Therefore, N treatments were applied in all experiments to test the dependence of the growth response to elevated $p_{\text{CO}_2}$ on restrictions of regrowth by nutrient supply. In L. perenne, there were pronounced effects of N treatments on growth both in the field (Hebeisen et al. 1997) and in the growth chamber (Part 3). Thus, N availability was certainly limiting growth at low N supply. Even though high N treatments were chosen with the intent to obtain non-limiting conditions, there was no stimulation of regrowth at elevated $p_{\text{CO}_2}$ in the growth chamber. However, a stimulation of growth at elevated $p_{\text{CO}_2}$ did occur during seedling development at moderately high N supply (most if not all of this effect was already apparent after 39 days of growth at 7.5 mM NO$_3^-$; not shown). Thus, N supply in the high N treatment in the growth chamber most likely did not limit the growth response to elevated $p_{\text{CO}_2}$. In contrast, there is some evidence that a further stimulation of yield in the field as well as a more pronounced yield response to elevated $p_{\text{CO}_2}$ can be obtained at extremely high levels of N supply (112 g m$^{-2}$ yr$^{-1}$ N; Markus Daep, Institute of Plant Sciences, ETH Zürich, personal communication). Results from another study also suggest that extremely high rates of N fertiliser application may be necessary for a maximal stimulation of yield in swards of L. perenne (Soussana et al. 1996). Further, very high rates of fertiliser application were necessary to obtain a stimulatory effect of elevated $p_{\text{CO}_2}$ on tiller density in L. perenne (Soussana et al. 1994; Schenk et al. 1996).

Unlike in L. perenne, N supply did not affect the growth of T. repens in the field (Hebeisen et al. 1997) and only had an effect in the growth chamber during what appeared as a phase of acclimation after imposing the N treatments (Part 4). It is known that symbiotic N$_2$ fixation in T. repens can compensate for differences in external N supply, apparently in proportion to the plant’s needs for N (Hartwig et al. 1994; Zanetti 1997). It was thus not surprising to find no difference between N treatments in the effect of elevated $p_{\text{CO}_2}$ on RGR in the growth chamber or on yield in the field (Hebeisen et al. 1997). In fact, in the growth chamber, a transient stimulation of RGR at elevated $p_{\text{CO}_2}$ was observed in both N treatments during a period when RGR differed between N treatments but was no longer apparent at the end of regrowth when RGR was similar in both N treatments. This further supports the interpretation that the absence of a persistent stimulatory effect of elevated $p_{\text{CO}_2}$ on RGR of T. repens did not depend on N supply.

While it has been proposed that the initial regrowth of forage plants after defoliation may depend on the mobilisation of N reserves (Volenc et al. 1996), the extent to
which rates of regrowth are affected by the availability of such reserves is still unknown. However, at least in the high N treatments, it is unlikely that regrowth was limited by insufficient N reserves. Further, in spite of the common drop in nutrient uptake after defoliation of plants at high N supply (Richards 1993), there is evidence that nutrient limited plants may continue to take up and/or assimilate N after defoliation, as discussed in Parts 3 & 4.

In Part 2, a significant correlation between the C/N ratio in structural leaf material and water-soluble carbohydrate (WSC) concentrations was observed, indicating a significant adjustment of leaf structural composition to the relative availability of C and N in *L. perenne* in the field. Both a decrease in nitrogenous compounds (particularly proteins) and an increase in C-rich leaf constituents (e.g. cell wall) may have contributed to this response (cf. Fig. 3-3). Extending this approach to all experiments in this study may give further insight into C and N relations at elevated $p_{\text{CO}_2}$. As shown in Fig. 5-1a (note that, unlike in Fig. 2-5, non-structural carbohydrate concentrations have been expressed per unit of structural leaf dry matter), such an adjustment was nearly absent in *T. repens* in the field. Leaf structural C/N ratio increased only slightly with increasing total non-structural carbohydrate (TNC) concentrations. The same was true for *T. repens* in the growth chamber (Fig. 5-1b, data shown for days 11–22/23). This supports the interpretation that the growth of *T. repens* was not N limited in the field and during late regrowth in the growth chamber.

However, the relation observed for *L. perenne* differed between the growth chamber and the field (Fig. 5-1b, data shown for days 7–28, compare with Fig. 5-1a). At high N supply in the growth chamber, leaf structural composition changed only very little in spite of large differences in the non-structural carbohydrate content (Fig. 5-1b). In fact, the relationship was highly similar to that observed in *T. repens*. However, at low N supply, structural C/N ratios in the growth chamber were increased, particularly at elevated $p_{\text{CO}_2}$. In addition, leaf structural C/N ratios at elevated $p_{\text{CO}_2}$ increased more strongly with increasing leaf non-structural carbohydrate concentrations than at ambient $p_{\text{CO}_2}$. At low N supply combined with elevated $p_{\text{CO}_2}$, the structural C/N ratio rose to values similar to those found in the corresponding treatment in the field when non-structural carbohydrate concentrations were high (compare Fig. 5-1b with Fig. 5-1a). This response in *L. perenne* supports the interpretation that, in the growth chamber experiment, only the low N treatment was limiting growth. More pronounced alterations of leaf structural composition suggest that the N-limitation was even aggravated at elevated $p_{\text{CO}_2}$. In contrast, all treatments in the field were apparently N-limited as differences in the response between treatments were small. A pronounced increase in the structural C/N ratio was observed already at low concentrations of non-structural carbohydrate.

As the growth of both *T. repens* and *L. perenne* at high N supply in the growth chamber was not limited N availability, other factors must have limited their growth response to elevated $p_{\text{CO}_2}$. 
Figure 5-1. Structural C/N-ratio plotted against non-structural carbohydrate concentrations. In (a) field experiments and (b) growth chamber experiments, blades of growing leaves and first fully grown leaves, respectively, of *L. perenne* (△ □ ■) were used. Blades of first fully expanded leaves of *T. repens* (▲ □ △ ▽) were used both in the field and in the growth chamber. Plants were subjected to two $p_{\text{co}_2}$ treatments (△ □ △ ▽: ambient $p_{\text{co}_2}$; ■ △ △ ▽: elevated $p_{\text{co}_2}$) and two N treatments (△ △ △ ▽: low N supply; ■ △ ▬ ▽: high N supply). Field data comprise all 1993 and 1994 harvests. Growth chamber data comprise days 7–28 for *L. perenne* and days 11–22/23 for *T. repens*. C/N-ratios were corrected for non-structural carbohydrate and, additionally, in (b) for nitrate. Non-structural carbohydrate is water-soluble carbohydrate and total non-structural carbohydrate in the case of *L. perenne* and *T. repens*, respectively, and is expressed as moles of hexose equivalents per unit of structural dry matter.
As discussed in Parts 3 & 4, the activation of buds (new sinks) is controlled by many environmental factors such as N availability and light quality. Similarly, leaf appearance rate, as an important determinant of the rate of plant development, depends on many factors and an upper limit may be determined by maximum rates of cell division and extension at the prevailing temperature. In fact, the rate of tillering of *L. perenne* in the growth chamber during early regrowth at high N supply may have been already close to the maximum possible for this species (van Loo 1993).

Further, plant hormones have been suggested to be involved in the characteristic growth responses to N supply (Beck 1994; Engels and Marschner 1995; critically reviewed by Jackson 1993) or light (Baraldi *et al*. 1995; Kraepiel *et al*. 1995; Chory *et al*. 1996). One reason why plant growth and development often fails to show a similarly characteristic response to elevated $p_{\text{CO}_2}$ might be that a comparably specific mechanism affecting the control of sink activation in response to atmospheric $p_{\text{CO}_2}$ is lacking. As atmospheric $p_{\text{CO}_2}$ is nearly the same in all habitats, there probably never was an appropriate selective pressure for such a mechanism to evolve. Instead, an acclimation to the prevailing level of atmospheric $p_{\text{CO}_2}$ may have proven sufficient.

### 5.1.5 Sink development rarely carbon limited

The present results indicate that in *L. perenne*, growth both after defoliation and following recovery was sink limited in the sense that growth rates did not change in response to changes in carbohydrate availability. In *T. repens*, the control of sink initiation was only transiently shared by C availability after defoliation.

Recent experiments have demonstrated the usefulness of short-term partitioning models based on sucrose concentration gradients and phloem transport properties (Minchin and Thorpe 1996). While the expression of several genes involved in sink metabolism has been shown to be sugar inducible (Koch 1996), evidence for an involvement of carbohydrate availability in the control of sink activation is scarce (Stitt and Schulze 1994; Koch 1996; Pollock and Farrar 1996). However, the present results do not support the model suggested by Farrar and Williams (1991) and elaborated by Farrar (1992) proposing that elevated $p_{\text{CO}_2}$ will promote sink growth through an enhanced availability of sucrose. Instead, it appears that the availability of C is rarely limiting sink development. Stimulatory effects of elevated $p_{\text{CO}_2}$ on sink development are likely observed mainly during seedling growth, as discussed in Part 3, and as observed by Zanetti (1997), or during brief periods following severe defoliation as observed in Part 4 (discussed above). Only in plants with large storage sinks (e.g. crops such as sugar beet, Ziska *et al*. 1995) is dry matter accumulation likely limited by C assimilation during later parts of their life cycle.

Published reports on enhanced plant growth at elevated $p_{\text{CO}_2}$ may thus reflect an increase in the amount of dry matter accumulated per morphological unit (leaf, internode, etc.) in the absence of a change in the rate of plant development – in experiments with established plants – or, alternatively, a stimulation of sink initiation during a source limited
phase of seedling development. Even the former effect can already lead to significant
differences in total plant dry mass (as discussed in Parts 3 & 4).

5.2 No acceleration of the recovery of carbohydrate reserves
after defoliation

5.2.1 Reduced rate of carbohydrate depletion linked to residual
leaf area?

A stimulation of photosynthesis at elevated $p_{\text{CO}_2}$ was expected to reduce the rates of car-
bohydrate mobilisation from storage tissue. However, reduced rates of carbohydrate re-
serve mobilisation were observed only in *L. perenne* in the field (Part 2). In *L. perenne*
and in *T. repens* grown in the growth chamber, rates of carbohydrate mobilisation from
storage tissue at elevated $p_{\text{CO}_2}$ were either similar to those observed at ambient $p_{\text{CO}_2}$ or
tended to be even higher (Parts 3 & 4). Thus, higher rates of photosynthesis at elevated
$p_{\text{CO}_2}$ by themselves were apparently not sufficient to cause a significant decrease in the
rate of carbohydrate reserve mobilisation.

However, the proportion of leaf dry matter remaining after defoliation was significantly
higher at elevated $p_{\text{CO}_2}$ in *L. perenne* in the field (Tab. 2-1), but not in the growth cham-
ber experiments (Figs. 3-1 & 4-2). Data for *T. repens* in the field are not available, but
as canopy height was well above the 5 cm cutting height at all but some late fall harvest
dates, significant differences in residual leaf area in *T. repens* in the field are highly un-
likely. Thus, it appears that the reduction in the rate of carbohydrate mobilisation ob-
served in *L. perenne* in the field was more likely a consequence of the higher residual
leaf area observed at elevated $p_{\text{CO}_2}$ than a direct effect of the stimulation of photosynthe-
sis at elevated $p_{\text{CO}_2}$.

What could be the reasons for the absence of an effect of the enhanced carbohydrate
availability at elevated $p_{\text{CO}_2}$ on rates of carbohydrate mobilisation? Carbohydrate re-
serves are needed not only for growth and growth-related respiration, but an important
fraction of the mobilised carbohydrate reserves is also needed to cover the needs of non-
photosynthetic tissues for maintenance respiration. LAR was lower in plants growing at
elevated $p_{\text{CO}_2}$ compared to plants growing at ambient $p_{\text{CO}_2}$. This likely compensated for at
least a part of the stimulation of photosynthesis per unit leaf area at elevated $p_{\text{CO}_2}$ (Parts
3 & 4). Thus, a true alleviation of the impact of defoliation on source-sink relations by
elevated $p_{\text{CO}_2}$ would require that the decrease of LAR at elevated $p_{\text{CO}_2}$ be reverted by de-
foliation. However, such an effect was only apparent in *L. perenne* in the field. In *L.
perenne* in the growth chamber (where no adjustment of pseudo-stem height in response
to previous defoliation had occurred), a higher proportion of shoot tissue was removed
at elevated than at ambient $p_{\text{CO}_2}$, leading to an over-proportional increase in the
root:shoot ratio and a further reduction of LAR (Part 3). Still, effects of elevated $p_{\text{CO}_2}$ on
LAR cannot entirely explain the similar rates of reserve carbohydrate mobilisation as
carbohydrate availability was higher throughout the experiment in *T. repens* (Part 4).
5.2.2 No shortening of the mobilisation phase

The duration of the lag period before growth of pseudo-stems (in L. perenne) or stolons (in T. repens) and roots resumed in the growth chamber was independent of treatments within the limits of resolution set by the harvest intervals (Parts 3 & 4). Similarly, the phase of reserve carbohydrate mobilisation was not shortened in plants growing at elevated $p_{CO_2}$ either in the growth chamber or in the field.

5.2.3 Control of reserve carbohydrate accumulation and mobilisation - a speculative model

To explain these unexpected results, a qualitative model based on published and emerging knowledge on the hormonal regulation of starch mobilisation in T. repens was applied to explain the time course of reserve starch concentrations and carbohydrate availability during regrowth after defoliation (Part 4). Amylase activity in stolons is assumed to depend on the balance of phytohormones: The mobilisation of starch is promoted by gibberellins produced by the apex and inhibited by ABA produced by leaves. The mobilisation of starch after defoliation does not start as a direct response to the drop in assimilate production after defoliation, but instead is triggered by the lack of inhibitory ABA. Similarly, the mobilisation of starch ceases only when newly formed leaves produce sufficient quantities of ABA.

To explore the features of that model into some more detail, it is further assumed that the rate of starch synthesis is related to carbohydrate availability. With these assumptions, time courses of reserve starch concentrations similar to those shown in Fig. 5-2 may be obtained. Points 1–4 represent different initial carbohydrate concentrations. Rates of carbohydrate mobilisation are controlled by the hormonal balance which is a function of the number of leaves remaining on the plant (for simplicity, time courses for only a single degree of defoliation are shown). As new leaves grow, carbohydrate availability from current photosynthesis increases and some starch synthesis occurs. As long as amylases are still active, potential starch accumulation (steady-state starch concentrations dependent on carbohydrate availability and amylase activity; dotted lines ending in points 5 & 6) remains low and an accumulation of soluble carbohydrates may occur (not shown). Large quantities of starch are only accumulated after the hormonal balance is fully restored. Actual starch concentrations follow the mobilisation curve until it intersects with the potential accumulation curve. This intersection determines the time point and magnitude of the minimal starch concentration (i.e. the end of the mobilisation phase).

5.2.3.1 Several features of the time course of reserve carbohydrates in T. repens reproduced

This model reproduces several features of stolon starch concentrations observed in T. repens in this study (Figs. 4-7c & 4-11e). Rates of starch mobilisation were independent of both the initial amount of starch reserves present and the availability of carbohydrates.
from current photosynthesis. Apparently, the duration of the mobilisation phase and the minimal starch concentration were largely determined by the initial starch concentrations and carbohydrate availability. However, the end of the mobilisation phase was not directly related to the apparent recovery from defoliation. Thus, plants with a high initial stolon starch concentration tended to reach a minimum later, even though carbohydrate availability was higher than in other plants with low initial starch concentrations. Finally, the time course of carbohydrate concentrations after recovery appeared to be only a function of treatment effects on rates of photosynthesis and C utilisation, but not of initial starch levels.

Patterns of stolon starch concentrations compatible with the above model were also reported by Scheidegger (1983, Fig. 21, p. 47). Different light intensities and cutting regimes were used to obtain a range of initial stolon starch concentrations. In the treatment with almost no stolon starch at the beginning of the experiment, starch concentrations followed a time course similar to the potential starch accumulation curves in Fig. 5-2. In the other treatments, starch concentrations decreased until they were close to concentrations in that first treatment and then followed a similar time course. Baur-Höch et al. (1990, Fig. 1) observed lower rates of starch mobilisation and a faster recov-

Figure 5-2. Generalised time course of carbohydrate mobilisation and replenishment. Different initial carbohydrate concentrations (1–4) were combined with different rates of carbohydrate accumulation (5–6). The dashed line represents a minimum carbohydrate concentration which is not available for mobilisation (for starch, this appears to be close to zero). The period during which mobilising enzyme activity is thought to be important is indicated. Different degrees of defoliation would result in different initial rates of carbohydrate mobilisation (for simplicity, only a single, initially constant rate with a decrease at low starch concentrations is shown).
ery in plants with a higher residual leaf area but not in plants with higher initial starch
reserve levels. This observation can readily be explained by the above model as the
plants with the higher residual leaf area would be able to restore their hormonal balance
earlier than the more severely defoliated plants.

While this model appears to have considerable explanatory value, it does not allow to
make quantitative predictions on the response of carbohydrate reserves to various treat-
ments during regrowth after defoliation. Clearly, a more thorough knowledge of all the
processes involved will be necessary before an attempt can be made to express the
model in quantitative terms.

5.2.3.2 Applicability of the model to *L. perenne*

Even much less is known about the regulation of carbohydrate reserve mobilisation in *L. perenne* – a fructan accumulator – than in the starch accumulator *T. repens*. In general, the regulation of fructan accumulation and mobilisation appears to be such as to balance fluctuations in carbohydrate availability (review by Simpson and Bonnett 1993). The regulation of fructan mobilisation may be accomplished in part by a direct inhibition of fructan exohydrolases by sucrose as demonstrated in *L. perenne* and other species (Simpson and Bonnett 1993; Marx *et al.* 1997). However, an increase in enzyme activity dependent on *de novo* protein synthesis has also been observed after defoliation (Simpson and Bonnett 1993). FEH activity in *L. perenne* was maximal two days after defoliation and only slowly declined thereafter (Prud’homme *et al.* 1992). According to Simpson and Bonnett (1993), an increase in fructose concentrations is often observed during fructan hydrolysis which may indicate that the rate of hydrolysis exceeds the export of mobilised sugars to sinks. However, no evidence for such a corresponding increase in fructose concentrations was observed in pseudo-stems of *L. perenne* during recovery from defoliation (Gonzales *et al.* 1989; Prud’homme *et al.* 1992). Instead, an increase in the concentration of fructans occurred at about the same time as the increase in hexoses and sucrose. Thus, the regulation of carbohydrate mobilisation in *L. perenne* may be different from the model proposed for *T. repens*. Still, the control of fructan metabolism does not only depend on carbohydrate availability but is also influenced by environmental factors as demonstrated e.g. by a rapid hydrolysis of fructans in stems of *Triticum aestivum* during drought (Virgona and Barlow 1991).

Even though there appears to be no evidence for a mobilisation of carbohydrate reserves in excess of the plant’s needs, other aspects of the time course of carbohydrate concentrations after defoliation of *L. perenne* were similar to those observed in *T. repens* and featured by the model discussed above. Minimum WSC concentrations in pseudo-stems appeared to depend primarily on initial carbohydrate concentrations and on N treatments (Fig. 3-4c), and the time course of later carbohydrate accumulation appeared to be entirely independent of initial WSC concentrations (similar to the generalised time course depicted in Fig. 5-2). Further, indirect evidence suggested that rates of reserve WSC mobilisation were reduced only when there was a greater residual leaf area (Parts 2 & 3
as discussed above). Clearly, more information on the regulation of fructan hydrolysis is needed to assess whether the rate and duration of WSC mobilisation in *L. perenne* is determined directly by carbohydrate availability or if other consequences of defoliation are more important.

5.3 Sink limitation reflected in greatly increased leaf carbohydrate concentrations at elevated $p_{\text{CO}_2}$ and in rates of apparent carbohydrate export.

In both *L. perenne* and *T. repens*, the apparent sink limitation of the growth response to elevated $p_{\text{CO}_2}$ was reflected in an accumulation of carbohydrates to varying degrees – not only in storage tissue but also in leaves (in excess of the transient stores needed to maintain export throughout a diurnal cycle). An accumulation of carbohydrates in leaves was observed both in *L. perenne* and *T. repens*, but was more pronounced in *L. perenne*, both in the field (particularly at low N supply) and in the growth chamber (compare Figs 3-3 & 4-6). Differences between species were also observed in the response of apparent night-time export to elevated $p_{\text{CO}_2}$. In *L. perenne*, apparent night-time export in the field was only transiently stimulated at elevated $p_{\text{CO}_2}$; towards the end of regrowth it decreased to low values, particularly at elevated $p_{\text{CO}_2}$ (Part 2). While variability was high, apparent night-time export from leaves of *L. perenne* in the growth chamber was mostly not significantly different from zero (not shown). Low rates of apparent night-time export indicate a sink limitation of C utilisation and growth (as discussed in Part 2). A pronounced effect of a growth limitation (depending on N supply) on the mobilisation of leaf carbohydrates during the night was also observed in *Nicotiana tabacum* (Fichtner et al. 1993). In contrast, pronounced diurnal differences in leaf starch concentrations were always observed in *T. repens*, both in the field and in the growth chamber (Part 4). To some extent, these differences between the two species may be related to differences in their growth response to elevated $p_{\text{CO}_2}$. Higher rates of export in *T. repens* likely served to cover the extra sink demand during the period of enhanced growth at elevated $p_{\text{CO}_2}$ in the growth chamber. Further, it is tempting to relate differences in apparent carbohydrate export observed in the field to the different responses of harvestable yield to elevated $p_{\text{CO}_2}$ in *L. perenne* and *T. repens*. However, there was no direct relationship to growth as pronounced differences in apparent night-time export persisted in *T. repens* even though RGR was no longer stimulated at the end of regrowth in the growth chamber. Nevertheless, the differences between *L. perenne* and *T. repens* in the response of apparent night-time export to elevated $p_{\text{CO}_2}$ suggest that different strategies were used to balance C relations in these two species.

5.4 Species differed in the contribution of various morphological and physiological adjustments to the balancing of C relations

Since the relative growth response to elevated $p_{\text{CO}_2}$ did not always reflect the effect of elevated $p_{\text{CO}_2}$ on carbohydrate availability in either species, some adjustments must have
occurred in both *L. perenne* and *T. repens* to maintain balanced C relations. As discussed in Part 1, RGR can be partitioned into a function of the rate of gross photosynthesis per unit leaf area $\pi$, the average specific respiration rate $\rho$ (rate of ‘dark’ respiration per unit of biomass) and LAR:

$$\text{RGR} = \pi \text{LAR} - \rho \quad (5-1)$$

with RGR and $\rho$ in g g$^{-1}$ d$^{-1}$ (as loss of dry matter per unit of dry matter and time), $\pi$ in g cm$^2$ d$^{-1}$ (as dry matter production per unit of leaf area and time) and LAR in cm$^2$ g$^{-1}$ (Causton and Venus 1981). Thus, RGR can remain unchanged at elevated $p_{\text{CO}_2}$ if adjustments occur in the gross rate of photosynthesis per unit leaf area $\pi$, in LAR or in the specific respiration rate $\rho$. The extent to which each of these components contributed to the adjustments in *L. perenne* and *T. repens* is discussed below.

### 5.4.1 Similar morphogenetic responses to elevated $p_{\text{CO}_2}$ in both *L. perenne* and *T. repens*

A reduction in LAR was observed in both species in the growth chamber (Parts 3 & 4) while LWR remained similar or was slightly increased (*T. repens* at low N supply). Thus, the decrease in LAR at elevated $p_{\text{CO}_2}$ was mainly due to a lower SLA. Such responses are typical for C$_3$ species (Poorter 1993; Poorter *et al.* 1996). Lower values of SLA at elevated $p_{\text{CO}_2}$ were mainly due directly to carbohydrate accumulation, but changes in the area density of leaf structure also contributed to this effect. While LAR cannot be determined directly in the field, generally reduced values of SLA in both *L. perenne* and *T. repens* (Stadelmann 1993) suggest that LAR was probably also reduced in the field. Therefore, it is suggested that LAR reduced RGR in both species and all experiments.

### 5.4.2 Different tendencies towards photosynthetic acclimation

Rates of net photosynthesis were generally increased at elevated $p_{\text{CO}_2}$ both in the field and in the growth chamber. Unless there were large changes in the rate of dark respiration per unit leaf area in response to elevated $p_{\text{CO}_2}$, these effects likely reflect changes in gross photosynthesis. A down-regulation of photosynthesis in *L. perenne* was observed at the end of regrowth in the growth chamber (Part 3) and in the field (Rogers *et al.* 1995), though this effect may also be influenced by environmental factors as it was not always observed in the field (Bryant 1994). At least in the growth chamber, plots of $A$ vs. $C_i$ measured at high PPFD suggested that photosynthetic acclimation led to reduced rates of photosynthesis in leaves of *L. perenne* grown and measured at 70 Pa $p_{\text{CO}_2}$ in comparison to leaves of plants grown at 35 Pa $p_{\text{CO}_2}$ and measured at 70 Pa $p_{\text{CO}_2}$ (not shown). No acclimation of photosynthesis was observed in *T. repens* during the first three weeks of regrowth (Part 4). Only a tendency towards reduced values of $V_{\text{C}_\text{max}}$ was observed in the field in the high defoliation frequency treatment examined in this study.
Thus, a down-regulation of photosynthesis reduced the stimulatory effect of elevated $p_{\text{CO}_2}$ on $\pi$ in *L. perenne*, but not in *T. repens*.

### 5.4.3 Were there alterations in the specific respiration rate?

As no measurements of respiration were made, only evidence from the literature can be used to consider likely effects of elevated $p_{\text{CO}_2}$. It is difficult to predict the effect of elevated $p_{\text{CO}_2}$ on respiration on the basis of plant biomass, as the net result may depend on a number of different and counteracting effects (Wullschleger *et al.* 1994). Elevated $P_{\text{CO}_2}$ has been shown to have a direct inhibitory short-term effect on mitochondrial respiration (Gonzàlez-Meler *et al.* 1996b). One might speculate that the nearly ubiquitous accumulation of at least moderate carbohydrate concentrations in plants or plant parts exposed to elevated $p_{\text{CO}_2}$ serves to counteract the direct inhibitory effect of elevated $p_{\text{CO}_2}$ on respiration and to ensure a sufficient supply of metabolic energy in the form of ATP. In addition, there may be a stimulation of CO$_2$ efflux in response to high tissue carbohydrate concentrations, possibly due to a stimulation of the alternative oxidase pathway (Gonzàlez-Meler *et al.* 1996a). Alterations of tissue composition at elevated $p_{\text{CO}_2}$ are likely to affect growth and maintenance respiration (Wullschleger *et al.* 1994). An accumulation of non-structural carbohydrates in combination with decreased leaf N concentrations is likely to reduce the specific construction and maintenance costs of plant tissue. However, there seems to be no information available on whether there are differences in the respiratory cost associated with the storage of carbohydrate as fructan versus starch. Any effect of elevated $p_{\text{CO}_2}$ on RGR will further affect rates of respiration. In young plants of *L. perenne* grown at 70 vs. 35 Pa $p_{\text{CO}_2}$, rates of whole plant carbon dioxide efflux per unit of biomass at the end of the night were reduced by 30–40% during a period when RGR was no longer significantly altered by elevated $p_{\text{CO}_2}$ (Bunce and Caulfield 1991). No comparable data are available for *T. repens*; however, results similar to those for *L. perenne* were also observed in the legumes *Glycine max* and *Medicago sativa* (Bunce 1990; Bunce and Caulfield 1991). Thus, these findings suggest that $\rho$ may have been reduced at elevated $p_{\text{CO}_2}$ at least during periods of unchanged RGR.

Differences between $p_{\text{CO}_2}$ treatments in the rate of some physiological processes may further influence mean specific respiration rates. In the field but not in the growth chamber, *T. repens* has been shown to obtain a greater proportion of its N from symbiotic N$_2$ fixation when grown at elevated $p_{\text{CO}_2}$ than when grown at ambient $p_{\text{CO}_2}$ (Zanetti *et al.* 1996; Zanetti 1997). Since a higher energy cost is associated with the assimilation of N from N$_2$ than from NO$_3^-$ or even from NH$_4^+$, an increase in the proportion of N derived from symbiotic N$_2$ fixation provides an extra C sink and will increase the average $\rho$. Nothing appears to be known about effects of elevated $p_{\text{CO}_2}$ on the respiratory cost of mineral nutrient uptake. However, recent evidence that grassland species including *Lolium multiflorum* growing at elevated $p_{\text{CO}_2}$ obtained a greater proportion of their N from NH$_4^+$ (Jackson and Reynolds 1996) suggests that the respiratory costs associated with N uptake may even be decreased in *L. perenne* grown in the field.
5.4.4 Species differences in the adjustment of carbon assimilation to relative growth rate

Several lines of evidence suggest that the effect of elevated $p_{\text{CO}_2}$ on the components of RGR differed between *T. repens* and *L. perenne*. While changes in LAR were similar between the two species, *L. perenne* appeared to adjust mainly its rate of photosynthesis ($\pi$) while *T. repens* exhibited a limited stimulation of RGR in the growth chamber. Additional experiments would be required to assess the contribution of the specific respiration rate ($\rho$); however, in the field, the effect of elevated $p_{\text{CO}_2}$ on the amount of energy expended for the uptake and assimilation of N may have differed between *T. repens* and *L. perenne*. These differences in the components of RGR may explain the different response of apparent night-time export to elevated $p_{\text{CO}_2}$ in the two species discussed above.

What could have been the reasons for these differences in the adjustment strategies of *T. repens* and *L. perenne*? Among others, these two species differ significantly in their morphology and growth habit, in their primary N source and in their main storage carbohydrate. Can differences in any of these characteristics be related to the response of RGR components to elevated $p_{\text{CO}_2}$?

Due to its morphology, *L. perenne* is limited to local expansion during vegetative growth while *T. repens* exhibits a foraging behaviour to exploit open spaces in the canopy. Clearly, these differences define very distinct strategies in competition with other species. However, for the response of monocultures or single plants to elevated $p_{\text{CO}_2}$, such differences were probably of little importance. In contrast, the different positioning of developing leaves in the canopy may be of greater significance. *T. repens* always tries to position its youngest fully grown leaves at the top of the canopy where they develop a high photosynthetic capacity (Dennis and Woledge 1982). In contrast, leaves of *L. perenne* mature close to the ground. With increasing LAI of the canopy, the photosynthetic capacity of subsequent leaves decreases (Woledge and Leafe 1976). If the canopy density increases at elevated $p_{\text{CO}_2}$, this effect by itself may already lead to an apparent photosynthetic acclimation to elevated $p_{\text{CO}_2}$. In the growth chamber, such a mechanism may have contributed to the observed decline of $V_{\text{c max}}$ at elevated $p_{\text{CO}_2}$ (Part 3). However, LAI of *L. perenne* in the field was lower at elevated than at ambient $p_{\text{CO}_2}$ for the regrowth periods studied in Part 2 (Hebeisen 1997).

The down-regulation of photosynthesis at elevated $p_{\text{CO}_2}$ has long been thought to be causally linked to the accumulation of non-structural carbohydrates in leaves (e.g. Stitt 1991). It has been suggested that starch storing species may be more prone to photosynthetic acclimation than sucrose storing species (Goldschmidt and Huber 1992). However, *L. perenne* and *T. repens* clearly do not fit this pattern. More recently, hexokinase has been associated with the sensing of sugar concentrations and with changes in gene expression leading to a down-regulation of photosynthesis (Jang and Sheen 1994; Jang *et al.* 1997). One might speculate that high rates of fructan turnover in grass leaves (Farrar 1989) due to significant basal activities of fructan hydrolases (Simpson and Bonnett 1993) lead to a continuous release of hexoses into the cytosol. As
carbohydrates accumulate in leaves at elevated $p_{\text{CO}_2}$, even higher concentrations of hexose may lead to photosynthetic acclimation. In contrast, starch turnover takes place within the chloroplast and is therefore unlikely to have a comparable effect on cytosolic hexose concentrations and on sugar-dependent gene expression. Clearly, detailed information on the contribution of individual sugars to WSC in *L. perenne* in relation to photosynthetic acclimation would be very valuable to assess the plausibility of this hypothesis.

While there is increasing evidence that carbohydrates are indeed involved in mediating photosynthetic acclimation, actual causes may also be found elsewhere. Sage (1994) suggested that photosynthetic acclimation is observed mainly when plant growth is restricted by nutrient availability or pot size. Thus, the true reason for the smaller tendency towards photosynthetic acclimation in *T. repens* than in *L. perenne* may be found in the ability of *T. repens* to increase its supply of N from symbiotic N$_2$ fixation in response to an enhanced N demand at elevated $p_{\text{CO}_2}$ (Zanetti *et al.* 1996).

### 5.5 Conclusions

In all experiments, elevated atmospheric $p_{\text{CO}_2}$ generally led to an enhanced availability of C building blocks as indicated by a pronounced accumulation of non-structural carbohydrates in both *L. perenne* and *T. repens*. Together with a stimulation of the production of structural tissue components, the carbohydrate accumulation resulted in a higher dry mass per growing point, as observed in the growth chamber. In fact, this increase in dry mass per growing point was the principal growth response to elevated $p_{\text{CO}_2}$ in the growth chamber in both species. Even though the higher C availability at elevated $p_{\text{CO}_2}$ might be expected to alleviate the impact of defoliation and thus to promote regrowth under favourable conditions, no such effect could be observed. The regrowth of leaves during the first four days after defoliation proceeded essentially in proportion to the existing dry mass and appeared to depend more directly on the vigour of meristems present at the time of defoliation than on either carbohydrate reserves or rates of current photosynthesis. Similarly, recovery from defoliation as assessed by the resumption of growth of all plant tissues and/or the start of replenishment of carbohydrate reserves was not accelerated. Rates of carbohydrate depletion from storage tissue after defoliation did not appear to depend directly on C availability, but may have been related to the residual leaf area.

Only about one week after defoliation, a transient stimulation of regrowth was observed in *T. repens* grown at elevated $p_{\text{CO}_2}$ in the growth chamber. Thus, it appeared that regrowth of *T. repens* was temporarily co-limited by C availability after a severe defoliation and the responsiveness of growth to elevated $p_{\text{CO}_2}$ – as it is typically observed in seedlings – was transiently restored after defoliation. In contrast, C availability never appeared to contribute to the control of regrowth in *L. perenne*. In either species, N supply did not affect the responsiveness of regrowth to an increased carbohydrate availability at elevated $p_{\text{CO}_2}$. Thus, it is suggested that the restricted responsiveness to elevated
was due to other – environmental or inherent – limitations. In both species, patterns of carbohydrate concentrations in leaves and storage tissue observed in the field were essentially similar to those found in the growth chamber, indicating that similar processes were governing source-sink relations in both systems. Therefore, both in the growth chamber and in the field, the higher leaf carbohydrate concentrations and the enhanced accumulation of carbohydrate reserves towards the end of regrowth reflected an apparent sink limitation of the growth response to elevated \( p_{\text{CO}_2} \). A pronounced \( p_{\text{CO}_2} \times N \) interaction in \( L. \ perenne \) in the field suggested that this sink limitation appeared to be particularly pronounced at low N supply. Further, a sink limitation of C utilisation was also indicated by the pattern of apparent night-time carbohydrate export from leaves of \( L. \ perenne \). It is suggested that not only differences between \( p_{\text{CO}_2} \) treatments in total plant biomass in the growth chamber, but also differences in yield in the field may have been caused primarily by an accumulation of more dry matter per growing point.

5.6 Outlook

5.6.1 Can limitations in the plants’ response to elevated \( p_{\text{CO}_2} \) be overcome by evolutionary adaptation?

The results described in this thesis suggest that the growth response of both \( L. \ perenne \) and \( T. \ repens \) to elevated \( p_{\text{CO}_2} \) was limited by the small contribution of C availability to the control of sink initiation and growth. Is it conceivable that evolutionary adaptation might produce plants which can take better advantage of the increased efficiency of photosynthesis at elevated \( p_{\text{CO}_2} \)? Up to present, the potential for evolutionary adaptation in response to elevated \( p_{\text{CO}_2} \) has been assessed primarily by screening selections of genotypes for significant differences in the response of various growth and yield parameters to elevated \( p_{\text{CO}_2} \) (Lüscher et al. 1996 and references therein). Only some of these studies have detected differences in the responsiveness of different genotypes to elevated \( p_{\text{CO}_2} \), indicating that the potential for a rapid evolutionary adaptation to elevated \( p_{\text{CO}_2} \) may be small (Lüscher et al. 1996). Such an approach is certainly appropriate to assess the potential for rapid changes in the frequency of existing genotypes. However, due to the limited number of genotypes examined, rare alterations of metabolism which may be of adaptive value at elevated \( p_{\text{CO}_2} \) may be missed. Thus, the formulation of precise hypotheses about the processes limiting the response of plants to elevated \( p_{\text{CO}_2} \) may allow to screen more specifically for genotypes with properties which might provide a selective advantage at elevated \( p_{\text{CO}_2} \).

5.6.1.1 Adaptation of the control of sink activation by light quality

Elevated \( p_{\text{CO}_2} \) is known to reduce the light compensation point of photosynthesis (Long et al. 1993) and thus one might expect that plant canopies will grow to a higher density. Such a response might be desirable in an agronomic context, as a higher standing biomass may allow for higher yields. A reduced sensitivity of bud activation to changes in light quality in the presence of neighbouring plants might allow for such a behaviour.
However, in various ecosystems with closed canopies, the leaf area index was not increased at elevated $p_{\text{CO}_2}$ (Körner 1996). Instead, a continuous redistribution of N to new leaves, which are positioned at the top of the canopy in most species, likely confers a greater advantage in a competitive situation under nutrient limited conditions. Thus, artificial selection may be required to obtain genotypes which produce denser canopies at elevated $p_{\text{CO}_2}$.

### 5.6.1.2 Adaptation of control of sink activation by nutrient availability

Theories of optimal nutrient usage suggest that a higher leaf C/N ratio would be optimal for plants growing at elevated $p_{\text{CO}_2}$ (Hilbert et al. 1991). In fact, such an increase in optimal tissue composition can actually be observed experimentally (e.g. Hocking and Meyer 1991; Rogers et al. 1993; Soussana et al. 1996). However, it appears that changes in tissue C/N ratio are largely due to an accumulation of non-structural carbohydrates. A reduced sensitivity of bud activation to the availability of N might therefore allow plants to produce more structural biomass at elevated $p_{\text{CO}_2}$ with the same amount of N. Such a modification might even confer a competitive advantage. If the control of bud activation by N availability is mediated through phytohormones, either the dependence of hormone production on N availability or the sensitivity of sink activation to hormones might be altered to allow the activation of sinks at lower levels of N availability.

### 5.6.1.3 Adaptation of the regulation of reserve carbohydrate mobilisation by phytohormones

Assuming that the mobilisation of carbohydrate reserves is indeed (at least partly) controlled by phytohormones, then plants with a reduced rate of carbohydrate mobilisation at a given residual leaf area may have a selective advantage. They would be able to take advantage of the increased photosynthetic efficiency of their residual and newly grown leaf area and resume the accumulation of carbohydrate reserves earlier at elevated $p_{\text{CO}_2}$ as long as higher rates of photosynthesis are not fully absorbed by a decrease in LAR. Both an increased sensitivity of amylase activity to ABA or a reduced sensitivity of amylase activity to gibberellins might produce such a phenotype.

### 5.6.2 Promising directions for future research

Clearly, more research is needed to establish firmly the factors controlling the responsiveness of growth in forage crops such as *L. perenne* and *T. repens* to elevated $p_{\text{CO}_2}$. The results of this investigation demonstrate that an understanding of the long-term response to elevated $p_{\text{CO}_2}$ can only be obtained by studying plants which are fully acclimatised to the level of $p_{\text{CO}_2}$ of interest. Unfortunately, this is not possible without accepting differences between treatments in the state of the plants at the start of an experiment. Ideally, initial conditions should be chosen such as to represent a relevant situation e.g. in the field. Experiments which start by exposing plants to a new level of $p_{\text{CO}_2}$ may allow...
valuable insight into the physiology of the plants before the change (Farrar 1996) and into acclimation processes; however, they do not reflect the long-term response of plants to elevated $p_{\text{CO}_2}$.

5.6.2.1 Research to evaluate potential growth limitations

Various approaches may be chosen to establish more firmly which processes do limit the growth response to elevated $p_{\text{CO}_2}$. A more direct estimate of the relative availability of C and N for growth processes may be obtained from a comparison of concentrations of readily available carbohydrates and amino acids (Buysse et al. 1993).

As already mentioned, changes in the sensitivity to light quality may affect the responsiveness of growth to elevated $p_{\text{CO}_2}$. It would be highly interesting to expose mutants which are insensitive to far-red light to elevated $p_{\text{CO}_2}$ and to investigate if a more pronounced increase in branching can be observed in such mutants relative to controls. However, before such experiments are attempted, it may be advisable to obtain more detailed morphological data and to determine whether the response of RGR to elevated $p_{\text{CO}_2}$ is limited by site filling in *L. perenne* (which may have been already close to maximal) and bud activation in *T. repens* or by leaf appearance rate. More detailed knowledge on this point may allow to assess further strategies to overcome limitations to a plant species’ response to elevated $p_{\text{CO}_2}$.

5.6.2.2 Can the mobilisation phase be shortened at elevated $p_{\text{CO}_2}$?

Some evidence put forward in this thesis suggests that the mobilisation of carbohydrate reserves at elevated $p_{\text{CO}_2}$ in *T. repens* is not regulated directly by carbohydrate availability and extends beyond the moment at which sufficient assimilates are available from current photosynthesis. A close monitoring of amylase activity (in *T. repens*) or FEH activity (in *L. perenne*) after defoliation in relation to a detailed C balance would allow a better comparison of reserve carbohydrate mobilisation with C demand for maintenance and growth not covered by current photosynthesis. The model of the regulation of starch mobilisation in *T. repens* outlined above suggests that the recovery phase until carbohydrate reserves start to be replenished after defoliation may be shortened by an external supply of the phytohormones (ABA) normally produced by the leaves. An experimental test of this prediction would therefore allow to assess the descriptive value of the model. A study of the response to elevated $p_{\text{CO}_2}$ of mutants in the regulation of carbohydrate mobilisation may provide further insight into the significance of these processes for the growth response to elevated $p_{\text{CO}_2}$ after defoliation.

Research along the lines suggested here may provide the foundation for a selection of forage plant genotypes with more desirable agronomic properties in future conditions of high atmospheric $p_{\text{CO}_2}$.
REFERENCES


ACKNOWLEDGEMENTS

I am very grateful to Prof. J. Nösberger for giving me the opportunity to work in his group and to participate in the FACE project. Many thanks go to my supervisor, Marco Frehner, for helping me whenever the going got tough and advice was in need and for all the good times we had together. With their never-ending optimism and support, both of them helped me to keep my spirits up, even when despair was close. I would like to thank Prof. Steve Long and for giving me the opportunity to collaborate with him and his group and for accepting to act as co-examiner for this thesis.

Many thanks go to all my colleagues from the FACE team – Herbert Blum, Ueli Hartwig, Thomas Hebeisen, Andi Lüscher and Silvia Zanetti (in alphabetical order) – for the good collaboration and the many interesting discussions we had. This research would not have been possible without their effort in setting up and maintaining the field experiment.

More thanks go to many others with whom I could enjoy so many good times and interesting discussions, particularly Zé Pedro Almeida, Dariusz Malinowski, Martin Messerli, Satya Pasumarty, Prof. Ravi Sangakkara and Jan Trommler. A special mention goes to Markus Daepf for doing a semester thesis with me and for helping me with the work and the analysis of the white clover growth chamber experiment. I would like to thank Steve P. Long, Jonathan B. Bryant, Phillip A. Davey, Colin Osborne and Alastair Rogers, for their collaboration and useful discussions on leaf gas-exchange and carbon assimilation.

Invaluable help of Anni Dürsteler in the laboratory and of Patrik Schlüssel, Irene Bläuenstein and many others who helped me with my harvests and analyses is also greatly appreciated.

Further, I really enjoyed the good company of Silvia Zanetti, Katja Jacot and Martin Messerli – work was a lot easier that way.

Special thanks go to Claudia Mühlhäuser for her support and patience when work was plentiful and nights were too short. Similarly, I am very grateful to my parents for giving me the opportunity to study biology and for their continuous support.

This research was made possible by grants from the NEFF and the ETH. The FACE system was developed by Brookhaven National Laboratory and operated in collaboration with ETH.
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