Importance of nitrogen for the CO₂ response of two grassland species with emphasis on symbiotic N₂ fixation in Trifolium repens L.

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Publication Date:
1997

Permanent Link:
https://doi.org/10.3929/ethz-a-001816471
IMPORTANCE OF NITROGEN FOR THE CO₂ RESPONSE OF TWO GRASSLAND SPECIES WITH EMPHASIS ON SYMBIOTIC N₂ FIXATION IN TRIFOLIUM REPENS L.

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

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1997
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List of Abbreviations:

- a: year (annum)
- atom%^{15}\text{N}-excess: atom%^{15}\text{N} in excess of natural abundance (=0.3663%)
- bv.: biovar
- C: carbon
- cv.: cultivar
- FACE: Free Air Carbon-dioxide Enrichment
- Gt: \(10^9\) tons
- IN: index of nitrogen nutrition
- K: potassium
- N: nitrogen
- %N_{sym}: percentage of plant N derived from symbiotic N\text{2} fixation
- %N_{trans}: percentage of plant N derived from apparent N transfer
- P: phosphorus
- Pa: Pascal
- pCO\text{2}: partial pressure of carbon dioxide
- PS: photosynthesis
- SE: standard error
- WUE: water use efficiency
SUMMARY

In a world of increasing atmospheric CO₂ partial pressure (pCO₂), concern is growing as to the extent to which the biosphere can act as a carbon (C) sink for the additionally available C. The CO₂ response of an ecosystem depends on the availability of resources other than CO₂ (light and nutrients) and on plant species. An important potential growth-limiting factor under field conditions is nitrogen (N). Therefore, an increased C storage in the biosphere may also depend on the ability of N uptake to parallel the enhanced C fixation. Symbiotic N₂ fixation, an important process introducing N into terrestrial ecosystems, may play a crucial role in the CO₂ response of ecosystems.

The effect of elevated pCO₂ (60 Pa) on a grassland model ecosystem was investigated in a three year field experiment using Free Air Carbon-dioxide Enrichment (FACE) technology. This work focused on the role of N nutrition (mineral N and symbiotic N₂ fixation (¹⁵N-isotope-dilution method)) in the CO₂ response of Lolium perenne L. and Trifolium repens L. grown under fertile field conditions. T. repens and L. perenne were cultivated as monoculture or as binary mixture. The established swards were exposed to two levels of N fertilization (10 or 42 g m⁻² a⁻¹ in 1993; 14 or 56 g m⁻² a⁻¹ in 1994 and 1995) and to two defoliation frequencies (5 or 3 defoliations during the fumigated period of 1993; 8 or 4 defoliations in 1994 and 1995). To gain more insight into the mechanistic impact of elevated pCO₂ on symbiotic N₂ fixation, an experiment was carried out in growth chambers. Seedlings of T. repens were grown in silicic sand and supplied with nutrient solution which contained either no or 7.5 mM N.

In the three year field experiment, a strong species-specific CO₂ response was observed. Under elevated pCO₂, the above-ground plant material of the L. perenne monocultures showed a consistent and significant decline in N concentration. As a result, the total above-ground N yield decreased in general under elevated pCO₂. This reduction was less pronounced in the infrequently defoliated grass than in the frequently defoliated ones. In 1995, the N yield of the infrequently defoliated grass in the high N treatment was 10% higher under elevated pCO₂. This was associated in part to the open sward structure in this
treatment, allowing the plant to increase its yield by forming more tillers. However, the total above-ground N yield of the frequently defoliated grass was strongly reduced by 31% and 14% in the low and high N treatments, respectively. Frequently defoliated grass monocultures, exposed to elevated pCO2, had a significantly lower index of N nutrition (ratio of the actual to the critical N concentration in the above-ground plant material) than those under ambient pCO2. This indicates a CO2-induced increase in N limitation, presumably due to a lower amount of available N under elevated pCO2.

In contrast to the grass, total above-ground N yield of monocropped T. repens increased by 6.8% under elevated pCO2 (averaged over all treatments and three growing seasons). The N concentration in the above-ground plant material of T. repens was less affected by elevated pCO2 in comparison to L. perenne. In T. repens, the percentage of plant N derived from symbiotic N2 fixation (%Nsym) increased under elevated pCO2 in both sward types in all management treatments and over the entire experimental period. In the monocultures, %Nsym increased in the low N treatment from 56% under ambient pCO2 to 65% under elevated pCO2 and in the high N treatment from 34% to 42% (averaged over all growing seasons and both defoliation treatments). In general, this CO2-induced stimulation of symbiotic N2 fixation was such that even more than all additionally yielded N derived from symbiotic N2 fixation.

T. repens seedlings grown in silica sand and depending solely on N2 fixation increased the amount of fixed N under elevated pCO2. This increase was not associated with an enhanced specific N2 fixation activity (amount of N fixed per root nodule dry weight) but with an increased nodule formation. In contrast to field-grown plants, however, elevated pCO2 had no effect on the relative contribution of symbiotically fixed N as opposed to N assimilated from the mineral N source to the total N yield of the T. repens seedlings supplied with N. These results illustrate that elevated pCO2 did not affect the performance of symbiotic association per se. It is suggested that symbiotic N2 fixation is adapted to the plants' demand for symbiotically fixed N. The plants' demand for symbiotically fixed N is co-determined by the N availability in the growth medium. This is confirmed by the data obtained in the field study, where, under elevated pCO2, symbiotic N2 fixation was enhanced probably as a consequence of a CO2-induced reduction in the amount of N available to the plants.
Symbiotic N$_2$ fixation is a significant competitive advantage for N$_2$-fixing plant species. This is especially stressed under low N availability which becomes, as suggested in the present study, apparently more pronounced under elevated pCO$_2$. This competitive advantage of T. repens is confirmed by a CO$_2$-induced increase in the clover proportion in mixed swards. As a result of the CO$_2$-induced stimulation of symbiotic N$_2$ fixation and of the enhanced yield proportion of T. repens in the mixed swards, the amount of symbiotically fixed N in the intercropped T. repens was increased by 65% under elevated pCO$_2$. In both sward types, the relative CO$_2$ effect on symbiotically fixed N was stronger than on the total above-ground N yield of T. repens. This indicates that the enhanced symbiotic N$_2$ fixation helped to compensate for the apparently smaller amount of N available to plants exposed to elevated pCO$_2$.

The suggested CO$_2$-induced reduction in the N availability is the result of an increase in N demand of the entire ecosystem exposed to elevated pCO$_2$. Both, the stimulated N$_2$ fixation and the higher clover proportion, resulted in an increased amount of N apparently transferred from the clover to the grass. As a result, the grass-clover mixtures showed a 13% higher N yield under elevated pCO$_2$ when averaged over all treatments. Based on the data of this three year field experiment, it is suggested that symbiotic N$_2$ fixation is a crucial process for maintaining the C:N balance in grassland ecosystems with an inadequate N supply. In such situations, symbiotic N$_2$ fixation is a prerequisite for an increased C sequestration in grassland ecosystems exposed to elevated pCO$_2$. 

8
ZUSAMMENFASSUNG


Im dreijährigen Feldversuch wurde eine ausgeprägte artspezifische CO₂ Reaktion festgestellt. Unter erhöhtem pCO₂ nahm die N-Konzentration im oberirdischen Erntegut der Grasreinbestände signifikant ab. Dadurch war im

Im Gegensatz zum Gras konnten die Weisskleereinbestände ihren gesamten oberirdischen N-Ertrag unter erhöhtem pCO₂ um 6.8% steigern (Mittelwert über alle Verfahren und drei Vegetationsperioden). Die N-Konzentration im oberirdischen Erntegut wurde im Vergleich zu jenen des Grases durch den erhöhten pCO₂ weniger stark beeinflusst. In allen Verfahren und in beiden Bestandesformen von T. repens stieg der prozentuale Anteil an symbiotisch fixiertem N im gesamten oberirdischen N-Ertrag (%Nsymb) an. Dieser CO₂ Effekt auf %Nsymb konnte während der ganzen Versuchsphase beobachtet werden. Unter erhöhtem pCO₂ stieg %Nsymb in den Reinbeständen des tiefen N-Verfahrens von 56% (Kontrolle) auf 65% und im hohen N-Verfahren von 34% auf 42% an (Mittelwerte über drei Jahre und beide Entblätterungsverfahren). Die CO₂-induzierte Erhöhung von %Nsymb war so groß, dass sogar mehr als der gesamte zusätzlich geerntete N-Ertrag aus der symbiotischen N₂ Fixierung stammte.

In Quarzsand kultivierte Weissklee-sämlinge, die ihren N-Bedarf ausschließlich über die symbiotische N₂ Fixierung deckten, stieg der symbiotisch fixierte N-Ertrag unter erhöhtem pCO₂ an. Dieser Anstieg wurde nicht über eine erhöhte spezifische Knöllchenaktivität (fixierte N-Menge pro Knöllchengewicht) sondern über eine vermehrte Knöllchenbildung erlangt. Bei Weissklee-sämlingen, die mit 7.5 mM N-haltiger Nährösung gegossen wurden, hatte der erhöhte pCO₂ keinen Einfluss auf den relativen Beitrag des symbiotisch


GENERAL INTRODUCTION

1 CULTIVATED GRASSLAND ECOSYSTEMS - SOME FACTORS AFFECTING ITS PRODUCTIVITY AND STABILITY

In humid temperate regions much of the cultivable land is covered by grassland. The term 'grassland' usually refers to a mostly multi-species plant community in which graminaceous plant species are abundant. However, there are also other functional groups (plant species sharing attributes which are relevant to dominant ecosystem processes) such as legumes and non-leguminous dicots. Shrubs and trees are rare in these communities. This vegetation type is a dynamic system. Grassland may be either extensively or intensively managed. Management regimes (fertilization and defoliation frequency) can affect the stability of such systems by changing the functioning of the ecosystem and species composition. The productivity of grassland communities is sensitive to any climatic changes, i.e. fluctuations in temperature, precipitation and radiation from one growing season to the next one and also within a season. Therefore, it is likely that the stability and functioning of grassland ecosystems may also be sensitive to increasing atmospheric CO₂ partial pressure (pCO₂) - as well as to concurrent increase in temperature and particulate pollution. Despite the fact that the last two mentioned factors are very important, they will not be an issue of this specific study.

The stability of grassland ecosystems is also important for the farmer. Any changes of the floristic composition of the grassland can influence the density of the sward and the forage quality and, therefore, indirectly the productivity of the cattle. Moreover, to produce a forage of high nutritive value is a target worth aiming for because it reduces the costs of concentrates for feeding the cattle and has ecological advantages.
1.1 Nitrogen, an important factor in grassland productivity

Nitrogen is one of the major factors ensuring vigorous plant growth. Unfortunately N - in terms of plant available N - is scarce and often limiting for the productivity of ecosystems despite the fact that aerial plant parts are exposed to an atmosphere composed of 78% of N.

There is intensive cycling of N through a variety of pathways within ecosystems. Cycling of N also occurs in the air and water due to the high mobility of N. A result of this high mobility is the economically undesirable loss which is also of environmental concern. Processes causing loss of N from ecosystems include leaching, runoff, volatilization and denitrification. The last process releases N from the biosphere to the atmosphere. Denitrification like the other processes mentioned above is further characterized by pronounced spatial and temporal variability (Knowles, 1981). The pathways of N-cycling in managed grassland ecosystems are the same as those in other vegetation types. However, a crucial difference is that managed grassland systems are exposed to repeated removal of the above-ground plant biomass by farmers or cattle. In prairie ecosystems, the below-ground plant biomass can be a dominant fraction (Woodmansee et al., 1981). This influences the distribution of N amongst the different pools and also the rate of the N-cycling (Woodmansee et al., 1981).

Grasslands are, in general, multi-species communities of perennials. These plant communities usually include legumes which improve significantly the nutritive value of the forage because of the high digestibility and protein content of legumes. Thanks to symbiotic N₂ fixation, legumes require few or even no mineral N supply. This characteristic contributes significantly to the sustainability of grassland ecosystems.

1.2 Symbiotic N₂ fixation is an important process introducing N into grassland ecosystems

The phenomenon of symbiotic N₂ fixation is based on the mutual interaction between soil-born *Rhizobium* bacteria and legumes. For legumes, this symbiotic association not only provides the possibility of a complete independence of any other N sources but also represents an important competitive advantage in mixed swards which is most pronounced under N limiting growth conditions. In return, the endosymbiotic form of N₂-fixing bacteria (bacteroids) are supplied with assimilates needed for the highly energy
demanding reduction of molecular N. Moreover, the symbiotic association provides a safe site for the proliferation of the microorganisms and also protection from drying out.

Thanks to symbiotic N$_2$ fixation, a significant amount of N can be introduced into grassland ecosystems. In cultivated grasslands, the presence of red or white clover channels an amount of symbiotically fixed N in the range of 100-200 kg ha$^{-1}$ a$^{-1}$ into the system (Robson et al., 1989). Expressed as white clover yield typical for a fertile agricultural soil in the Swiss plateau, the amount of fixed N is approximately thirty kilos per ton dry-matter. This amount is independent of the extent of mineral N fertilization from 0 to 80 kg ha$^{-1}$ per regrowth-period (Zanetti and Boller, unpublished data).

Symbiotically fixed N can also become available to other plant species not able to symbiotically fix N (Boller and Nösberger, 1987). This apparent N transfer from legumes to non-fixing plants has been ascribed to various processes (Haystead et al., 1988; Dubach and Russelle, 1994; Hatch and Murray, 1994; Murray and Hatch, 1994). Hence the presence of a legume species in a community increases the supply of available N in the root medium. This is the result not only of the apparent N transfer but also of the lower N requirement of legumes for mineral N. As a consequence of these processes, the mixed swards are characterized by a general improvement in the N concentration of the non-fixing plant species and in the N utilization in comparison to those in monocultures (Boller and Nösberger, 1987). Based on these facts legume/grass mixtures require less mineral N in comparison to swards exclusively consisting of non-fixing plant species.

In addition to the symbiotic fixation of molecular N there is also a N$_2$ fixation by non-symbiotic and associative prokaryotic organisms. In some ecosystems (desert, rice paddies) these organisms may be the predominant N source for the system (Sprent and Sprent, 1990). However, in cultivated grassland ecosystems they are of minor importance (1-2 kg of N ha$^{-1}$ a$^{-1}$) in comparison to the N input by the symbiotic N$_2$ fixation (Woodmansee et al., 1981). Besides the symbiotic interaction between legumes and the eubacterium _Rhizobium_, non-legumes can also form N$_2$-fixing symbioses. One of the best known woody representatives is _Alnus_ sp. which interacts symbiotically with actinomycetes (_Frankia_).
1.3 Response of symbiotic N$_2$ fixation to environmental and management factors

It is well known that symbiotic N$_2$ fixation can be inhibited by stress and that the macrosymbiont has a wider ecological range than the symbiotic association itself. Stress originates from plant defoliation, nutrient limitation, extreme temperatures, and rapidly changing oxygen or water availability in the soil. Such extreme conditions may change nodule number by directly affecting nodule formation. Under controlled conditions, it was shown that very low potassium (K) supply results in a complete failure of nodule formation on *Vicia faba* and *Phaseolus vulgaris* roots (Sangakkara et al., 1996a;b). In field-grown crops, the percentage of N from the symbiosis (%N$_{sym}$) was reduced due to a low phosphorus (P) supply (Cadisch et al., 1993). Therefore, a good performance of the symbiotic N$_2$-fixing association requires an adequate supply of P and K to the plant. A further factor reducing nodule formation and %N$_{sym}$ is the supply of mineral N. The high availability of mineral N in the root zone results in a decline in the plants' demand for symbiotically fixed N. It has been suggested, that the plant's demand for symbiotically fixed N is a crucial parameter in the regulation of N$_2$ fixation (reviewed by Hartwig and Nösberger, 1994; Hartwig and Nösberger, 1996).

The physiological mechanism which tunes N$_2$ fixation to the plants demand for symbiotically fixed N is not fully understood. However it is known, that the activity of the N$_2$-fixing enzyme nitrogenase drops within minutes when plants are exposed to stressful events (e.g. plant defoliation, nitrate application). This reduction has been related to an increase in oxygen diffusion resistance within the legume nodules (Minchin et al., 1985; Hartwig et al., 1987). Hence, it has been suggested that nitrogenase activity is regulated by a rapid and reversible control mechanism. A varying oxygen permeability is controlling the oxygen availability in the central zone of the nodule.

At the plant level, the plant demand for symbiotically fixed N is the important parameter for the regulation of N$_2$ fixation. Any changes in this parameter due to management regimes or altered environmental conditions (e.g. elevated pCO$_2$) will, therefore, be transformed into a changed performance of symbiotic N$_2$ fixation. Moreover, any harm to the symbiotic association may affect the stability and the productivity of grasslands. Thus, the performance of symbiotic N$_2$ fixation and the stability of a grassland ecosystem are closely related to each other.
2 INCREASING CO$_2$ PARTIAL PRESSURE IN THE ATMOSPHERE AND POSSIBLE IMPACTS ON THE BIOSPHERE

2.1 General aspects of elevated CO$_2$ partial pressure at a global scale

The global change phenomenon is an issue of increasing political, social and scientific concern. It includes changes in global temperature and precipitation as well as changes in the composition of atmospheric gases. The prediction of the magnitude of these changes is uncertain. However, there is no doubt that human activities produce measurable changes in the earth system and cause the increasing pCO$_2$ in the atmosphere.

Carbon is a key element for life. It contributes 40% to 50% to the dry mass of plants and animals. In its oxidized form (as CO$_2$) it is one of the critical parameter involved in global change. Since the industrial revolution the increase in deforestation and in the consumption of fossil C have added 7 to 9 Gt annually of C to the atmosphere (Gifford, 1994). Both processes, deforestation and burning of fossil C, transfer C from an inactive, C 'storing' pool into an actively C exchanging reservoir, i.e. the atmosphere. This net addition of C resulted in a 21% increase in pCO$_2$ over the past 150 years (from 29 to 35 Pa pCO$_2$). The most rapid increase has occurred since 1950, when the concentration was still 31 Pa (Mooney et al., 1991). The actual annual increase in pCO$_2$ is 0.15 Pa (Gifford, 1992). The accelerating growth rate of pCO$_2$ and the awareness that elevated pCO$_2$ will affect the entire globe has caused increasing interest in the ecological aspects of the impact of elevated pCO$_2$. In fact, the effect of a doubling of the today's pCO$_2$ (expected to be achieved around 2050) has been estimated to result in a 10 to 40% increase in net global terrestrial productivity (Gifford, 1992).

However, atmospheric pCO$_2$ does not increase as fast as could be expected from the extent of CO$_2$-producing processes (7 to 9 Gt a$^{-1}$ of C), deforestation and burning of fossil C. CO$_2$ absorption by the ocean is estimated to account for 1.3 to 3 Gt a$^{-1}$ of C. The amount of 0.4 to 4.0 Gt of C of the annually added C to the atmosphere can neither be explained by an increase in the atmospheric pCO$_2$ nor by an oceanic uptake. Therefore, it has been suggested that enhanced C storage in the terrestrial biosphere (vegetation and soil) can account for the 'missing C-sink' (Gifford, 1994). The concept of the terrestrial biosphere acting as a C sink is based on the assumption that the CO$_2$-induced increase in photosynthesis observed at plant and/or leaf level will
be transformed into an enhanced terrestrial productivity. However this assumption seems to have only limited validity (see below).

2.2 Is 'scaling up' from single plant to ecosystem level a promising approach?

Some of the commonly occurring, short-term effects of elevated pCO$_2$ on single plants are summarized in Table I. The effects of elevated pCO$_2$ at the level of cell, leaf or plant are well understood and described (e.g. Long and Hutchin, 1991). However, the effects of multivariate interactions represent major barriers to the extrapolation of the result from the cell, leaf or plant level to the level of complex, natural communities (Long and Hutchin, 1991). For example, the stimulatory effect of elevated pCO$_2$ on plant photosynthesis is not necessarily translated into an increase in productivity at the community or ecosystem level. This reflects a discrepancy between the increased C fixation and the effective C yield. Moreover, the increased water use efficiency (WUE) observed at the single plant level under elevated pCO$_2$ (Table I), may become nil when expressed on a ground-area basis due to an increase in leaf area index. In most of the CO$_2$-enrichment studies, the plants were exposed to non-limiting resource availability. In contrast, the productivity of most natural ecosystems is co-limited by various resources (nutrients, water, light). Therefore, the CO$_2$ response of ecosystems may interact with environmental limitations (Mooney et al., 1991).

The 'scaling up' from single plant to community level may, thus, not reflect the reality. In fact, the reverse process (scaling down) should be the dominating approach in CO$_2$ research (Diaz, 1995). Such a concept definitely requires an experimental approach under field conditions. Key processes should be identified at the plant community level and thereafter examined at the single plant level (Diaz, 1995). In addition, there is only limited information available concerning the potential effects of elevated pCO$_2$ on a long-term scale (Mooney et al., 1991). An example of the potentially misleading and, thus, unsatisfactory results of 'scaling-up' from single-plant experiments carried out under controlled conditions is the unexpectedly strong CO$_2$ response of the C$_4$ plants in the tallgrass prairie (Table II). Based on the difference in the pathway of C fixation between C$_4$ and C$_3$ plants, a stronger CO$_2$ effect on the C$_3$ species would have been expected. However, in the tallgrass prairie, other factors
Table I  Some commonly observed CO₂ effects on single plants cultivated under controlled conditions; reviewed by Strain and Cure (1985), Long and Hutchin (1991), Coleman et al. (1993), Conroy and Hocking (1993) and Bunce (1994).

<table>
<thead>
<tr>
<th>Physiological processes</th>
<th>CO₂ effects on specific physiological processes</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Rate of photosynthesis (PS)</td>
<td>stimulation, often reduced after long-term CO₂ exposure due to photosynthetic acclimatization or adjustment (decline in Rubisco activation/quantity, in stomata density, end-product inhibition)</td>
</tr>
<tr>
<td>• Stomatal conductance</td>
<td>reduction due to a constant ratio of intercellular to external pCO₂ (0.6-0.8 in C₃ plants) resulting in a lower transpiration</td>
</tr>
<tr>
<td>• Water use efficiency (WUE)</td>
<td>34% less water used per dry matter unit due to the reduced transpiration of the plants (on ground-area basis: impact depends on changes in leaf area index)</td>
</tr>
<tr>
<td>• Dark respiration</td>
<td>increasing evidence for reduction in respiration. This may improve the ability of the plants to sequester more carbon</td>
</tr>
<tr>
<td>• Chemical composition of leaves</td>
<td>enhanced C:N ratio; this may result from increased N dilution (by enhanced carbohydrate accumulation), lower N investment in PS involved enzymes, reduced N uptake by plants (due to lower transpiration), and accelerated growth (size effect)</td>
</tr>
<tr>
<td>• Biomass production</td>
<td>stimulation in most experiments; inconsistent response pattern of the root-shoot ratio</td>
</tr>
<tr>
<td>• PS pathway</td>
<td>CO₂ effect on C₃ &gt; CO₂ effect on C₄</td>
</tr>
<tr>
<td>• Nutrient use efficiency</td>
<td>often enhanced</td>
</tr>
</tbody>
</table>

interfered to such an extent that a reverse and unexpected CO₂ response occurred in the investigated grass species (Owensby et al., 1993a;b). Poa pratensis, a C₃ species, did not respond to elevated pCO₂. This was ascribed
The response of some grassland ecosystems to elevated atmospheric pCO$_2$: the ecosystems are characterized by different environmental constraints; exclusively long-term field, non-single-species experiments were considered for this overview.

<table>
<thead>
<tr>
<th>Eco-systems</th>
<th>Major growth limitations</th>
<th>Botanical composition</th>
<th>Some of the most important, reported CO$_2$ effects in these ecosystems</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arctic tundra (Alaska)</td>
<td>temperature and nutrient availability, short growing season growth potential</td>
<td>arctic sedge (<em>Eriophorum vaginatum</em>), mosses, deciduous shrubs</td>
<td>loss of the stimulation of PS (3 weeks) in <em>E. vaginatum</em> and of net ecosystem CO$_2$ uptake (3 years) due to low N and lack of sinks (genetic limitation); net C sequestration is maintained if temperature is also increased</td>
</tr>
<tr>
<td>Estuarine salt marsh (Chesapeake Bay)</td>
<td>considered as highly productive, nutrient-rich, infrequently flooded site</td>
<td>pure stand of C$_3$ (<em>Scirpus olneyi</em>) and C$_4$ (<em>Spartina patens</em>) plants; C$_3$ &amp; C$_4$ mixture (with <em>Distichlis spicata</em>, C$_4$)</td>
<td><em>Scirpus</em>: increase in PS, yield (below- and above-ground) and shoot density, decrease in rate of senescence; <em>Spartina</em>: no response; composition may shift at the expense of <em>Spartina</em>; stimulated C accumulation (55% in <em>Scirpus</em>) and reduced water loss in all stands</td>
</tr>
<tr>
<td>Tallgrass prairie (Kansas)</td>
<td>dry temperate grassland, low N availability, periodic moisture stress</td>
<td>mixture of C$_3$ (<em>Poa pratensis</em>) and C$_4$ (e.g. <em>Andropogon gerardii</em>) plant species</td>
<td>CO$_2$ response in dry years due to improved water relations (increased WUE); response of C$_4$ species &gt; of C$_3$ grass, altered yield proportion; N limits magnitude of CO$_2$ response, no direct effect on litter decomposition, higher microbial activity and biomass with additional N</td>
</tr>
<tr>
<td>Annual grassland (Jasper Ridge)</td>
<td>low to modest soil productivity, high variation in precipitation</td>
<td>multi-species communities (annuals), native forbs*, grasses, and legumes</td>
<td>no PS downregulation (<em>Avena barbata</em>), increase in above-ground biomass except for the late season, shift in plant composition (late-season annuals), increase in litter, improved WUE</td>
</tr>
<tr>
<td>Eco-systems</td>
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<tr>
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</tr>
<tr>
<td>Alpine grassland</td>
<td>growth potential (but high PS efficiency), low night temperature, short growing season</td>
<td>alpine, sedge dominated vegetation (Carex curvula)</td>
<td>higher NCE** (despite downward adjustment), improved WUE, no stimulation of above-ground biomass (even with additional N), increased root biomass, below-ground C sink, species-specific response (Poa alpina) may alter community structure, no effect on microbial and soil organic C and N, N and climatic conditions are stronger determinants of biomass production than pCO₂</td>
</tr>
<tr>
<td>Calcareous grassland</td>
<td>N, P and K availability, periodically water stress</td>
<td>undisturbed and artificially established (3 levels of plant diversity) communities dominated by Bromus erectus</td>
<td>no CO₂ response of total above-ground biomass, CO₂ effect was greatest in the high diversity treatment (higher graminoid fraction), minor shift between functional groups (low and medium diversity treatments), no response of legumes, species-specific CO₂ response within functional groups occurred but were statistically insignificant</td>
</tr>
<tr>
<td>Fertile grassland</td>
<td>N and light (depending on imposed management), periodically water or temperature</td>
<td>A) T. repens, L. perenne monoculture, binary mixture; B) grass, legume &amp; forb* genotypes</td>
<td>A&amp;B) species-specific CO₂ response: strong above-ground response of legumes, a weak one of grasses, reverse below-ground response (A), higher clover proportion in mixture (A), no genotype x CO₂ interaction (B)</td>
</tr>
</tbody>
</table>

* forbs: non-leguminous dicots; **NCE: net CO₂ exchange of the ecosystem canopy

to both an initial nutrient and a subsequent light limitation due to the rapidly developing C_4 tallgrass canopy (Owensby et al., 1993a).

The extent of the CO_2 response of a given ecosystem is co-determined by growth factors such as nutrients, light and temperature and their interactions. The different grassland ecosystems (Table II) are restricted by various factors and exposed to distinct degrees of limitations. As a result, not all ecosystems respond similarly to elevated pCO_2. Limitation by resources may become accentuated under elevated pCO_2 by a general intensification of the competition for resources other than CO_2 among plants (Bazzaz and McConnaughay, 1992). In addition, the genetic background of the plant and year-to-year fluctuations of climatic parameters (rainfall, temperature and radiation) may interact with a potential pCO_2 response of perennial grassland ecosystems exposed to more or less intensive management regimes.

Most of the experiments show that, elevated pCO_2 changes also the competitive interaction between species (Table II, citations therein). This results in shifts of the species composition in a given community. Bazzaz (1990) argued that the impact of elevated pCO_2 on productivity of ecosystems results mainly from changes in species composition brought about by differential species response to elevated pCO_2. Therefore, it is necessary to identify the traits of plants which are contributing to low or high CO_2 responsiveness of different plant species and, thus, contributing to shifts in species composition. All experiments (Table II) intended to solve the following questions:

(A) Are the effects of elevated pCO_2 transient; will the biosphere remain a C sink for the extra atmospheric C on the long-term scale?

(B) Does the enhanced C fixation increase the efficiency of other key processes providing additional sinks for the extra C?

The results of the experiments show that the initial CO_2-induced stimulation of biomass production may disappear over time due to a multitude of other interacting restrictive parameters (Oechel et al., 1994). Moreover, CO_2-induced increases in photosynthesis do not necessarily translate into a higher productivity at the ecosystem level. The crucial question whether or not a given ecosystem may act as a C sink is very complex. A CO_2-induced increase in C sequestration in the biosphere would be a result of various interacting processes (e.g. photosynthetic parameters, leaf area, senescence, water balance, dark respiration, CO_2 efflux from the soil). This may further be influenced by environmental conditions and management factors.
2.3 How important is N for the CO$_2$ response of a grassland ecosystem?

There is not much known about the degree to which the CO$_2$ response depends on the availability of other resources. It is hypothesized that elevated pCO$_2$ may help plants to cope with limitations on growth imposed by insufficient light, water or nutrients (Table I; Idso and Idso, 1994). Despite generally lower relative requirements for nutrients (Table I) a compilation of various data sets showed that the CO$_2$ response is stronger if high quantities of nutrients are added to the systems (Poorter et al., 1996). For this reason relatively small CO$_2$ responses are expected under field conditions. This is in line with the concept of Field et al. (1992) arguing that the resource which is most limiting to ecosystem productivity would dominate even under elevated pCO$_2$. If so, then we need to focus mainly on N as one of the predominant growth limiting factor for productivity of ecosystems with adequate P and K supply in the humid, temperate region. Hence, an inadequate N availability could restrict C fixation and, thus, be a major constraint on C storage in the biosphere especially under conditions of elevated pCO$_2$.

The CO$_2$-induced stimulation of the ecosystem productivity would need an increased amount of N (per ground area) despite a lower N requirement per unit dry matter. However, the amount of N available to plants may be reduced under elevated pCO$_2$ due to the following processes. Increases in the C:N ratio of the plant material (Table I), an indirect CO$_2$ effect and/or a result of low N availability, may reduce forage and litter quality. The latter may ultimately slow down the decomposition rate and reduce the amount of N released into the system. In addition, the suggested CO$_2$-induced increase in rhizodeposition (van Veen et al., 1991) may stimulate the microbiomass and thus N immobilization (Díaz et al., 1993). Moreover, the higher soil moisture resulting from the improved WUE of sward exposed to elevated pCO$_2$ (Table I and II) would favor the activity of denitrifying bacteria. These soil processes would reduce the amount of N available to plants exposed to elevated pCO$_2$ and accentuate a possible N limitation.

Therefore, under elevated pCO$_2$, the increased N demand of the sward may be confronted with a decline in N supply and N uptake of plants. The latter may be induced by the lower transpiration of the plants (Table I). An inadequate N supply of grasses would ultimately reduce tiller formation and leaf development in grasses resulting in a delayed achievement of the optimal leaf area index and in a reduced productivity. Such processes may strongly
curb a potential CO₂ effect of plant species with high N requirements. However, legumes may be less affected by these processes thanks to their symbiotic association.

It seems likely that increased C fixation must be paralleled by an increase in N addition to the system, otherwise further C fixation by the ecosystem will be curbed. This means, that elevated pCO₂ can only stimulate biomass productivity and/or certain processes in an ecosystem (e.g. denitrification) if the enhanced N demand of the enlarged C sinks under elevated pCO₂ can be satisfied. The increased N demand of the ecosystem can potentially be satisfied by an increased application of fertilizer N and/or by the continuously increasing input of wet and dry N deposition (Vitousek, 1994).

2.4 How important is symbiotically fixed N for the CO₂ response of a grassland ecosystem?

At the plant level, symbiotic N₂ fixation is considered to be tuned according to the plants demand for symbiotically fixed N (Hartwig and Nösberger, 1994). The CO₂-induced stimulation of plant growth enhances the demand for N (Ingestad, 1982) and, thus, may induce a paralleled increase in the amount of symbiotically fixed N.

At the level of the entire ecosystem, symbiotic N₂ fixation introduces significant amounts of N into the grassland. Granhall (1981) proposed that the performance of N₂ fixation in an ecosystem is inverse to the N content of the specific system. This concept suggests that the widening of the C:N ratio which results from the increased C fixation under elevated pCO₂ would ultimately evoke an enhanced N₂ fixation. Thus, in a CO₂-rich environment, symbiotic N₂ fixation may play a crucial role in maintaining the C to N balance within an ecosystem and therefore may indirectly permit the increased C storage in the vegetation and/or the soil as suggested by several authors (Luxmoore, 1981; Bazzaz, 1990). However, there are no data available about the impact of elevated pCO₂ on the performance of symbiotic N₂ fixation in field-grown legumes.

CO₂-induced alteration in symbiotic N₂ fixation could cause changes in ecosystem functioning by, for example, altering competitive interaction between species. Symbiotic N₂ fixation could represent a relevant trait associated with high CO₂ responsiveness of legumes and hence be the cause for the species-specific CO₂ effect in grassland (Lüscher et al., 1996; Hebeisen et al., 1997).
2.5 The objectives of this study

The general aim of the field experiment was to investigate the impact of elevated pCO₂ on a fertile grassland ecosystem. To ensure an even CO₂ fumigation of large field plots without artefacts related to microclimatic conditions such as temperature, irradiation and wind, we used the Free Air Carbon-dioxide Enrichment (FACE) technology. The term 'ecosystem' is defined as a system of interacting organisms in a particular habitat (Harris, 1990). In order to study the processes involved in the CO₂ response in grasslands, we reduced the complexity of the system by focusing on two important, well characterized grassland species, Lolium perenne L. and Trifolium repens L. The two plant species show differences not only in morphology but also in their mode of satisfying their N needs. L. perenne is highly N demanding and exclusively dependent on the supply of N from the soil (mineralized N and fertilizer N). In contrast, T. repens can use molecular N as an additional N source thanks to its symbiotic association with Rhizobium leguminosarum bv. trifolii.

The aim of this work was to investigate the importance of N nutrition for the CO₂ response of the two plant species. Special attention was given to the role of symbiotic N₂ fixation. To approach these experimental questions, a model ecosystem consisting of monocultures and binary mixture of the two species was studied (Chapters IV and V of this work). In order to investigate the interaction between elevated pCO₂ and the availability of other resources two N fertilization regimes and two defoliation frequencies were applied. We investigated the N content of the above-ground biomass of the swards. The contribution of symbiotic N₂ fixation to the total N yield of T. repens was determined using the ¹⁵N-isotope-dilution method. In order to dissect the direct CO₂ effects on symbiotic N₂ fixation from indirect ecosystem-induced ones we examined the impact of elevated pCO₂ on T. repens seedlings grown under controlled conditions (Chapter VI). The experiments were based on the following hypothesis:

(A) symbiotic N₂ fixation in T. repens is a crucial process explaining the species-specific CO₂ response
(B) elevated pCO₂ enhances the amount of symbiotically fixed N due to the increased N-demand related to the higher productivity of T. repens
(C) elevated pCO$_2$ results in a shift in the relative contribution of symbiotically fixed N as opposed to N assimilated from mineral sources if the amount of mineral N is insufficient to cover the CO$_2$-induced increase in the plant's N demand.

(D) elevated pCO$_2$ will reduce the forage quality resulting from alterations in the C:N ratio in the plant material.

The results of these experiments illustrate a strong link between C and N metabolism, especially in a CO$_2$-rich world. Based on the data of these experiments, it is suggested that a CO$_2$-induced stimulation of symbiotic N$_2$ fixation in white clover is a crucial process for an increase in C storage in fertile grassland ecosystems.
STIMULATION OF SYMBIOTIC N₂ FIXATION IN TRIFOLIUM REPENS L. UNDER ELEVATED ATMOSPHERIC CO₂ IN A FERTILE GRASSLAND ECOSYSTEM

1 ABSTRACT

Symbiotic N₂ fixation is one of the main processes introducing nitrogen (N) into terrestrial ecosystems. As such, it may be crucial for the sequestration of the extra carbon (C) available in a world of continuously increasing atmospheric pCO₂. The effect of elevated pCO₂ (60 Pa) on symbiotic N₂ fixation (¹⁵N-isotope-dilution method) was investigated using Free Air Carbon-dioxide Enrichment (FACE) technology over a period of three years. Trifolium repens L. was cultivated either alone or in mixed swards together with Lolium perenne L. (non-fixing reference crop). Two N fertilization levels (in 1993: 10 or 42 g m⁻² a⁻¹; in 1994 and 1995: 14 or 56 g m⁻² a⁻¹) and two defoliation frequencies (5 or 3 defoliations in 1993; 8 or 4 defoliations in 1994 and 1995) were applied. Total N yield of T. repens increased consistently under elevated pCO₂. The percentage of plant N derived from symbiotic N₂ fixation (%Nsym) increased under elevated pCO₂ in both sward types and in all management treatments over the entire experimental period. In the monocultures, %Nsym increased from 56% under ambient pCO₂ to 65% under elevated pCO₂ in the low N treatment and from 34% to 42% in the high N treatment (averaged over all growing seasons and both defoliation treatments). All additionally assimilated N in the above-ground plant material was derived from symbiotic N₂ fixation and none from the soil. Averaged over all three years of the experiment, total N₂ fixation increased by 40%. It is suggested that the increase in symbiotic N₂ fixation is a competitive advantage for T. repens in mixed swards with pasture grasses resulting in altered species composition in mixed swards. Symbiotic N₂ fixation, a N introducing process, is considered to be a crucial factor in maintaining the C:N ratio in the ecosystem, in particular in those exposed to elevated pCO₂.
2 INTRODUCTION

In recent years significant attention has been paid to the possible impact of increasing atmospheric pCO$_2$, one of the major factors in the global climate change phenomenon, on ecosystems. Elevated pCO$_2$ is likely to affect C cycling by stimulating photosynthesis and primary productivity of terrestrial ecosystems. This may result in increasing sequestration of C into the biosphere. However, primary productivity of an ecosystem may be limited by other environmental factors such as irradiation, temperature or availability of water and mineral nutrients (Bazzaz, 1990; Gifford, 1992). Nitrogen availability is one of the key factors limiting crop yield of most ecosystems (Kirkby, 1981). If greater CO$_2$ availability results in increased plant growth then elevated pCO$_2$ will ultimately lead to increased N demand at the single plant level (Ingestad, 1982). Thus, the extent of the CO$_2$ response at the plant level could be limited by N availability. If so, then legumes, which can fix atmospheric N in symbiosis, are in a favored position.

Symbiotic N$_2$ fixation is considered to be the major process for the introduction of N into most terrestrial ecosystems. It can be assumed that sequestration of C and N into an ecosystem occurs in concert (Granhall, 1981; Gifford, 1992; Hartwig et al., 1996). Hence, symbiotic N$_2$ fixation may not only be regulated by the N demand of the individual plant but also indirectly by the N demand of the ecosystem as a whole (Hartwig et al., 1996). Numerous studies covering a wide range of N$_2$-fixing legumes and woody species have demonstrated an increase in total nitrogenase activity per individual plant under elevated pCO$_2$ (Hardy and Havelka, 1976; Phillips et al., 1976; Masterson and Sherwood, 1978; Finn and Brun, 1982; Williams et al., 1982; Murphy, 1986; Norby, 1987; Arnone and Gordon, 1990). However, instantaneous measurements of nitrogenase activity provide no information about the relative contribution of N assimilation from symbiosis as opposed to N from soil and/or fertilizer. Moreover, these measurements do not provide an estimate of the amount of N introduced by symbiotic N$_2$ fixation into an ecosystem. In a world of increasing atmospheric pCO$_2$, such information is needed in order to predict how individual plants and the ecosystem as a whole will respond to an alteration in the C:N balance.

Integrated measurements of N$_2$ fixation using the $^{15}$N-isotope-dilution method will ultimately lead to a better understanding of the processes
regulating C sequestration into ecosystems. The results of various CO$_2$-enrichment experiments have shown that the stimulation of the above-ground biomass production is stronger in legumes than in non-legumes (Newton et al., 1994; Lüscher et al., 1996; Hebeisen et al., 1997). However, the below-ground response of *Trifolium repens* is relatively weak compared with *Lolium perenne* (Jongen et al., 1995; Hebeisen et al., 1997). All these facts are consistent with the possibility that symbiotic N$_2$ fixation plays a key role in the response of grassland ecosystems to elevated pCO$_2$.

The aim of these experiments was to examine symbiotic N$_2$ fixation of field-grown *T. repens* under long-term elevated CO$_2$ conditions. To satisfy this aim we used the FACE technology. This technology allows investigations of the CO$_2$ response of large field plots without artifacts related to microclimatic conditions such as temperature, irradiation and wind. The results indicate that there is an increase in symbiotic N$_2$ fixation in response to the CO$_2$-induced increase in the N demand in the individual plant and in the ecosystem as a whole.

3 MATERIALS AND METHODS

3.1 Free Air Carbon-dioxide Enrichment (FACE) experiment and plant material

FACE technology (Hendrey et al., 1992; Lewin et al., 1994) was used to investigate the long-term effects of elevated pCO$_2$ on a grassland model ecosystem in the field. The study was carried out at 550 m above sea level in Eschikon, which is near Zurich, Switzerland. Each of the three blocks consisted of a CO$_2$-enriched (60 Pa pCO$_2$) and a control (ambient pCO$_2$) ring (18 m diameter). The rings were situated at least 100 m apart from one another. Within each block, the plots had been subjected to the same crop rotation before the establishment of the experiment. The CO$_2$ fumigation was begun May 31, 1993, and continued until the end of the growing season of that year. In 1994 and 1995, however, the CO$_2$ enrichment lasted for the entire growing season during the daytime with a reliability of a one-minute average of 60 Pa ±10% within 92% of the fumigated time (±20% within 99% of the time) for the three rings.
The soil at the experimental site was a fertile, eutric cambisol with a potassium and phosphorus content adequate for intensively managed grassland. In the early spring of each year, the plots of the first two blocks were fertilized with P$_2$O$_5$ (12 g m$^{-2}$), K$_2$O (29 g m$^{-2}$) and MgO (1.6 g m$^{-2}$). In the third block the amount of fertilizer was increased by 35% to counterbalance differences in nutrient availability.

In mid-August 1992, *Trifolium repens* cv. Milkanova was sown either as a monoculture (0.8 g m$^{-2}$) or as a mixture (0.4 g m$^{-2}$) with *Lolium perenne* cv. Bastion (1.6 g m$^{-2}$). Each plot measured 2.8 x 1.9 m. Two different nitrogen treatments and two different harvesting regimes were applied. In 1993, the swards were harvested either five (frequent defoliation) or three (infrequent defoliation) times during the fumigation period. Before the start of CO$_2$ fumigation (May 31, 1993) the swards were harvested in mid-April and in mid-May. These cuts were not considered for the analysis of the 1993 data. In 1994 and 1995, defoliation frequency was increased to eight and four harvests, respectively. Cutting height was approximately 5 cm above ground level.

Each year, in the autumn, the plots were treated with vinclozolin (Rovilan, Du Pont, SA, Geneva, Switzerland) (0.1 g m$^{-2}$) to eradicate *Sclerotinia trifoliorum*. In spring, benomyl (Benlate, Siegfried, Zofingen, Switzerland) (5 g m$^{-2}$) was applied to provide protection against *Fusarium* spp.

### 3.2 Nitrogen and $^{15}$N application

Nitrogen was applied at two different rates (low N; high N): either 10 or 42 g m$^{-2}$ a$^{-1}$ (equivalent to 100 or 420 kg ha$^{-1}$ a$^{-1}$) in 1993 and either 14 or 56 g m$^{-2}$ a$^{-1}$ (140 or 560 kg ha$^{-1}$ a$^{-1}$) in 1994 and 1995. The amount of N for one regrowth period was adjusted to the biomass production. This was expected to be highest in the spring and progressively lower towards the end of the growing season. In the frequently defoliated, low N treatment the annual dosage in 1993 was split in 25%, 20%, 17%, 15%, 15% and 8% for the first through the sixth regrowth period and in 24%, 19%, 17%, 14%, 14% and 12% in high N treatment, respectively. In 1994 and 1995 the corresponding values were 16%, 15%, 12.5%, 12.5%, 12.5%, 12.5%, 10% and 9% for the eight regrowth periods. The infrequently defoliated treatment received 30%, 25%, 25% and 20% of the annually applied N in all three years. Applications were made on the day after harvesting and for the first regrowth period at the beginning of April (1994, 1995) or in mid-April (1993).
N was supplied as a solution of NH$_4$NO$_3$ (1 l m$^{-2}$). All plots were watered with 1 l m$^{-2}$ of water following the N application. The harvest area (0.9 x 1.1 m) was fertilized with $^{15}$N-enriched NH$_4$NO$_3$ (ammonium and nitrate were equally labelled) (Isotec, Miamisburg, OH, and Matheson, Secaucus, NJ, USA). In the first two experimental seasons, the atom%$^{15}$N-excess was 0.4% for the high N treatment and 1.6% for the low N treatment. In the third year the atom%$^{15}$N-excess was decreased to 0.3% and 1.3% for the high and low N treatments, respectively. The remainder of each plot (4.33 m$^2$) was supplied with an equal amount of unlabelled NH$_4$NO$_3$ (Fluka Chemie AG, 9470 Buchs, Switzerland).

3.3 Sampling

Plant material from the central part (0.45 x 0.55 m) of the $^{15}$N-labelled area (0.9 x 1.1 m) was separated into _T. repens_, _L. perenne_ and unsown species. _T. repens_ and _L. perenne_ were dried at 65°C for 48 h. After the last harvest in 1993 and 1994, the remaining above-ground (below cutting height) and below-ground biomass was harvested with three above-ground samples (100 cm$^2$) and two soil cores (5 cm diameter, 50 cm depth) being taken in all of the _T. repens_ monocultures. Before drying, the root material was washed using Gillison's hydropneumatic elutriation system (Smucker et al., 1982).

All dried plant material was chopped into small pieces (Fuchs-Mühle, Type M.M.125H, Fuchs AG, Wien, Austria) and then ground (sequentially by a Cyclotec 1093 sample mill, Tecator AB, Höganäs, Sweden and by a ball mill [Type MM2, Retsch, Arlesheim, Switzerland]) to a very fine powder. After re-drying (35°C for 24 h), the samples (1 mg) were weighed into tin caps (0.04 ml, Lüdi AG, Flawil, Switzerland). The samples were analyzed for $^{15}$N- and N-concentration by a continuous flow mass spectrometer (Europa Scientific, Cambridge, UK) in the laboratory of Dr. C. van Kessel, University of Saskatchewan, Saskatoon, Canada. Leaf material (50 mg) from two harvests (May and July 1994) prepared in the same way was analyzed for N concentration on an elemental analyzer (LECO CHN-1000, LECO Corporation, St. Joseph, MI, USA).
3.4 Calculation of the percentage of N derived from the symbiosis (%Nsym)

%Nsym, a yield-independent parameter, was calculated for each regrowth period according to McAuliffe et al. (1958).

\[
%Nsym = \left(1 - \frac{^{15}N \text{ atom}\% \text{-excess in fixing crop in monoculture or mixture}}{^{15}N \text{ atom}\% \text{-excess in intercropped non-fixing reference crop}}\right) \times 100
\]

\(L. \ perenne\) in the mixtures of the appropriate treatment served as reference crop. \(L. \ perenne\), which has similar N uptake and rooting patterns as \(T. \ repens\) (Boller and Nösberger, 1988) is therefore a suitable reference plant for studies on \(N_2\) fixation by \(T. \ repens\).

Elevated \(pCO_2\) may induce changes in rooting depth. Thus, a homogeneously labelled soil profile is a prerequisite for an accurate application of the \(^{15}N\)-dilution method (Danso et al., 1993). This was achieved by applying \(^{15}N\) in solution to the same area at the beginning of each regrowth period. This procedure is considered to minimize errors due to mismatch between the reference and the \(N_2\)-fixing crop (Danso et al., 1993). To further check the suitability of the selected reference plant, plant material from unsown, non-fixing plants was collected in the same area and analyzed as described above. Only small differences were observed in atom%\(^{15}\)N-excess between the different non-fixing plant species. These are most likely due to differences in N uptake and rooting patterns between species (Witty, 1983). However, these differences were not influenced by the \(CO_2\) treatments, indicating that possible \(CO_2\)-induced changes in rooting depth were insignificant for the N-uptake pattern. In November 1994, four weeks after the last fertilization, the atom%\(^{15}\)N-excess of the plant-available N in different soil horizons (0-15 cm; 15-30 cm; 30-45 cm) was examined in separate plots of monocropped \(L. \ perenne\). They had been treated exactly as the \(T. \ repens\) monocultures supplied with high N. Mineral N was extracted from 100 g of sieved soil with 1 M KCl-solution (200 ml) according to the procedure described by Keeney and Nelson (1982) and analyzed for atom%\(^{15}\)N-excess. The results showed that the \(^{15}\)N-label was evenly distributed throughout the profile. This ensures that any possible \(CO_2\)-induced change in the rooting depth of the plants would not influence the accuracy of the determination of \(N_2\) fixation in \(T. \ repens\).
3.5 Statistical analysis

The experimental design was a split-split-plot design. The pCO₂ was the main-plot factor and the defoliation frequency was the sub-plot factor. Because the blocks and interactions between three or four experimental factors were statistically insignificant they were pooled into the specific error terms. Weed growth was particularly severe in 1995, and thus yields were corrected proportionally when weed dry matter was higher than 5% of the total yield. Analysis of variance was carried out using the statistical analysis package SAS (SAS Institute, Cary, NC, USA).

4 RESULTS

4.1 Effect of elevated pCO₂ on N yield and N concentration of T. repens as influenced by N supply, defoliation treatment and sward type

In general, elevated pCO₂ increased the annual above-ground N yield of T. repens (Figs. 1 and 2). When averaged across all treatments, the N yield under elevated pCO₂ increased by 24%, 18% and 12% in 1993, 1994 and 1995, respectively. Only the increase in 1993 was statistically significant (Table III). The N yield response to pCO₂ in T. repens was not affected by N supply, defoliation treatment or sward type (Table III). In all three years the effect of pCO₂ on the N yield of T. repens was more pronounced in the mixed swards (52%, 44% and 46% in 1993, 1994 and 1995, respectively) than in the monocultures, where the initially strong CO₂ effect (12% in 1993) declined to 7.5% and 1% in 1994 and 1995, respectively (Figs. 1 and 2). However, there was no significant interaction between CO₂ and sward type (Table III). Although the total N yield of T. repens increased under elevated pCO₂, the N concentration in the above-ground plant material produced under elevated pCO₂ was significantly reduced (Table IV). Infrequently defoliated T. repens material showed a reduced N concentration as compared to frequently defoliated material. Regardless of which CO₂ treatment was applied, there was always a significant effect of N supply, defoliation regime and sward type on the N yield of T. repens (exception: N supply in 1995) (Table III).
Figure 1. The contribution of soil and symbiotically derived N to the annual above-ground N yield of *T. repens* under ambient (35 Pa) and elevated (60 Pa) pCO$_2$ in infrequently defoliated monocropped and mixed swards over three growing seasons. Means and standard errors (SE) of total and symbiotically fixed N yield of three replicates are shown.
Figure 2. The contribution of soil and symbiotically derived N to the annual above-ground N yield of *T. repens* under ambient (35 Pa) and elevated (60 Pa) pCO$_2$ in frequently defoliated monocropped and mixed swards over three growing seasons. Means and SE of total and symbiotically fixed N yield of three replicates are shown.
Table III  Mean squares (MS) of an analysis of variance of the annual N yield and the annual N yield originating from symbiotic N\textsubscript{2} fixation (Nfix yield) in T. repens in three growing seasons.

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<tbody>
<tr>
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<td>MS 1)</td>
<td>MS 1)</td>
<td>MS 1)</td>
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<tr>
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<tr>
<td>D x Sw</td>
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<td>Error C</td>
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1) Level of significance: *, p < 0.1; **, p < 0.05; ***, p < 0.01; ns: not significant.
2) df: degree of freedom.
Table IV  
*N concentration in above-ground plant material of T. repens under ambient and elevated pCO₂ in frequently and infrequently defoliated monocropped and mixed swards at two nitrogen supplies during three growing seasons. Means and SE of three replicates are shown.*

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<td>35 Pa</td>
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<td>48.5</td>
<td>48.3</td>
<td>43.7</td>
<td>47.1</td>
<td>50.2</td>
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<tr>
<td></td>
<td></td>
<td>60 Pa</td>
<td>41.6</td>
<td>42.6</td>
<td>45.0</td>
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<td>46.3</td>
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<tr>
<td></td>
<td>60 Pa</td>
<td>45.6</td>
<td>41.7</td>
<td>43.2</td>
<td>41.8</td>
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<td>38.8</td>
<td>48.8</td>
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<td></td>
<td></td>
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<td>1.8</td>
<td>1.0</td>
<td>1.6</td>
<td>1.8</td>
</tr>
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</table>

[mg g⁻¹ dry weight]
Figure 3. %Nsym of infrequently defoliated *T. repens* grown in monocropped and mixed swards under ambient (35 Pa) and elevated (60 Pa) pCO₂ over three growing seasons. Annual average (Ø) is shown at the right side of each graph. Means and SE of three replicates are shown.
Figure 4. %Nsym of frequently defoliated T. repens grown in monocropped and mixed swards under ambient (35 Pa) and elevated (60 Pa) pCO₂ over three growing seasons. Annual average (Ø) is shown at the right side of each graph. Means and SE of three replicates are shown.
4.2 Effect of elevated pCO₂ on percentage of N derived from symbiosis (%Nsym) in T. repens as influenced by N supply, defoliation treatment and sward type

For all three years, the annually averaged %Nsym, a yield-independent parameter, was significantly higher in T. repens grown under elevated pCO₂ than in that grown under ambient pCO₂ (Figs. 3 and 4; Table V). This effect of pCO₂ on %Nsym was observed in all N treatments, defoliation regimes and sward types. In 1993, the annually averaged %Nsym increased from 53.7% under ambient to 63.2% under elevated pCO₂. In 1994 and 1995, the values were generally lower for %Nsym (Figs. 3 and 4). Furthermore the response of %Nsym to CO₂ was lower in 1994 (43.2% vs. 50.4%) and 1995 (45.9% vs. 51.0%) than in 1993 (53.7% vs. 63.2%). Except in 1993, when elevated pCO₂ evoked a more pronounced increase in %Nsym in the monocultures (from 42% to 54%) than in the mixtures (from 65% to 72%), no interactions were found between CO₂ treatment and sward type, N supply or defoliation frequency (Table V). Out of 128 data pairs, %Nsym was higher under elevated pCO₂ in a total of 114 cases (Figs. 3 and 4). The CO₂ effect on %Nsym appeared to be strongest during the mid-summer period in the frequently defoliated T. repens (Fig. 4). The %Nsym for all treatments declined towards the end of the growing season in all three years (Figs. 3 and 4). High N supply decreased the %Nsym significantly in all three years (Figs. 3 and 4; Table V). The %Nsym for T. repens grown in mixtures was significantly higher than for T. repens grown in monocultures in all three years (Figs. 3 and 4; Table V). There was no consistent effect of defoliation treatment on %Nsym in any of the three growing seasons (Table V).

4.3 Effect of elevated pCO₂ on N yield from symbiosis as opposed to N from the soil in T. repens

Averaged across all treatments, the amount of N derived from symbiosis under elevated pCO₂ was increased by 53%, 38% and 28% in 1993, 1994 and 1995, respectively. The increase in N yield derived from the symbiosis under elevated pCO₂ was statistically significant only in 1993 (Table III). In the monocultures there was a tendency for above-ground N yield originating from the soil (fertilizer and soil N) to decrease under elevated pCO₂ (Figs. 1 and 2) (exception: infrequently defoliated monocultures in 1995). Thus, any additional
N incorporated under elevated pCO$_2$ was derived from the symbiosis (Figs. 1 and 2). This effect was less evident in the intercropped clover (Figs. 1 and 2).

N yield of roots and stolons harvested at the end of the growing seasons in 1993 and 1994 (early November) showed a similar pattern for the contribution of N derived from symbiosis as opposed to N from the soil, as was seen for the above-ground plant material (Figs. 1, 2 and 5). Total N yield of stolon and root fractions tended to increase under elevated pCO$_2$. The major contributor to this was symbiotic N$_2$ fixation (Fig. 5).

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**Table V**  
*Mean squares (MS) of an analysis of variance of %N$_{sym}$ in *T. repens* in three growing seasons.*

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<td>1</td>
</tr>
<tr>
<td></td>
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<td>49</td>
<td>4</td>
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<td>Sub plot:</td>
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<td>4</td>
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<td>4</td>
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<td>Sw x N</td>
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<td>1</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>85</td>
<td>4</td>
</tr>
<tr>
<td>D x N</td>
<td>1</td>
<td>66 ns</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>85</td>
<td>4</td>
</tr>
<tr>
<td>D x Sw</td>
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<td>507**</td>
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</tr>
<tr>
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</tr>
<tr>
<td>Total</td>
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<td>29</td>
</tr>
</tbody>
</table>

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1) Level of significance: *, p< 0.1; **, p< 0.05; ***, p< 0.01; ns: not significant.
2) df: degree of freedom.
**Figure 5.** Contribution of soil and symbiotically derived N to the N yield of *T. repens* roots and stolons under ambient (35 Pa) and elevated (60 Pa) pCO$_2$ in infrequently and frequently defoliated monocultures. Means and SE of total and symbiotically fixed N yield of three replicates are shown.
5 DISCUSSION

5.1 Effect of elevated pCO₂ on N yield of *T. repens* and on the N concentration in the above-ground biomass

Under elevated pCO₂, the total above-ground N yield of *T. repens* tended to increase. The CO₂ effect was statistically significant in 1993 (Figs. 1 and 2; Table III). This is partly attributed to the fact that the error term A (Table III) has only four degrees of freedom and thus the factor CO₂ was only weakly tested. The CO₂-induced increase in N yield was more pronounced and persistent in the intercropped *T. repens* than in the monocropped *T. repens*. The difference in CO₂ response between the mixtures and the monocultures can be attributed at least in part to the increased yield proportion of *T. repens* in the mixtures exposed to elevated pCO₂ (Hebeisen et al., 1997).

The N concentration in the above-ground plant material was reduced under elevated pCO₂ (Table IV). This commonly documented CO₂ effect (Conroy and Hocking, 1993) is attributed to carbon-assimilated accumulation in leaves and/or an improvement in N use efficiency under elevated pCO₂ (Andrews and Lorimer, 1987; Badger, 1992; Morell et al., 1992). However, the reduction in N concentration appears to be less pronounced in legumes than in other grassland species (Luo et al., 1994). Carbohydrate accumulation in plant organs and/or shifts in the composition of the harvested material in favor of plant fractions of low N concentration may also contribute to a reduction in the N concentration in the above-ground biomass. In fact, a consistent decrease in the leaf:petiole ratio, known to correspond to a reduced N concentration in the above-ground material in *T. repens* (Soussana and Arregui, 1995), was also observed under elevated pCO₂ in our experiment. In line with this observation are the results of specific harvests (May 17 and July 12, 1994) that showed that under elevated pCO₂ the N concentration of leaf blade material was only reduced by 7% compared with the above-ground material, which was reduced by 10%.

5.2 Why does elevated pCO₂ stimulate symbiotic N₂ fixation?

Under elevated pCO₂, one might expect that the increased N yield in the above-ground plant material would be the result of a concerted increase in both N from the symbiosis and N from the soil/fertilizer. Such a response has been observed in a growth chamber experiment in which plants were grown in sand.
However, in the present field experiment we observed a consistent increase in %Nsym under elevated pCO2 (Figs. 3 and 4; Table V).

The N availability in the soil affects the performance of N2 fixation in legumes. A low supply of N from fertilizer as well as the presence of associated non-symbiotic plants competing for N decrease the soil-N availability, which is positively correlated with %Nsym in T. repens (Boller and Nössberger, 1987; Nesheim and Oyen, 1994; Seresinhe et al., 1994) and in many other legume species (Hardarson et al., 1991; Nesheim and Oyen, 1994). These observations are clearly confirmed by the present study (Figs. 3 and 4). Therefore, the increased %Nsym under elevated pCO2 must be the result of reduced soil-N availability evoked by the CO2-induced increased N demand in the system. Hence, the increased competitive ability of T. repens under elevated pCO2 (Newton et al., 1994; Lüscher et al., 1996; Hebeisen et al., 1997) is attributed to its ability to fix N and thus to compensate for the apparently reduced availability of mineral N.

Apart from the amount of N supply and the presence of non-fixing associated plant species, other processes such as leaching, mineralization, denitrification and N immobilization influence the amount of mineral N available to T. repens and thus affect %Nsym. All of these processes may be affected by pCO2. Under elevated pCO2, increases in rhizodeposition as well as in the total quantity and the C:N ratio of litter and root material (van Veen et al., 1991; Jongen et al., 1995), may ultimately alter below-ground processes involved in nutrient cycles (Curtis et al., 1994; Norby, 1994). Along with the suggested increase of N immobilization into the expanded microbial biomass (Díaz et al., 1993), enhanced denitrification may also reduce N availability. Higher soil moisture resulting from the lowered water use of plants exposed to elevated pCO2 (Morison, 1985; Goudriaan and Unsworth, 1990) and elevated oxygen consumption by the increased microbial activity and root biomass (Jongen et al., 1995) may lower the oxygen partial pressure in the soil and hence favor denitrification activity. Preliminary denitrification measurements suggested higher gaseous N losses from soil under elevated pCO2 (Ineson et al., 1997). Further support for a decrease in below-ground N availability comes from the observation that nitrate leaching from soil is reduced during winter from a CO2-fumigated perennial ryegrass plot (Soussana et al., 1996).

The particularly strong CO2 response of %Nsym in mid-summer (Figs. 3 and 4) results from the changes in plant growth and the soil processes outlined
above, which are most pronounced at this time of the year. These factors combine most strongly to reduce the N availability at this period.

Assimilation of soil N was either unchanged or decreased under elevated pCO$_2$ (Figs. 1, 2 and 5), even though Jongen et al. (1995) reported up to a 48% increase in clover root biomass in the same experiment. This observation is consistent with an unchanged or reduced soil-N availability under elevated pCO$_2$. Moreover, in the mixtures, there was little difference in the amount of N derived from the soil (Figs. 1, 2 and 5), despite the fact that the yield of _T. repens_ as a proportion of total forage yield was greater under elevated pCO$_2$ (Hebeisen et al., 1997). Under elevated pCO$_2$, the increase in the total amount of N fixed accounted for all additionally yielded N and compensated for the apparently reduced N availability in the soil under elevated pCO$_2$ (Figs. 1, 2 and 5). In addition, CO$_2$ effect on %N$_{sym}$ was observed in the high N treatment and thus the inhibiting effect of mineral N on %N$_{sym}$ was alleviated under elevated pCO$_2$ (Figs. 3 and 4).

It is known from short-term studies in growth chambers, greenhouses and open-top chambers that nitrogenase activity is enhanced under elevated pCO$_2$ in grain legumes (Hardy and Havelka, 1976; Phillips et al., 1976; Masterson and Sherwood, 1978; Finn and Brun, 1982; Williams et al., 1982), N$_2$-fixing trees (Norby, 1987; Arnone and Gordon, 1990), and forage legumes (Masterson and Sherwood, 1978; Murphy, 1986). Hardy and Havelka (1976) suggested that the CO$_2$-induced increase in nitrogenase activity is the result of an enhanced carbon availability for the high-energy-demanding nitrogen reduction in the nodules. However, there is little evidence in the literature for an increase in specific nitrogenase activity (activity per unit of nodule weight) under elevated pCO$_2$ (Finn and Brun, 1982; Murphy, 1986; Norby 1987). Moreover, recent studies showed that symbiotic N$_2$ fixation is not directly regulated by the availability of carbon assimilates either in the whole plant (Hartwig et al., 1990; Denison et al., 1992) or in the nodules (Weisbach et al., 1996). Nodule oxygen permeability (Hartwig and Nösberger, 1994) and plant N-sink strength (Heim et al., 1993; Oti-Boateng and Silsbury, 1993; Parsons et al., 1993; Oti-Boateng et al., 1994) are likely to be the direct regulators of nitrogenase activity. Increased plant biomass production results in an increased N-sink strength (Ingestad, 1982) and hence in an increased N$_2$ fixation. The increase in total nitrogenase activity under elevated pCO$_2$ reported by Finn and Brun (1982), Murphy (1986) and Norby (1987) resulted from an increase in nodule weight and number and was not apparent until
several days after exposure to elevated pCO₂. Therefore, this increase was induced by a stimulation of plant growth under elevated pCO₂, leading to a higher N demand and, thus, a higher nitrogenase activity per plant. The higher nitrogenase activity per plant is possible because of an increased nodulation and this in turn may lead to the higher N assimilation from the symbiosis, as shown in the present experiment. A two-fold increase in the number of free-living *Rhizobium leguminosarum* bv. *trifolii* cells in the rhizosphere of *T. repens* exposed to elevated pCO₂ (Schortemeyer et al., 1996) suggests that the roots were successfully and extensively nodulated.

5.3 Symbiotic N₂ fixation can correct the C:N imbalance in the ecosystem caused by elevated pCO₂

It is known that the C:N ratio in *T. repens* is lower than in non-fixing species. This difference is especially pronounced under elevated pCO₂ (Table IV; Table VI in Chapter V; Luo et al., 1994; Zanetti et al., 1995). As a result, *T. repens* litter decomposes more rapidly than litter from grasses. Thus, re-cycling of N would be expected to be quicker in a pasture ecosystem in which *T. repens* is the dominant species.

The strong initial CO₂ effect on N₂ fixation and on the amount of symbiotically fixed N (Figs. 1 to 5) in the grassland ecosystem may be a result from the abrupt increase in atmospheric pCO₂ at the beginning of the experiment in 1993 and consequently in a similarly abrupt increase in the C:N ratio in the grassland ecosystem. This suggests a shift from a predominantly C-limited to a predominantly N-limited ecosystem. Therefore, we expected that the mixtures would exhibit a stronger CO₂-induced increase in %Nsym due to a more pronounced reduction in soil N caused by the associated, N-demanding *L. perenne*. One reason this did not occur is that the increased N₂ fixation under elevated pCO₂ improved the competitive ability of *T. repens*, resulting in an increased legume and in a decreased grass yield proportion (Hebeisen et al., 1997). Thus, total demand and uptake of mineral N per area decreased. Consequently, the mineral N concentration in the soil decreased less and, therefore, the stimulation of N₂ fixation was less pronounced. In summary, the CO₂-induced increased sink for symbiotically fixed N in the mixtures was not only satisfied by an enhanced N₂ fixation, but also by an increased clover yield proportion.
Our results are consistent with the hypothesis that the amount of fixed N, which is inversely related to the N content of an ecosystem (Granhall, 1981), increases under elevated pCO$_2$ as a result of the increased C:N ratio in the ecosystem (Gifford, 1992). Indeed, more symbiotically fixed N (60 kg ha$^{-1}$ a$^{-1}$ averaged over all years) was introduced in the mixtures exposed to elevated pCO$_2$ compared with those of the control.

The ability of legumes to respond to elevated pCO$_2$ by assimilating more N (increased total N$_2$ fixation per plant) enhances their competitive advantage over non-legumes. Thus, over several years, the increased N input from N$_2$ fixation and from mineral fertilizer will introduce significant amounts of N into the ecosystem and the previously postulated increase in the C:N ratio will return to a new balanced value. Thus, under elevated pCO$_2$ legumes may enable additional carbon sequestration into the plant biomass and subsequently into the ecosystem as a whole.

Published in:
Plant Physiology (1996): 112; 575-583
DOES NITROGEN NUTRITION RESTRICT THE CO₂ RESPONSE OF FERTILE GRASSLAND LACKING LEGUMES?

1 ABSTRACT

The extent of the response of plant growth to atmospheric CO₂ enrichment depends on the availability of resources other than CO₂. An important growth limiting resource under field conditions is nitrogen (N). N may, therefore, influence the CO₂ response of plants. The effect of elevated CO₂ (60 Pa) partial pressure (pCO₂) on the N nutrition of field grown Lolium perenne swards, cultivated alone or in association with Trifolium repens, was investigated using the Free Air Carbon-dioxide Enrichment (FACE) technology over a period of three years. The established grassland ecosystems were supplied with two N-fertilization levels and were defoliated at two frequencies. Under elevated pCO₂, the above-ground plant material of L. perenne monoculture showed a consistent and significant decline in N concentration which, in general, led to a lower total annual N yield. Despite the decline in the critical N concentration (minimum N concentration required for non N-limited biomass production) under elevated pCO₂, the index of N nutrition (ratio of actual N concentration and critical N concentration) was lower under elevated pCO₂ than under ambient pCO₂ in frequently defoliated L. perenne monocultures. Thus, we suggest that reduced N yield under elevated pCO₂ was evoked indirectly by a reduction of plant available N. For L. perenne grown in association with T. repens and exposed to elevated pCO₂, there was an increase in the contribution of symbiotically fixed N to the total N yield of the grass. This can be explained by an increased apparent transfer of N from the associated N₂-fixing legume species to the non-fixing grass. The total annual N yield of the mixed grass/legumes swards increased under elevated pCO₂. All additionally yielded N was symbiotically fixed N. Through the presence of an N₂-fixing plant species more symbiotically fixed N was introduced into the system and consequently helped to overcome the N limitation under elevated pCO₂.
2 INTRODUCTION

It is generally accepted that the continuously increasing atmospheric pCO$_2$ should have a stimulatory effect on plant biomass production as a consequence of the rise in net photosynthesis (e.g. Bazzaz 1990; Poorter 1993). However, as is evident from the survey by Poorter (1993), the magnitude of the plant response varies greatly. Nutrient availability is an important factor influencing the extent of the CO$_2$ response of the plants (Poorter et al., 1996). Under field conditions, nutrients and in particular N are often scarce. This may lead to an intensification of competition for resources under elevated pCO$_2$ (Bazzaz and McConnaughay 1992) which would ultimately affect the composition of plant communities.

In temperate regions, large areas of cultivated land are covered by grassland. Therefore, any changes in the ecological stability of these systems, evoked by increasing atmospheric pCO$_2$, are of increasing ecological, agricultural and political concern. The results of a long-term FACE experiment showed that legumes and grasses respond differently to elevated atmospheric pCO$_2$. Elevated pCO$_2$ caused only a weak above-ground dry matter increase in grass species, whereas the response of legumes was much stronger (Lüscher et al., 1996; Hebeisen et al., 1997). These results are in line with those of previous studies carried out under controlled conditions (Newton et al., 1994; Schenk et al., 1995). The CO$_2$ stimulation of the above-ground dry matter production of T. repens under fertile field conditions (ample potassium and phosphorus supply) coincided with a stimulation of symbiotic N$_2$ fixation (%N$_{sym}$) (Zanetti et al., 1996; Zanetti and Hartwig 1997). This may lead to an alteration in the competitive interaction for N between L. perenne and T. repens under elevated pCO$_2$ and, thus, to the reported shift in the species composition in favor of the legume (Newton et al., 1994; Hebeisen et al., 1997).

Furthermore, the stability and functioning of a grassland ecosystem may also be affected by a CO$_2$-induced alteration in nutrient cycling in the ecosystem. In the long run, such changes will be reflected by sward structure and productivity (Mooney et al., 1991). In addition, a CO$_2$-induced increase in plant growth enhances the N demand of each individual plant (Ingestad 1982) and of the ecosystem as a whole (Hartwig et al., 1996). This could, therefore, result in a decrease in the amount of available N and, in turn, limit plant growth and any CO$_2$ response.
The objective of this three year field experiment was to investigate the effect of elevated pCO₂ (60 Pa) on the N nutrition of two different ecosystems (L. perenne monocultures and a binary mixture of L. perenne and T. repens). We hypothesized that an ecosystem depending solely on fertilizer and soil N differs in its CO₂ response from one that includes an N₂-fixing species. Because of the symbiotic N₂ fixation, legumes introduce additional amounts of N into the system. By various processes, the symbiotically fixed N (N_{sym}) of the legumes is apparently transferred to other non-legumous plants (N_{trans}) (Boller and Nösberger 1987) or other non-N₂-fixing organisms. This phenomenon could alleviate a possible inadequate N supply by the fertilizer and/or soil N (N_{fert}, N_{soil}) in ecosystems exposed to elevated pCO₂. The FACE technology should be free of any artifacts related to microclimatic conditions (temperature, wind, precipitation and irradiation) and, thus, suitable for an investigation in the large field plots needed for ecosystem approaches.

3 MATERIALS AND METHODS

3.1 Plant material and growth conditions

The FACE technology (Hendrey et al., 1992) was used to investigate the long-term effects of elevated pCO₂ on a grassland ecosystem in the field in Eschikon (550 m above sea level), near Zurich, Switzerland. Three blocks, each consisting of a CO₂-enriched (60 Pa CO₂) and a control (ambient pCO₂) area (18 m diameter) were investigated from 1993 to 1995. The CO₂ enrichment started on 31 May, 1993 and continued until the end of the growing season of that year. In 1994 and 1995, the period of CO₂ enrichment covered the entire growing season, i.e. from March to November (threshold: air temperature of 5°C). The CO₂ enrichment was activated during the daytime only. The experimental site has been described in detail elsewhere (Zanetti et al., 1996; Hebeisen et al., 1997).

In mid-August 1992, Lolium perenne cv. Bastion was sown either as a monoculture (3.2 g m⁻²) or as a mixture (1.6 g m⁻²) with Trifolium repens cv. Milkanova (0.4 g m⁻²). The size of each plot was 2.8 x 1.9 m. Both grassland ecosystems were exposed to two different N treatments and defoliation regimes. In 1993, the swards were defoliated five times (frequent defoliation) or three times (infrequent defoliation) during the period of CO₂ enrichment at a
cutting height approximately 5 cm above ground level. In mid-April of 1993, the
swards were cut to the same canopy height, and in mid-May one harvest was
carried out before the start of CO₂ enrichment. These cuts were not considered
in the analysis of the 1993 data (yield including the harvest carried out in mid-
May is indicated in parentheses for each treatment (Tables VI, VII and VIII)). In
1994 and 1995, the swards were defoliated either eight times (frequent
defoliation) or four times (infrequent defoliation) during the growing season.

Nitrogen was applied at two rates (low N: 10 in 1993 and 14 g m⁻² a⁻¹ in
1994 and 1995; high N: 42 g m⁻² a⁻¹ in 1993 and 56 g m⁻² a⁻¹ in 1994 and
1995). The harvest area (0.9 x 1.1 m) was fertilized with double labelled ¹⁵N-
enriched NH₄NO₃ (Isotec, Miamisburg, OH, and Matheson, Secaucus, NJ,
USA) at the beginning of the growing season and after each defoliation. In the
first two growing seasons, the atom% ¹⁵N-excess (¹⁵N concentration in excess
of the natural abundance) was 0.4% for the high N treatment and 1.6% for the
low N treatment. In the third year, the atom% ¹⁵N-excess was reduced to 0.3%
and 1.3% for the high and low N treatments respectively. The remaining area
was fertilized with an equal amount of unlabelled NH₄NO₃ (Fluka Chemie AG,
Buchs, Switzerland). For the last regrowth in 1995, the entire plots (2.8 x 1.9 m)
were fertilized with unlabelled NH₄NO₃.

In 1995, the area to which ¹⁵N-enriched NH₄NO₃ was applied was
enlarged by 0.55 m² in the frequently defoliated L. perenne monocultures.
From this newly labelled area, a representative sample of leaf material was
taken at each harvest and analyzed for its ¹⁵N concentration (see below).

3.2 Sampling

The plant material from the central part (0.45 x 0.55 m) of the ¹⁵N-labelled
area (0.9 x 1.1 m) was separated into L. perenne, T. repens and unsown
species. The dried plant material (65°C for 48 h) of L. perenne was ground into
powder and prepared for ¹⁵N analysis as described by Zanetti et al. (1996).
¹⁵N and N concentrations were analyzed by continuous flow mass
spectrometry (Europa Scientific, Cambridge, UK).

After the last harvest in 1993 and 1994, stubbles (three sub-samples,
100 cm² each) and root biomass (two soil cores of 5 cm diameter and 50 cm
depth) were sampled in the L. perenne monocultures. This plant material and
leaf blades of monocropped L. perenne from two harvests (May and July 1994)
were analyzed separately for N concentration using an elemental analyzer (LECO CHN-1000, LECO Corp., St. Joseph, MI, USA).

3.3 Calculation of N derived from fertilizer (Nfert) and of the percentage of N derived from apparent transfer (%Ntrans)

N concentration refers here to the ratio between the total annual N yield and the total annual dry matter yield. Nfert is the accumulation of Nfert\textsubscript{x} at each individual harvest 'x'. Nfert\textsubscript{x} was calculated according to Vose and Victoria (1986).

\[
\text{Nfert}_x = \left( \frac{\text{atom}\%^{15}\text{N-excess in plant material}}{\text{atom}\%^{15}\text{N-excess in fertilizer}} \right) \times \text{Ntot}_x
\]

Ntot\textsubscript{x} stands for the total harvestable N yield of a specific harvest 'x'. %Ntrans, a yield independent parameter, was calculated for each regrowth period according to Vallis et al. (1977).

\[
\%\text{Ntrans} = \left( 1 - \frac{\text{atom}\%^{15}\text{N-excess in the intercropped ryegrass}}{\text{atom}\%^{15}\text{N-excess in the monocropped ryegrass}} \right) \times 100
\]

\textit{L. perenne} in the monoculture of the corresponding treatment (same N and defoliation regime) served as a reference crop.

3.4 Calculation of N nutrition index (IN)

The N nutrition index IN is the ratio between the measured N concentration of a given sward and the corresponding calculated critical N concentration. The critical N concentration is the N concentration that ensures the maximum, non N-limited biomass production for a given sward (Lemaire and Salette 1984). The critical N concentration (Nc) was calculated according to the following functions proposed by Soussana et al. (1996): Nc= 4.9 DM \textsuperscript{-0.38} for swards exposed to ambient pCO\textsubscript{2}; Nc= 5.3 DM \textsuperscript{-0.52} for swards exposed to elevated pCO\textsubscript{2}. DM stands for the above-ground dry-matter yield (t ha\textsuperscript{-1}).

3.5 Statistical analysis

The experimental design was a split-split-plot design. The pCO\textsubscript{2} was the main-plot factor, and the defoliation frequency was the sub-plot factor. Because the block and interactions between three or four experimental factors were not
significant statistically, they were pooled into the specific error terms. Analysis of variance was carried out using the statistical analysis package SAS (SAS Institute, Cary, NC, USA).

4 RESULTS

4.1 Effects of elevated pCO₂ on N yield and N concentration of L. perenne grown alone or with T. repens under two N and defoliation regimes

L. perenne grown in monoculture

In most L. perenne monocultures, the annual above-ground N yield was reduced significantly under elevated pCO₂ (Table VI). In the frequently defoliated monocultures, the reductions in N yield of L. perenne under elevated pCO₂ were 16% (1993), 21% (1994) and 17% (1995). However, this consistent and pronounced CO₂ effect on the frequently defoliated swards was not observed under infrequent cutting regimes except in 1993 where the CO₂-induced reduction in N yield was 11%. The differing CO₂ responses to the two defoliation frequencies resulted in a significant CO₂ x defoliation interaction in 1995 (p<0.05). The CO₂-response pattern was similar in both N treatments (exception: 1995).

In the L. perenne monoculture, the N concentration of the above-ground plant material was always significantly lower under elevated pCO₂ than under ambient pCO₂ (Table VI). Averaged across all three growing seasons and all treatments, the CO₂-induced reduction in the N concentration of the L. perenne monoculture was 20% in the frequently defoliated swards and 15% in the infrequently defoliated swards.

L. perenne grown in association with T. repens

Under elevated pCO₂, total N yield of L. perenne grown in mixture decreased by 10% (1993) and 14% (1994) (Table VII). The mean N-yield reducing effect of elevated pCO₂ disappeared in 1995. This was due solely to the positive CO₂ response (18%) of the infrequently defoliated, high N treatment (Table VII).

In contrast to the results for L. perenne in monoculture, elevated pCO₂ had a weaker or no effect on the N concentration in the above-ground plant
Table VI  The contribution of fertilizer N (Nfert) to the total harvestable annual N yield (Ntot) and the N concentration of L. perenne monoculture grown under ambient (35 Pa) and elevated (60 Pa) pCO₂ at two defoliation frequencies and two N supplies over three growing seasons. Means and SE of three replicates are shown.

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¹⁾ number in parenthesis: total annual N yield including first unfumigated harvest
Table VII  *The contribution of apparently transferred N* (*N_{trans}* to the total annual harvestable N yield (*N_{tot}*) and the N concentration of *L. perenne* grown in mixture and exposed to ambient (35 Pa) and elevated (60 Pa) pCO$_2$, at two defoliation frequencies and two N supplies over three growing seasons. Means and SE of three replicates are shown.*

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$^1$) see Table VI
Table VIII  The contribution of symbiotically fixed and transferred N (Nsymb & Ntrans), fertilizer N (Nfert) and soil N (Nsoil) to the total (L. perenne and T. repens) annual harvestable N yield (Ntot) of mixed swards grown under ambient (35 Pa) and elevated (60 Pa) pCO$_2$ at two defoliation frequencies and two N supplies over three growing seasons. Means and SE of three replicates are shown.

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1) see Table VI
Figure 6. %Ntrans in L. perenne grown in mixture as determined from the above-ground plant material under ambient (35 Pa) and elevated (60 Pa) pCO₂, exposed to two N fertilization levels and two defoliation frequencies. Means and SE of three replicates are shown.
material of *L. perenne* grown in mixture (Table VII). In addition, the N concentration of *L. perenne* grown in mixture was, in general, significantly higher than that of the monoculture (Tables VI and VII).

4.2 Differences in CO$_2$ effects between N yield of monoculture and N yield of mixture

The effect of elevated pCO$_2$ on the total annual N yield of *L. perenne* monocultures (Table VI) was contrary to the effect on that of the sum of both species in the mixture (Table VIII). This was evident by the significant CO$_2$ x ecosystem interaction in all three growing seasons (p<0.05). The N yield of the mixture increased by 17% (1993), 9% (1994) and 13% (1995) under elevated pCO$_2$ (Table VIII). This CO$_2$-induced yield increase in the mixture was observed both in the defoliation and N treatments. Furthermore, the N yield of the two ecosystems differed in its response to N fertilization and defoliation frequency (ecosystem x N and ecosystem x defoliation interaction, p< 0.05).

4.3 Origin of the N yield as affected by elevated pCO$_2$, ecosystem, N supply and defoliation frequency

In all *L. perenne* monocultures, showing reduced total annual N yield under elevated pCO$_2$, the absolute amount of assimilated fertilizer N (Nfert) decreased (Table VI). Under most management regimes, this decrease under elevated pCO$_2$ was proportional to the reduction in the total N yield.

For *L. perenne*, grown in mixture and exposed to elevated pCO$_2$, the %Ntrans, a yield-independent parameter, tended to be higher than under ambient pCO$_2$ (Fig. 6). Elevated pCO$_2$ increased the %Ntrans in most regrowth periods in all growing seasons. In 58 out of 66 cases, the %Ntrans was higher under elevated pCO$_2$.

The N treatments had a significant effect on the %Ntrans. High N supply caused a significant decrease in %Ntrans in all three years (Fig. 6). The %Ntrans was virtually zero in the high N treatment exposed to ambient pCO$_2$ (Fig. 6), resulting in a consistently lower amount of N derived from apparent transfer under ambient pCO$_2$ compared to elevated pCO$_2$ (Table VII). In the low N treatment, the amount of N derived from apparent transfer was similar in both CO$_2$ treatments.

In the mixture, the contribution of fertilizer N and soil N to the total N yield was either similar in both CO$_2$ treatments or reduced in those exposed to
elevated pCO₂ (Table VIII). The N yield, originally derived from symbiotic N₂ fixation (Nsymb and Ntrans), increased strongly under elevated pCO₂ (Table VIII). This effect was independent of N supply and defoliation frequency (Table VIII).

5 DISCUSSION

The most important result of this study is that the CO₂ effect on N yield of the grassland ecosystems differed significantly in all three growing seasons (Tables VI and VIII; significant CO₂ x ecosystem interaction, p<0.05). The ecosystem including a legume (L. perenne / T. repens mixture) showed consistent N yield stimulation under elevated pCO₂, whereas N yield of the L. perenne monoculture responded negatively to CO₂ enrichment in 1993 and 1994 (Tables VI and VIII). The CO₂ response of the dry matter production of these two ecosystems is in line with the data presented here; Hebeisen et al. (1997) showed that the above-ground biomass of L. perenne grown in monoculture was stimulated only weakly by the additional CO₂ supply (+7%) in comparison to the 18% increase in the productivity of the mixtures. It was suggested that the productivity of a system exposed to elevated pCO₂ may be limited by resources other than CO₂ (Bazzaz 1990). N limitation could be involved in the poor dry matter response of L. perenne to CO₂ enrichment (Lüscher et al., 1996; Hebeisen et al., 1997) as observed even at a high rate of N fertilization (Hebeisen et al., 1997). In contrast, the presence of a legume in the ecosystem may help to overcome the situation of an unsatisfied N demand of the grass and of the entire system, because the legume may introduce an increased amount of symbiotically fixed N into the system (Zanetti et al., 1996; Zanetti and Hartwig 1997).

In general, the total annual N yield of the L. perenne monoculture was lower under elevated pCO₂, with no shifts in the relative contribution of fertilizer and soil N to the N yield (Table VI). In 1995, the CO₂ response of the two defoliation frequencies differed. This significant interaction (p<0.05) occurred because of a CO₂-induced stimulation in the N yield of the infrequently defoliated, high N monoculture (Table VI). The low cutting frequency, lasting three years, produced a relatively open sward structure (in the frequently defoliated swards the tiller number per ground area was increased by 77% in
comparison to that in the infrequently defoliated swards) which may allow for
the expansion, provided that nutrients are not limiting. CO₂ yield stimulation
has been ascribed to the ability to increase tiller number (Schenk et al., 1995).
Indeed, swards with spaced plants were reported to respond more strongly to
CO₂ enrichment than dense swards (Lüscher et al., 1996). Moreover lower
plant density may result in an increased amount of N being available to the
individual plants. Therefore, both more N per plant and the possibility of
increased tillering may have contributed to the CO₂-induced N-yield stimulation
observed in the infrequently defoliated swards in 1995 (Table VI). An additional
explanation for the distinct CO₂ response of the two cutting frequencies could
be that the lower tiller numbers under low cutting frequency resulted in heavier
tillers possibly representing stronger sinks for additionally fixed carbon and
assimilated nitrogen. This is in agreement with the higher biomass production
under the low cutting frequency (Hebeisen et al., 1997).

The generally observed lower N yield of the L. perenne monoculture
resulted from a significant reduction in the N concentration of the above-ground
plant material (Table VI). A CO₂-induced reduction in the N concentration was
observed at every harvest over all three growing seasons (data not shown).
Reduced N concentration may be a dilution effect due to the accumulation of
carbohydrates in leaves exposed to elevated pCO₂. Indeed, under the same
experimental conditions, Fischer et al. (1997) reported increased carbohydrate
concentrations in leaves of the L. perenne monoculture under elevated pCO₂.

5.1 Does a reduced N requirement explain the reduced N concentration of
L. perenne monocultures exposed to elevated pCO₂?

It has been reported that the critical N concentration decreases with an
increase in above-ground biomass production of grass swards with a,
presumably, non-limiting N supply (Lemaire and Salette 1984). In a recent
study, Soussana et al. (1996) showed clearly that this relationship changes in
glass swards exposed to elevated pCO₂ in plastic tunnels. The critical N
concentration declines at a faster rate with increasing shoot biomass at
elevated pCO₂ than at ambient pCO₂. Thus, under elevated pCO₂, the relative
N requirement of the grass for maximal growth is reduced. The ratio between
the measured N concentration and the corresponding critical N concentration is
called 'index of N nutrition' (IN) for a given sward and is independent of yield
Figure 7. Index of N nutrition (IN) under ambient pCO₂ (IN₃₅) plotted against IN under elevated pCO₂ (IN₆₀) of frequently and infrequently defoliated *L. perenne* monocultures supplied with low N or high N fertilization levels in three growing seasons. Means of each individual harvest are shown. The solid line represents the regression (dotted line: IN₃₅ = IN₆₀).
Figure 8. N concentration of total annual above-ground biomass in relation to root fraction of total annual above-ground biomass of *L. perenne* monoculture grown under ambient (35 Pa) (open symbols) and elevated (60 Pa) pCO₂ (filled symbols). Swards were either frequently (cross in symbols) or infrequently (no pattern) defoliated and supplied with two N fertilization levels.
parameters (Lemaire et al., 1989). If IN equals one, then grass growth should not be limited by N.

In the frequently defoliated swards, the L. perenne monocultures exposed to elevated pCO$_2$ showed significantly (Wilcoxon's paired rank test, p<0.05) lower IN values than those under ambient pCO$_2$ (Fig. 7). Under these regrowth conditions, which were similar to those reported by Soussana et al. (1996), the IN under elevated pCO$_2$ was reduced by 36% in the low N treatment and by 17% in the high N treatment. This occurred, despite the correction of the critical N concentration under elevated pCO$_2$ (Soussana et al., 1996). The distance of a specific point below the dotted line (IN$_{75}$=IN$_{80}$) reflects a CO$_2$-induced decline in the N nutrition of the particular sward. This decline cannot be explained by the lower N requirement under elevated pCO$_2$. L. perenne swards showed a similar but less pronounced CO$_2$ effect when cultivated in CO$_2$-enriched plastic tunnels (Soussana et al., 1996). This indicates that resource limitations may have a greater impact under field conditions as compared with controlled conditions. The observed reduction in IN under elevated pCO$_2$ in the frequently defoliated, monocropped L. perenne sward indicates that changes in C and N metabolism are not solely responsible for the reduction in the N concentration of the above-ground plant material. Moreover, the increase in dry matter production per unit N under elevated pCO$_2$ (Field et al., 1992) suggests that, for a given plant size, the N requirement is lower and can, therefore, be satisfied by a smaller root system, assuming an unchanged N availability in the soil. This contradicts the increased root biomass reported by Jongen et al. (1995) and Hebeisen et al. (1997).

We suggest that the lower IN values under elevated pCO$_2$ resulted from an imbalance between C and N nutrition which may be induced by a reduction in the amount of plant available N. This caused a reduction in the N concentration and the N yield of the above-ground monocropped L. perenne plant material exposed to elevated pCO$_2$ (Table VI). To counteract this imbalance, the plants invested more in the root systems, as evident from the relationship between the N concentration in the above-ground plant material and the root fraction (root biomass as a percentage of the total above-ground biomass) (Fig. 8). This relationship is similar to that found in a short-term experiment (Schenk et al., 1995) and is unaffected by elevated pCO$_2$. Therefore, the increased investment of assimilates in the root (Jongen et al., 1995; Hebeisen et al., 1997) is not a CO$_2$ effect per se but a response to an unsatisfied N demand. This presumably results from a CO$_2$-induced decrease
in the amount of plant available N in the soil. In line with these results, an investigation of the carbohydrate concentrations in leaves of *L. perenne* during regrowth indicated an increased C availability together with an N-dependent sink limitation (Fischer et al., 1997).

In the infrequently defoliated swards, no differences between the IN values of the two CO₂ treatments were observed (Fig. 7). This may be related to the fact that the relationship between biomass production and critical N concentration (Soussana et al., 1996) was established in swards where regrowth periods were similar to those of the frequently defoliated swards. Therefore, the relationship may be less representative of these conditions. However, the generally high IN values indicate that factors other than N, i.e. light, may be the predominant growth-determining parameters during the long (eight weeks) regrowth periods. In 1995, the high IN values in the infrequently defoliated, high N monoculture, exposed to elevated pCO₂ (Fig. 7), coincided with a relatively small CO₂-induced stimulation of root biomass production (Hebeisen et al., 1997). Both parameters suggest improved N nutrition in this treatment in 1995. This is in line with the previously discussed processes involved in the CO₂-induced N yield stimulation of this specific treatment (Table VI).

### 5.2 Possible processes involved in the decreasing amount of plant available N under elevated pCO₂

A reduced amount of plant available N may reflect a CO₂-induced alteration in nutrient cycling. Qualitative and/or quantitative changes in exudates and dead plant material may cause an alteration in below-ground processes under elevated pCO₂ (van Veen et al., 1991; Jongen et al., 1995). In the newly labelled area (1995), the ¹⁵N-excess in the harvested plant material of the low N *L. perenne* monocultures was greater under elevated pCO₂ during the first three regrowth periods (Fig. 9). This indicates that, under elevated pCO₂, the fertilizer labelling was not as diluted by the unlabelled soil N. Gorissen et al. (1995) reported that root material produced under elevated pCO₂ is more resistant to decomposition. The same may be true for plant litter resulting in the accumulation of litter (Cotrufo and Ineson 1995). The N retained in the litter or in the soil organic matter may explain the higher ¹⁵N concentration of *L. perenne* monocultures, grown in the newly labelled area, exposed to elevated pCO₂. The effect of elevated pCO₂ on the ¹⁵N
concentration in the *L. perenne* plant material was not apparent in the high N treatment (data not shown). This may be due to the fact that the C:N ratio of the *L. perenne* litter under high N fertilization was so low that any CO₂ effect (Blum et al., 1997) was probably irrelevant for decomposition. A reduction in the amount of N available to plants under elevated pCO₂ may be induced initially by an expanding microbial biomass, temporarily immobilizing more N (Díaz et al., 1993). Data from this experiment provided no evidence of an increase in the microbial biomass due to elevated pCO₂ (Schortemeyer et al., 1996). However, enhanced denitrification, observed at our experimental site (Ineson et al., 1997), may have a more important and enduring effect on N availability in the soil. The possibility that N availability may change is supported by the fact that there was a reduction in the initial nitrate concentration and in the amount of nitrate drained in autumn and winter in *L. perenne* swards under elevated pCO₂ (Soussana et al., 1996). Therefore, soil N availability in swards of *L. perenne* monocultures may have been lower under elevated pCO₂. In turn, this may have contributed to the negative CO₂ response of the grass (Table VI). Such results are in agreement with the concept of a CO₂-induced increase in N demand of the whole grassland ecosystem (Hartwig et al., 1996).

![Figure 9](image)

**Figure 9.** Atom%¹⁵N-excess of monocropped *L. perenne*. Means and SE of three replicates are shown.
5.3 CO₂ response of an ecosystem with a N₂-fixing species

Nitrogen from symbiotic N₂ fixation is an important source of N in grasslands. It was shown that symbiotic N₂ fixation was stimulated under elevated pCO₂ (Zanetti et al., 1996; Zanetti and Hartwig 1997). This contributed to the improved competitive ability of *T. repens* in mixed swards resulting in a CO₂-induced increase in clover yield proportion from 30% to 42% (Hebeisen et al., 1997). Symbiotically fixed N can be transferred to other plants or organisms that are unable to fix N (Table VII) (Boller and Nösberger 1987; Seresinhe et al., 1994). In *L. perenne* grown in mixtures, the %Ntrans tended to be consistently higher under elevated pCO₂ (Fig. 6). Therefore, the increased symbiotic N₂ fixation of *T. repens* (Zanetti et al., 1996; Zanetti and Hartwig 1997) and the shift towards a higher clover proportion (from 30% under ambient pCO₂ to 42% under elevated pCO₂; Hebeisen et al., 1997) contributed to a greater amount of apparently transferred N observed under elevated pCO₂ (Table VII). Clover litter may decompose more easily because it has a higher N concentration; moreover the clover litter was less affected by CO₂ enrichment than the *L. perenne* litter (Zanetti et al., 1996). The amount of transferred N seems to be moderate (Table II). However, assuming the same proportional uptake of transferred N as of Nfert (Table VI) the amount of transferred N available to the system would be 5 g m⁻² for swards fertilized with high amounts of N and 16 g m⁻² for swards under low N. The latter amount is similar to the level of fertilizer supply in the specific treatment. Moreover, N₂-fixing species depend less on fertilizer/soil N than do non-fixing plants. This dependency is regulated by the availability of mineral N (Boller and Nösberger 1987; Zanetti et al., 1996). *T. repens* has a low requirement for fertilizer and/or soil N. As a result, the amounts of N derived from the fertilizer or the soil in the mixed *L. perenne* swards (i.e., Ntot minus Ntrans; Table VII) were similar to those in the *L. perenne* monoculture swards (i.e., Ntot; Table VI). Both apparent N transfer and the lower requirement of *T. repens* for mineral N improved the N nutrition of *L. perenne* grown in mixture, which was unaffected by elevated pCO₂ (Table VII). Even though N nutrition was improved in ecosystems with an N₂-fixing species, *L. perenne* grown in mixtures did not respond to elevated pCO₂ due to the CO₂-induced increase in the competitive ability of *T. repens* (Hebeiesen et al., 1997).

*L. perenne* / *T. repens* mixtures responded to elevated pCO₂ with an increase in yield (Table VIII) in both N and defoliation treatments. The CO₂-induced increase in N₂ fixation and in the competitive ability of *T. repens*
buffered the effect of elevated pCO₂ in the grass and in the whole ecosystem. The increase in N yield under elevated pCO₂ was due exclusively to symbiotically fixed N (Table VIII). This result is consistent with data from monocropped *T. repens* where all additionally yielded N under elevated pCO₂ was also derived from symbiotic N₂ fixation (Zanetti et al., 1996). The increased introduction of symbiotically fixed N may reflect an enhanced N demand within the entire grassland ecosystem exposed to elevated pCO₂.

6 CONCLUSION

The CO₂ effect on the N yield depended on the presence or absence of a N₂-fixing plant species. The CO₂ response of *L. perenne* monocultures was restricted by N even at high N-fertilization. Changes in N cycling may, over time, amplify the situation of an insufficient N supply. In contrast, grass/legume mixtures showed an N-yield stimulation under elevated pCO₂. All additionally yielded N was fixed symbiotically. Therefore, symbiotic N₂ fixation seems to be a crucial process in the equilibration of C and N in fertile grassland ecosystems, especially in those exposed to elevated pCO₂.

Published in:  
Oecologia: in press
ELEVATED ATMOSPHERIC CO\textsubscript{2} DOES NOT AFFECT PER SE THE PREFERENCE OF TRIFOLIUM REPENS L. FOR SYMBIOTIC NITROGEN AS OPPOSED TO MINERAL NITROGEN

1 ABSTRACT

Increasing atmospheric CO\textsubscript{2} partial pressure (pCO\textsubscript{2}) will affect the C:N balance of ecosystems. Symbiotic N\textsubscript{2} fixation is a main source of N and, thus, an important factor in maintaining the C:N balance of terrestrial ecosystems. Hence, an understanding of the physiological response of symbiotic N\textsubscript{2} fixation to increased pCO\textsubscript{2} is indispensable. The objective of this investigation was to examine the effect of a CO\textsubscript{2}-induced alteration in the plants' N demand on the performance of symbiotic N\textsubscript{2} fixation in Trifolium repens L. cv. Milkanova. Highly expanding T. repens seedlings were transferred after five weeks of pre-growth under ambient pCO\textsubscript{2} and N-free nutrient conditions to two pCO\textsubscript{2} atmospheres (35 Pa pCO\textsubscript{2} and 60 Pa pCO\textsubscript{2}) and two levels of N (N-free and 7.5 mM N [\textsuperscript{15}N enriched] nutrient solution) for 36 days. Elevated pCO\textsubscript{2} evoked a significant increase in plant dry matter production from day 12 after the CO\textsubscript{2} enrichment started. Due to the CO\textsubscript{2}-induced reduction in the N concentration of the plant material, the CO\textsubscript{2}-induced stimulation of plant growth was paralleled by a less pronounced but still significant increase in N yield. For plants supplied with 7.5 mM N, symbiotic N\textsubscript{2} fixation (determined by the \textsuperscript{15}N-isotope-dilution method), i.e. the relative contribution of symbiotically fixed N (%N\textsubscript{sym}) as opposed to N assimilated from mineral sources, dropped to 40\% immediately after the onset of the N treatment. However, %N\textsubscript{sym} was unaffected by atmospheric pCO\textsubscript{2} over the entire experimental period. In plants fully dependent on N\textsubscript{2} fixation, the increase in N yield reflects a stimulation of symbiotic N\textsubscript{2} fixation. In these plants, N\textsubscript{2} fixation was increased by the formation of more nodules rather than by higher specific N\textsubscript{2} fixation. This suggests that symbiotic N\textsubscript{2} fixation is not limited by the supply of assimilates to the nodules but is regulated by the plants' requirement for symbiotically fixed N.
The plants' demand for symbiotically fixed N was increased under elevated pCO₂ but reduced in plants supplied with 7.5 mM N. The results will also be discussed in the relation to the response of symbiotic N₂ fixation to elevated pCO₂ in field-grown T. repens plants.
2 INTRODUCTION

Under field or field-like conditions it was shown that N is the main limiting growth factor affecting the response of fertile grassland model ecosystems to an elevated atmospheric partial pressure of CO₂ (pCO₂) (Soussana et al., 1996; Zanetti et al., 1997). In these experiments, symbiotic N₂ fixation in Trifolium repens was higher under elevated pCO₂. The enhancement was in such a manner that all additionally yielded N under elevated pCO₂ was derived from the symbiotic N₂ fixation (Zanetti et al., 1996; Zanetti and Hartwig, 1997).

Elevated pCO₂ results in increased assimilate availability. The enhanced assimilate supply to the nodules reflects an increase in energy supply for the reduction of molecular N known to require much energy (e.g. Pate and Layzell, 1990). Besides the CO₂-induced increase in assimilate availability, elevated pCO₂ also stimulates plant growth and, therefore, induces a stronger N sink in the plants (Hartwig and Nösberger, 1994). Despite the well-documented stimulation of nitrogenase activity, due to elevated pCO₂, in a wide range of plants which fix N symbiotically (Hardy and Havelka, 1976; Phillips et al., 1976; Masterson and Sherwood, 1978; Finn and Brun, 1982; Williams et al., 1982; Murphy, 1986; Norby, 1987; Arnone and Gordon, 1990), the enzymatic response provides no information about the relative contribution of symbiotically fixed N as opposed to N nutrition from mineral sources to the total plant N yield. Moreover, CO₂-induced stimulation of N₂ fixation in field-grown T. repens (Zanetti et al., 1996; Zanetti and Hartwig, 1997) can not show whether elevated pCO₂ stimulates symbiotic N₂ fixation per se or indirectly by altering the plants' demand for symbiotically fixed N, since this parameter may also be influenced by a CO₂-induced decrease in the relative availability of mineral N to the plants.

The aim of this study was to investigate the factors which regulate symbiotic N₂ fixation under elevated pCO₂. In view of the observed CO₂- induced increase in the contribution of N₂ fixation to plant nutrition under field-like or field conditions (Soussana and Hartwig, 1996; Zanetti et al., 1996; Zanetti and Hartwig, 1997), it was of particular interest to monitor the relative contribution of symbiotically fixed N as opposed to the assimilation of N from mineral sources in plants supplied with a N-rich nutrient solution under controlled conditions. White clover seedlings, characterized by a strong N-sink during the exponential growth stage, were used as experimental plants. The plantlets were either solely dependent on symbiotic N₂ fixation or were irrigated
with a N-rich nutrient solution containing $^{15}$N-enriched N. The plants were exposed to two levels of atmospheric pCO$_2$ to manipulate both assimilate availability and plant growth, thus inducing an altered N sink strength.

3 MATERIALS AND METHODS

3.1 Plant material and growth conditions

White clover (Trifolium repens L. cv. Milkanova) seedlings were cultivated in pots (height: 25 cm; diameter: 7 cm) containing silica sand (diameter: 0.8-1.2 mm). Before planting, the seeds were surface sterilized in 70% ethanol for three minutes and pre-germinated on water agar (15 g l$^{-1}$; Fluka Chemie AG, Buchs, Switzerland) at room temperature for 24 h. Three seedlings were planted in each pot; two seedlings were removed ten days after planting. Plants were cultivated in two growth chambers (PGR-15, Conviron Instruments Co., Winnipeg, Canada) under ambient pCO$_2$ at 18/13°C (day/night) and a relative humidity of 80/90%. The 16 h photoperiod was maintained at a photosynthetic photon flux density of 500 μmol quanta PAR m$^{-2}$ s$^{-1}$ (Fluorescent [Cool White 160 W]; incandescent [138 L 100 W] Sylvania GTE SA, Geneva, Switzerland) at plant height. To avoid chamber and position effects, the plants were rotated weekly between and within the chambers. During the first five weeks, the pots were inoculated weekly with Rhizobium leguminosarum bv. trifolii (strain RBL 5020 (Leiden, the Netherlands)). Plants were well watered twice a day with N-free nutrient solution, similar to that of Hammer et al. (1978).

Six weeks after planting, the plants were exposed to two CO$_2$ treatments (35 ± 0.5 Pa pCO$_2$ vs. 60 ± 0.5 Pa pCO$_2$). The CO$_2$ levels were monitored throughout the photoperiod by an IR CO$_2$ analyzer (Model WMA-2, PP Systems, Stotfold Hitchin, Herts, UK). A microprocessor controlled the valve so that small amounts of CO$_2$ were injected into the growth chamber. Denitrogenation of CO$_2$ in excess was controlled by threshold value switch and provided by a ventilator filled with soda lime granulate containing an indicator (Merck, Darmstadt, Germany) absorbing the extra CO$_2$. After six weeks of pre-growth, the plants either kept receiving the N-free nutrient solution or, from now on, received 7.5 mM N. The 7.5 mM N was supplied as 1% $^{15}$N-enriched NH$_4$NO$_3$ (ammonium and nitrate were equally labelled [Isotec, Miamisburg, OH, and Matheson, Secaucus, NJ, USA]).
3.2 Plant sampling

Plants were harvested just before starting the treatments and on days 6, 12, 24 and 36 after the start of the treatments. Selected plants were irrigated with ice-cooled water three hours after the beginning of the photoperiod and transferred to a cool room (4°C) to avoid effects of diurnal fluctuations. At each harvest, the clover plants were separated into roots and shoots. The shoot was divided into stolons, petioles and leaves. Leaf number and area (Model LI-3000 A, Li-Cor Inc., Lincoln, NE, USA) were determined. All plant parts were snap-frozen in liquid N and stored at -80°C before freeze-drying. After freeze-drying the weight of all fractions was recorded. Nodules were removed from freeze-dried roots, counted, dried again at 40°C for 24 h and weighed.

All plant fractions were ground with a ball mill (type MM2, RETSCH, Arlesheim, Switzerland) and then proportionally and homogeneously mixed. After re-drying at 35°C for 24 h, samples (1 mg) were weighed and transferred to tin caps (0.04 ml, Lüdi, Flawil, Switzerland). The samples were analyzed for concentration of 15N and N by a continuous-flow mass spectrometer (Europa Scientific, Cambridge, UK) in the laboratory of Dr. C. van Kessel (University of Saskatchewan, Saskatoon, Canada).

3.3 Calculation of symbiotic N₂ fixation (%Nsym)

Plants treated with N-free nutrient solution depended totally on symbiotic N₂ fixation. For plants irrigated with a nutrient solution containing 7.5 mM N, the relative contribution of symbiotically fixed N to the total plant N yield (%Nsym) was determined according to the 15N-isotope-dilution method. %Nsym was calculated for the N assimilated after the beginning of the treatments according to the following equation:

\[
\%N_{sym} = \frac{N_{tot}(x) (1 - R) - N_{tot}(0)}{N_{tot}(x) - N_{tot}(0)} \times 100
\]

\(N_{tot}(0)\) and \(N_{tot}(x)\) stand for the total N yield (mg plant⁻¹) at time zero (zero harvest) and at subsequent points in time respectively. \(R\) is the ratio of the atom% 15N-excess in the plant material to that in the nutrient solution.
3.4 Statistical analysis

Before starting the experiment the plants were grouped according to estimated leaf area (Williams et al., 1964). Each size group represents a replication serie. The individual plants of a specific size group were classified randomly according to treatment and time of harvest. The data were analyzed as a block design. Apart from the zero harvest (15 replicates), the number of replicates increased during the experimental period. Five plants were investigated (five replicates) at the harvests on day 6 and 12. The number of replicates was increased to eight and ten replicates for the harvests on day 24 and 36 respectively. Data were subjected to factorial analyses of variance using SAS (Statistical Analysis System Institute, Cary, North Carolina, USA).

4 RESULTS

4.1 Effect of elevated pCO₂ on dry matter production and allocation of T. repens

Elevated atmospheric pCO₂ resulted in an increased total plant weight of T. repens in both N treatments (Table IX). This effect was significant at all harvests except for the first one (six days after the start of the treatments). Averaged over all harvests and both N treatments, elevated pCO₂ resulted in a 43% increase in plant weight. The CO₂-induced increase in the total plant weight was the result of a CO₂-induced stimulation in all plant fractions (Table IX). The leaf fraction tended to respond stronger to elevated pCO₂ as compared to the other plant parts. As a result, under elevated pCO₂, the shoot-root ratio shifted slightly in favor of the shoot parts (Table X). Over the entire experimental period, the stolon portion strongly increased from 7% on day six to 21% at the final harvest, independent of the treatment (Table IX). This reflects the plant habitus of the expanding clover seedling. The two N treatments did not differ in their pattern of response to elevated pCO₂ (no interaction was found between CO₂ and N at any harvest).

The supply of 7.5 mM N to the plants was a less powerful growth stimulator for T. repens than was the experimental factor CO₂ (Table IX). Only at the final harvest was a significant N effect observed on dry matter of all plant fractions (Table IX).
Table IX

Dry mass of the whole plant (total) as well as of leaves, petioles, stolons, and roots of T. repens L. exposed to two N levels (N-free and 7.5 mM N) and two pCO₂ levels (35/60 Pa CO₂). Means and SE are shown (n=at least five; see Materials and Methods).

<table>
<thead>
<tr>
<th>N level</th>
<th>pCO₂</th>
<th>days after exposure to the treatments</th>
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<td>leaves</td>
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<tr>
<td></td>
<td>SE</td>
<td></td>
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<tr>
<td>stolons</td>
<td>N-free</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td></td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>7.5mM N</td>
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<td></td>
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<tr>
<td></td>
<td>SE</td>
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<tr>
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<tr>
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<td></td>
<td>SE</td>
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<td>total</td>
<td>N-free</td>
<td>35</td>
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<td></td>
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<tr>
<td></td>
<td>7.5mM N</td>
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<td></td>
<td>SE</td>
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</tr>
</tbody>
</table>
Table X  
Shoot/root ratio (SRR), leaf area, leaf number and specific leaf weight (sLW) of T. repens L. exposed to two N levels (N-free and 7.5 mM N) and two pCO\textsubscript{2} levels (35/60 Pa). Means and SE are shown (n= at least five; see Materials and Methods).

<table>
<thead>
<tr>
<th>NL</th>
<th>pCO\textsubscript{2}</th>
<th>days after exposure to the treatments</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>SRR</td>
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<td>35</td>
</tr>
<tr>
<td></td>
<td></td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>7.5mM N</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td></td>
<td>60</td>
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<tr>
<td></td>
<td>SE</td>
<td></td>
</tr>
<tr>
<td>leaf</td>
<td>N-free</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td></td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>7.5mM N</td>
<td>35</td>
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<td></td>
<td></td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>SE</td>
<td></td>
</tr>
<tr>
<td>leaf</td>
<td>N-free</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td></td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>7.5mM N</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td></td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>SE</td>
<td></td>
</tr>
<tr>
<td>sLW</td>
<td>N-free</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td></td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>7.5mM N</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td></td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>SE</td>
<td></td>
</tr>
</tbody>
</table>
4.2 Effect of elevated pCO$_2$ on leaf area, leaf number and specific leaf weight of $T$. repens

$T$. repens increased its leaf area under elevated pCO$_2$ and was statistically significant from day 12 on (Table X). This CO$_2$-induced increase in leaf area was proportional to the enhanced total plant weight (Tables IX and X). The leaf number showed a weaker and slower response to CO$_2$ enrichment than did leaf area (Table X). A significant CO$_2$-induced increase in leaf number was observed only at the final harvest. Elevated pCO$_2$ resulted in heavier leaves as expressed on a leaf area basis (specific leaf weight) (Table X).

Leaf area and leaf number responded slowly to the supply of 7.5 mM N, which had a significant effect on these parameters only at the end of the experiment (Table X). Specific leaf weight was also insensitive to the N supply (Table X).

4.3 Effect of elevated pCO$_2$ on N concentration, N yield and percentage of N$_2$ fixation (%N$_{sym}$) in $T$. repens

Nitrogen concentration in the plant material of $T$. repens was significantly reduced by elevated pCO$_2$ in both N treatments (Table XI). The CO$_2$-induced reduction was 17% for the 7.5 mM N and 15% for the N-free treatment, averaged over the entire experimental period. No N x CO$_2$ interaction was observed. As a result of the reduction in the N concentration, elevated pCO$_2$ resulted in a weaker stimulation of the N yield as compared to the CO$_2$-induced increase in plant dry matter. At the final harvest, the stimulating effect on the N yield was almost nil in the 7.5 mM N treatment (Table XI), reflecting the declining CO$_2$ effect on total plant weight with increasing plant size (Table IX). The contribution of symbiotically fixed N (%N$_{sym}$) to the total N yield was not affected by elevated pCO$_2$ over the entire experimental period (Table XI).

The 7.5 mM N supply always caused a significant increase in the N concentration and N yield of $T$. repens in comparison to the N-free treatment. The contribution of symbiotically fixed N to the total N yield was strongly reduced by the supply of 7.5 mM N. In the N-free treatment, all N assimilated by $T$. repens was symbiotically fixed N (Table XI). $T$. repens, supplied with 7.5 mM N, depended more on the mineral N source than on N$_2$ fixation for its newly assimilated N (Table XI). This is shown by the sudden and sharp drop of %N$_{sym}$ to values around 40% in the plants supplied with 7.5 mM N (Table XI). %N$_{sym}$ reached 31% by the end of the experiment.
Table XI  \(N\) concentration \([N]\), \(N\) yield per plant and \%Nsym of \(T.\) repens exposed to two \(N\) levels (\(N\)-free and 7.5 mM \(N\)) and two \(p\)\(CO_2\) levels (35/60 Pa \(CO_2\)). Means and SE are shown (\(n=\) at least five; see Materials and Methods).

<table>
<thead>
<tr>
<th>NL</th>
<th>p(CO_2)</th>
<th>days after exposure to the treatments</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>[(N)]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(mg g(^{-1}) dry weight)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N-free</td>
<td>35</td>
<td>50.0</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>34.9</td>
</tr>
<tr>
<td>7.5mM N</td>
<td>35</td>
<td>54.1</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>45.9</td>
</tr>
<tr>
<td>SE</td>
<td></td>
<td>1.0</td>
</tr>
<tr>
<td>N yield</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(mg)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N-free</td>
<td>35</td>
<td>1.6</td>
</tr>
<tr>
<td></td>
<td>60</td>
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<td>SE</td>
<td></td>
<td>0.2</td>
</tr>
<tr>
<td>%Nsym</td>
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<td></td>
</tr>
<tr>
<td>N-free</td>
<td>35</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>100</td>
</tr>
<tr>
<td>7.5mM N</td>
<td>35</td>
<td>40.1</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>41.9</td>
</tr>
<tr>
<td>SE</td>
<td></td>
<td>4.1</td>
</tr>
</tbody>
</table>

4.4 Effect of \(N\) supply on number and dry weight of the nodules of \(T.\) repens

The response of nodule dry weight to elevated \(p\)\(CO_2\) differed for the two \(N\) treatments (\(CO_2\) x \(N\) interaction at the last two harvests; \(p<0.05\)). \(T.\) repens, supplied with 7.5 mM \(N\), showed no difference between the two \(CO_2\) treatments in respect to nodule dry weight, except at the last harvest (Table XII). The nodule dry weight of \(T.\) repens which received no additional \(N\) tended to be stimulated by elevated \(p\)\(CO_2\); this was significant at the final harvest (Table XII). Nodule number differed too in its \(CO_2\) response in the two \(N\)
Table XII  
**Nodule dry weight (nod-dw), nodule number (nods) per plant and specific (sp.) N\textsubscript{2} fixation of T. repens exposed to two N levels (N-free and 7.5 mM N) and two pCO\textsubscript{2} levels (35/60 Pa CO\textsubscript{2}). Means and SE are shown (n= at least five; see Materials and Methods).**

<table>
<thead>
<tr>
<th>N level</th>
<th>pCO\textsubscript{2}</th>
<th>days after exposure to the treatments</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>nod-dw (mg)</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>7.5mM N</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td></td>
<td>60</td>
</tr>
<tr>
<td>SE</td>
<td></td>
<td>0.9</td>
</tr>
<tr>
<td>nods</td>
<td>N-free</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td></td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>7.5mM N</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td></td>
<td>60</td>
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<tr>
<td>SE</td>
<td></td>
<td>1.5</td>
</tr>
<tr>
<td>sp. N\textsubscript{2} fixation (mg nod-dw\textsuperscript{-1})</td>
<td>N-free</td>
<td>35</td>
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<tr>
<td></td>
<td></td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>7.5mM N</td>
<td>35</td>
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<td></td>
<td></td>
<td>60</td>
</tr>
<tr>
<td>SE</td>
<td></td>
<td>0.08</td>
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</table>

Additional N supply reduced nodule number and nodule dry weight significantly but induced a three-fold increase in specific N\textsubscript{2} fixation at the end of the experiment (Table XII).
5 DISCUSSION

5.1 Effect of elevated pCO$_2$ on the performance of symbiotic N$_2$ fixation in
_T. repens_

To understand the mechanisms involved in the regulation of symbiotic N$_2$
fixation under elevated pCO$_2$, we investigated plant systems which differ in
growth rate and therefore their degree of N demand. After CO$_2$ fumigation for
12 days, elevated pCO$_2$ resulted in increased plant dry matter (Table X). The
observed increased plant growth under elevated pCO$_2$ (Table X) is the result of
a CO$_2$-induced increase in the rate of photosynthesis. This is consistent with
other studies reporting that the rate of photosynthesis of _T. repens_ did not
acclimate to enhanced level of pCO$_2$, neither at the plant level (Ryle et al.,
1992b) nor at the whole canopy level (Nijs et al., 1988).

Increased plant growth - in this particular case evoked by elevated pCO$_2$ -
induced an increased demand for N (Ingestad, 1982). This resulted in a greater
plant N yield (Table XI). In plants supplied with 7.5 mM N, the proportional
contribution of the two N sources (symbiotic N$_2$ fixation and N from mineral
source [nutrient solution]) to the total plant N yield was unaffected by the CO$_2$
treatments. This means that the CO$_2$-induced increase in the plant N demand
was satisfied by a proportional increase in N assimilation from both N sources
(Table XI). This result clearly illustrates that elevated pCO$_2$ alone did not alter
the relative preference for symbiotically fixed N as opposed to mineral N.

This, to our knowledge new finding, is in contrast to the CO$_2$ response of
symbiotic N$_2$ fixation in field-grown _T. repens_. Under field-like and field
conditions, the relative contribution of symbiotically fixed N to the total N yield
increased consistently in _T. repens_ in two independent three year studies
(Soussana and Hartwig, 1996; Zanetti et al., 1996; Zanetti and Hartwig, 1997).
In these studies, insufficient soil-N availability was considered to be the reason
for this CO$_2$-induced shift in the relative contribution of N$_2$ fixation (Soussana et
al., 1996; Zanetti et al., 1997). A CO$_2$-induced increase in the N demand of the
entire ecosystem (plants and micro- and macro-organisms) was considered to
be the cause of a reduction in the amount of mineral N available to the plants
(Hartwig et al., 1996; Zanetti et al., 1996; Zanetti and Hartwig, 1997). Thus, the
competition for the available N due to the presence of such a soil interface has
increased under elevated pCO$_2$. In the experiment reported here, both the
ample N supply to the plants and the absence of a soil interface may account
for the different CO₂ effects on symbiotic N₂ fixation between field and controlled conditions (Table XI).

This interpretation is in line with the concept that the demand of the plants for symbiotically fixed N is the key factor in the regulation of symbiotic N₂ fixation, since the plants' demand for symbiotically fixed N is not only determined by the plants' N sink itself but also by the availability of N in the growth medium of the plants. As a result, if the availability of mineral N is unlimited we indeed must not expect a CO₂-induced change in the relative preference of the plants for symbiotically fixed N as opposed to N assimilated from a mineral source (Table XI). This is a very important finding since the well documented CO₂-induced increase in nitrogenase activity (Hardy and Havelka, 1976; Phillips et al., 1976; Masterson and Sherwood, 1978; Finn and Brun, 1982; Williams et al., 1982; Murphy, 1986; Norby, 1987; Arnone and Gordon, 1990) gives no information about the relative contribution of symbiotically fixed N to the total plant N yield. This finding is important to understand the plants' response to elevated pCO₂ under field conditions.

The reduction of molecular N and, thus, the N₂ fixation too require considerable amount of energy (Gutschick, 1978). Respiration measurements showed that approximately 4.2 mol C is needed for the reduction of one mol of N (Ryle et al., 1984); 5.8 mol C is required per mol N reduced if the costs for the export of the fixed N are included (Gordon et al., 1985). The costs for N assimilation from nitrate seem to be lower than those for symbiotic N₂ fixation (Pate et al., 1979; Pate and Layzell, 1990). If only NO₃⁻ reduction is considered, the costs are considerably lower but vary according to the site where reduction takes place (Marschner, 1995). The absence of a CO₂-induced quick and lasting increase in specific N₂ fixation activity (nitrogenase activity per unit nodule dry weight) under elevated pCO₂ (Table XII), however, contradicts the concept that the energy-demanding N₂ fixation is primarily limited by the assimilate supply to the nodules (Hardy and Havelka, 1976). In addition, in the N-free treatment, the CO₂-induced enhancement of the amount of N fixed was not the result of an increased specific N₂ fixation activity but the result of a greater number of nodules (Table XII). These results are in line with the observation that, under elevated pCO₂, total nitrogenase activity increased at the whole plant level but that the specific nitrogenase activity remained unchanged (nitrogenase activity per nodule dry weight) (Phillips et al., 1976; Masterson and Sherwood, 1978; Finn and Brun, 1982; Norby, 1987). Furthermore, the well documented decrease in nodule carbohydrate content
with N supply (reviewed by Streeter, 1988) does not correspond to the higher specific N\textsubscript{2} fixation activity compared to that in the N-free treatment (Table XII). Thus, this is in agreement with the concept that C availability is not the major factor governing symbiotic N\textsubscript{2} fixation, neither at the whole plant (Williams et al., 1982; Hartwig et al., 1990) nor at the nodule level (Denison et al., 1992; Weisbach et al., 1996).

Under field conditions, the availability of mineral N often restricts plant growth. Under these conditions, the plants' demand for symbiotically fixed N will be high and, thus, symbiotic N\textsubscript{2} fixation will be stimulated. This will result in a competitive advantage for plants with the N\textsubscript{2}-fixing association when grown in mixed pasture communities. Moreover, in a world where levels of CO\textsubscript{2} are high, this competitive advantage and the CO\textsubscript{2}-induced growth of white clover seedlings (Tables IX and X) suggest that elevated pCO\textsubscript{2} may result in a faster sward establishment of white clover swards over a wide range of N availability. Results of a field (ETH-Swiss-FACE) experiment support this conclusion (Lüscher et al., 1996).

**5.2 Does elevated pCO\textsubscript{2} influence the nodule formation of T. repens roots?**

Under elevated pCO\textsubscript{2}, *T. repens*, depending solely on N\textsubscript{2} fixation, invested C in the formation of new nodules (Table XII). This ensured an increased N\textsubscript{2}-fixing capacity to satisfy the increased demand for symbiotically fixed N. The increase in root biomass (Table IX) increased the number of possible infection sites (Table XII). This may help to explain the strong CO\textsubscript{2}-induced increase in nodule formation (Table XII).

Plants supplied with 7.5 mM N showed a significantly lower nodule number as compared to plants depending solely on N\textsubscript{2} fixation (Table XII). Nodules of plants supplied with 7.5 mM N were heavier than those of plants in the N-free treatment (Table XII). This confirms that nodules may act as a strong sink for fixed C (Finn and Brun, 1982; Gordon et al., 1987). Despite the strong repression of nodule formation, it is very interesting that the once formed nodules were more active than those in the N-free treatment. This is evident from the three-fold higher specific nodule activity at the final harvest (Table XII). This may be further evidence that symbiotic N\textsubscript{2} fixation is not limited by the assimilates available in the nodule (Denison et al., 1992; Weisbach et al., 1996).
5.3 Was the N nutrition in *T. repens* adequate to meet the CO$_2$-induced increase in the N demand of the plants?

More leaves were formed under elevated pCO$_2$ (Table X). This is attributed mainly to increased branching, as evident from a parallel CO$_2$-induced increase in the number of apices (data not shown). Thus, additional sinks were available for the additional amount of fixed carbon, an important prerequisite for the plant's response to elevated pCO$_2$ (Conroy and Hocking, 1993; Diaz, 1995). These additional C sinks also induce an increase in the N sink of the plants.

Was N assimilation adequate to meet the increased demand for N of the plants exposed to elevated pCO$_2$? In plants which depended solely on symbiotic N, symbiotic N$_2$ fixation was very responsive to the increased strength of the N sink under elevated pCO$_2$ (Table XI). The fact that a supply of 7.5 mM N resulted neither in a significant stimulation of growth (Table IX) nor in a stronger response of *T. repens* biomass production to elevated pCO$_2$ (no significant CO$_2$ x N interaction) (Table IX) suggests that symbiotic N$_2$ fixation did not limit the growth or the CO$_2$ response of these exponentially developing *T. repens* plants. This conclusion is consistent with data reported by Ryle et al. (1992a). It illustrates the importance of symbiotic N$_2$ fixation as a phenomenon which allows the plant to be independent of mineral N supply.

Nevertheless, elevated pCO$_2$ resulted in a significant reduction in the N concentration in the plant material (Table XI). However, the fact that the CO$_2$-induced reduction in the N concentration was similar in both N treatments (no CO$_2$ x N interaction) (Table XI) underlines the previously made suggestion that *T. repens*, depending solely on N$_2$ fixation, received an adequate supply of N. Important factors, which may be partly or solely responsible for this commonly reported CO$_2$-induced reduction in the N concentration are first, a dilution effect of N attributed to increased assimilate accumulation (Wong, 1979; Wong 1990), second, a suggested decline in the N investment in enzymes related to photosynthesis (i.e. Rubisco) (Conroy and Hocking, 1993) and third, an inadequate supply of available N in soil (Soussana et al., 1996; Zanetti et al., 1997). The latter is irrelevant for the CO$_2$-induced reduction in the N concentration presented here because of the twice daily supply of N, to the plants and because of the lack of soil interface. In addition, the larger plants, due to the increased growth rate under elevated pCO$_2$, thus causing a change in physiological age, may further contribute to the lower N concentration. Since N concentration in plants generally decreases with age, the reduced N
concentration under elevated pCO₂ may simply be an effect of age as suggested by Coleman et al. (1993). In addition, increased plant growth under elevated pCO₂ occurs parallel to a decline in the proportional contribution of relatively N-rich leaf material to the biomass of the whole plant which contributes further to a reduced N concentration.

In conclusion, the results of this study clearly illustrate the importance of symbiotic N₂ fixation as a determining factor in the CO₂ response of T. repens. The data suggest that the additional C supply under elevated pCO₂ stimulated N₂ fixation indirectly, in accordance with the plant's increased need for symbiotically fixed N to maintain the internal C:N balance. As long as the mineral N supply satisfies the CO₂-induced increase in the plant's N demand, the relative contribution of symbiotically fixed N as opposed to mineral N is unaffected by the CO₂ enrichment. In a world in which CO₂ is increasing, the ability of the plant to regulate the symbiotic N₂ fixation according to its N requirement results in a strong competitive advantage of N₂-fixing species. Thus, symbiotic N₂ fixation may be responsible for adequate N import into whole ecosystems.
GENERAL DISCUSSION

1 IMPACT OF ELEVATED CO₂ ON A FERTILE GRASSLAND MODEL ECOSYSTEM: FACTS AND IMPLICATIONS

The data of this three year field experiment demonstrate the crucial role of N in the CO₂ response of two important grassland species, *Lolium perenne* L. and *Trifolium repens* L. Their distinct ability to cover their N demand is concluded to be a determining factor of their species-specific CO₂ response (Hebeisen et al., 1997). It is suggested that, under elevated pCO₂, the amount of mineral N available to the plants is reduced as a consequence of an increased N demand of the entire ecosystem. Thus, an insufficient N supply restricted the potential CO₂ response of the grass and resulted in the reduction of its annual above-ground N yield under elevated pCO₂ (Table VI; Zanetti et al., 1997). In contrast, *T. repens* has access to an additional, unlimited N source as a result of its symbiotic association with the N₂-fixing *Rhizobium*. This extra N source is considered to be a relevant trait, associated with CO₂-induced stimulation of the above-ground N yield of *T. repens* (Figs. 1 to 2; Zanetti et al., 1996; Zanetti and Hartwig, 1997).

1.1 The CO₂ response of *L. perenne* is restricted by the availability of N

It has been shown that the magnitude of the growth response of plants to elevated pCO₂ decreases with declining nutrient availability (Poorter et al., 1996), due in part to the suggested intensification of competition for resources under elevated pCO₂ (Bazzaz and McConnaughay, 1992). Thus, the plant response to elevated pCO₂ may be limited by N, a factor which often restricts growth in the field. Indeed, the results of this experiment show that the N concentration in the above-ground plant material of the monocropped *L. perenne* was strongly reduced under elevated pCO₂ (Table VI). This suggests that the weak CO₂-induced yield stimulation of the frequently defoliated *L. perenne* monocultures (Hebeisen et al., 1997) may be caused by an insufficient N supply. The conclusion is supported by calculated values of
indices of N nutrition (IN; ratio between the effective N concentration and the critical N concentration; see page 51 for detailed explanations and for a definition of the critical N concentration). The IN of the frequently defoliated grass swards was significantly lower under elevated pCO₂ than in the grass swards cultivated under ambient pCO₂ (Fig. 7; Zanetti et al., 1997) which indicates a more pronounced N limitation of the grass swards exposed to elevated pCO₂. This is an important result, because it occurred despite the fact that, under elevated pCO₂, less N is required for a given dry matter production. The lower N requirement may explain why, at some specific harvests in our experiment, a CO₂-induced reduction in the N concentration (data not shown) was observed even though the above-ground biomass was not affected by elevated pCO₂ (Hebeisen et al., unpublished data). This response pattern is confirmed by data from natural ecosystems (Owensby et al., 1993a). The lower N requirement under elevated pCO₂ is the result of both CO₂-induced processes, a lower N investment in enzymes related to photosynthesis (e.g. Bowes, 1993) and a stronger N dilution due to increased carbohydrate accumulation (e.g. Fischer et al., 1997, data of the same experiment). The fact that these CO₂-induced effects have been considered in calculating the critical N concentration (Soussana et al., 1996) suggests that a reduced N availability in the soil must have contributed to the strong reduction in the N concentration and thus to the lower annual above-ground N yield of the frequently defoliated L. perenne monocultures exposed to elevated pCO₂ (Table VI; Zanetti et al., 1997).

A common plant response to a low N supply is to increase the root to shoot dry weight ratio (Marschner, 1995). This results in a greater soil probing for mineral N. Biomass allocation to roots has been shown to be negatively correlated to the N status of the plant in an exponential manner (van der Werf et al., 1993). Indeed, a similar relationship was found in this study (Fig. 8; Zanetti et al., 1997) and in a growth chamber experiment (Schenk et al., 1995). Interestingly, this relationship was not influenced by the CO₂ treatment per se (Fig. 8; Zanetti et al., 1997). Hence, it is suggested that the increased C allocation to the root biomass (Jongen et al., 1995; Hebeisen et al., 1997) was also a consequence of an insufficient N supply which probably occurs under elevated pCO₂.
1.2 Symbiotic N₂ fixation is a key process in the CO₂ response of *T. repens*

A further important result of this experiment is that symbiotic N₂ fixation in *T. repens* increased in all treatments under elevated pCO₂ (Figs. 3 and 4) in such a manner that more than all additionally yielded N was derived from symbiosis (Figs. 1 and 2; Zanetti et al., 1996; Zanetti and Hartwig, 1997). In a growth chamber experiment in which plants were grown in sand and exposed to an ample N supply, the relative contribution of symbiotically fixed N as opposed to mineral N was not affected by elevated pCO₂ (Table XI). The difference in the results of experiments under controlled and field conditions suggests that the CO₂-induced enhancement of N₂ fixation in the field is an indirect effect: The CO₂-induced increase in clover growth is paralleled by an increase in its demand for N. Because the amount of N available in the soil could not cover the increased N demand, the increased N demand was met by an enhanced symbiotic N₂ fixation.

It has been shown in this experiment, and in many other field studies that symbiotic N₂ fixation is positively correlated with low soil-N availability (Figs. 3 and 4; Boller and Nösberger, 1987; Neshem and Oyen, 1994; Seresinhe et al., 1994; Zanetti et al., 1996; Zanetti and Hartwig, 1997). Therefore, it seems likely that symbiotic N₂ fixation in legumes is tuned according to the plant's demand for symbiotically fixed N, which is co-influenced by the availability of N in the growth medium.

An increased demand for symbiotically fixed N, suggested to occur under elevated pCO₂, was probably not met by an increase in specific nodule activity but by an increase in nodule number (Table XII; Phillips et al., 1976; Masterson and Sherwood, 1978; Finn and Brun, 1982; Norby, 1987). In fact, the enhanced root biomass under elevated pCO₂ (Hebeisen et al., 1997) reflects an increase in sites which may become infected by *Rhizobium* bacteria. The number of *Rhizobium leguminosarum* bv. *trifolii* in the field has been reported to be far from limiting and high enough to provide a high potential for infection (Schortemeyer et al., 1996).

This led to the conclusion that the CO₂-induced increase in N demand, which could not be met by the soil (fertilizer and mineralized N), was satisfied by the stimulated N₂ fixation. Therefore, symbiotic N₂ fixation was a key process for the strong CO₂-induced yield stimulation of *T. repens* (Figs. 1 and 2; Zanetti et al., 1996; Hebeisen et al., 1997; Zanetti and Hartwig, 1997) in fertile grassland ecosystems. This conclusion is supported by other
investigations: a CO$_2$-induced stimulation of symbiotic N$_2$ fixation was also measured in _T. repens_ grown in soil monoliths in plastic tunnels (Soussana and Hartwig, 1996). In addition, results of several related investigations (the same environmental conditions as in this study) showed that the CO$_2$-induced stimulation of the biomass production of legumes (_T. repens_ and _T. pratense_) was more pronounced than that of other functional groups (grasses and non-N$_2$-fixing dicots) (Lüscher et al., 1996). In addition, ineffectively nodulated _Medicago sativa_ L. and non-inoculated _Galega orientalis_ L. plants showed a weak CO$_2$-induced yield stimulation in comparison to that of either effectively nodulated or inoculated plants (Lüscher et al. and Hartwig et al., unpublished data). In a related study with four deciduous tree species, elevated pCO$_2$ had a significant impact only on the total biomass production of _Alnus incana_, a plant species with a symbiotic N$_2$-fixing association (actinomycetes _Frankia_) (Bucher, personal communication).

Even though symbiotic N$_2$ fixation was not measured in most of the above-mentioned experiments, the strong CO$_2$-induced biomass stimulation of various plants in symbiotic association with actinomycetes or with _Rhizobium_ supports the conclusion that symbiotic N$_2$ fixation is a crucial plant trait involved in a strong CO$_2$ responsiveness under fertile soil conditions. The results of the growth chamber experiment (Chapter VI) clearly illustrate that CO$_2$-induced alteration in symbiotic N$_2$ fixation is not a CO$_2$ effect _per se_ but an indirect one which is induced by changes in ecosystem functioning.

### 1.3 CO$_2$-induced stimulation of symbiotic N$_2$ fixation results in a greater amount of symbiotically fixed N being introduced into the system

The impact of elevated pCO$_2$ on the productivity of ecosystems has been ascribed to changes in species composition brought about by differential species response to elevated pCO$_2$ (Bazzaz, 1990). In the present experiment, the CO$_2$-induced yield stimulation in mixed swards was mainly a result of the strong response of _T. repens_ to elevated pCO$_2$ (Hebeisen et al., 1997). The suggested reduction in N availability under elevated pCO$_2$ and the resulting induced stimulation of symbiotic N$_2$ fixation improved the competitive ability of _T. repens_ grown in mixture under elevated pCO$_2$. The improved competitive ability of _T. repens_ led to an increased clover proportion in the mixed swards exposed to elevated pCO$_2$ (Newton et al., 1994; Hebeisen et al., 1997). In _T. repens_ grown in mixtures, all additionally yielded N under elevated pCO$_2$.
was derived from symbiosis despite increasing surface covering by white clover. Due to both processes, the increased clover proportion in the mixtures (Hebeisen et al., 1997) and the higher N2 fixation of each individual plant (Figs. 3 and 4) the amount of symbiotically fixed N in the mixed clover increased strongly under elevated pCO2 (60 kg ha\(^{-1}\) a\(^{-1}\) averaged over all years) (Figs. 1 and 2; Zanetti et al., 1996; Zanetti and Hartwig, 1997). Some of the symbiotically fixed N is known to become available to other organisms. This phenomenon, apparent N transfer, transfers symbiotically fixed N from the legume to non-fixing plants through various processes.

The apparent N transfer was attributed both to a direct N exchange along the hyphen of vesicular-arbuscular mycorrhiza (Haystead et al., 1988) as well as to an indirect N exchange by the release of N-containing compounds by damaged roots, caused by a common weevil pest feeding on white clover roots (Hatch and Murray, 1994; Murray and Hatch, 1994). However, the direct N excretion or the release of fixed N from living (damaged or not) roots or nodules is generally considered to be too small to explain the phenomenon of the N transfer. Moreover, it was shown that the decomposition of nodules and roots was insufficient to explain the apparent transfer of N (Dubach and Russelle, 1994). Therefore, above-ground plant residues (also harvesting residues) may, quantitively, be the main process involved in the apparent N transfer.

A further aspect which contributes to the generally higher N yield in mixtures as compared to monocultures is that mixed swards are characterized by improved N utilization. This is also reflected in the higher nutrient status of the non-fixing plant species cultivated in mixtures in comparison to those in monocultures (Table VII; Zanetti et al., 1997). More \(^{15}\)N was recovered in the mixtures (sum of both species) than in the monocultures (data not shown). This result may reflect a better root distribution and niche occupation in mixed swards than in the corresponding grass monocultures as suggested by Bolier and Nösberger (1987).

The increased amount of symbiotically fixed N under elevated pCO2 became at least partly available to the grass, as evident from the higher amount of apparently transferred N (Table VII; Zanetti et al., 1997). It seems likely that the transferred N in the \textit{L. perenne} is only a fraction of the effective amount of transferred N. Part of the symbiotically fixed N may be taken up by the clover itself or by other organisms in the soil and/or be subject to any of the forms of N loss (e.g. leaching, denitrification). All processes (the higher performance of symbiotic N\(_2\) fixation, the higher clover proportion and the
therefrom resulting increased amount of apparently transferred N) resulted in the fact that more than the total of 13% increase in the N yield of the mixed swards exposed to elevated pCO$_2$ was derived from symbiotic N$_2$ fixation.

These results illustrate the important role of symbiotic N$_2$ fixation in a grassland ecosystem especially when exposed to elevated pCO$_2$. The introduction of symbiotically fixed N into the ecosystem is tuned directly by the performance of the symbiotic N$_2$ fixation and indirectly by an altered species composition. Both processes help maintaining the C:N balance in a system. As evident from the growth chamber experiment, elevated pCO$_2$ had no direct effect on the symbiotic N$_2$ fixation if the plants were adequately supplied with N (Chapter VI). Thus, changes in symbiotic N$_2$ fixation is induced by the plant's demand for symbiotically fixed N which is co-determined by the N availability in the growth medium of the plant. This is in line with a model suggesting that the efficiency of the N$_2$ fixation in an ecosystem is the reverse of the N status within the same system (Granhall, 1981). Therefore, an increased C fixation by the vegetation exposed to elevated pCO$_2$ can be maintained only when paralleled by an increase in the introduction of N into the system (Hartwig et al., 1996).

1.4 Processes which may contribute to the suggested CO$_2$-induced reduction in the amount of mineral N available to the plants

It is suggested that the N demand of the ecosystem as a whole increases under elevated pCO$_2$. This results in an intensification of the competition for mineral N and ultimately in a reduction of N supply to the plants. This concept is supported by data illustrating a CO$_2$-induced reduction in nitrate leaching in perennial ryegrass swards (Soussana et al., 1996).

Increases in rhizodeposition, suggested to occur under elevated pCO$_2$ (van Veen et al., 1991), may ultimately lead to an expansion of the microbial biomass paralleled by an increased N immobilization (Díaz et al., 1993). The latter may have occurred at the very beginning of the CO$_2$ fumigation, evoking an abrupt enhancement of the C input into the system. Such a process may be temporarily important. In the tallgrass prairie, the response of microbial biomass to CO$_2$ enrichment was dependent upon soil water conditions. Increase in microbial biomass was observed only in a dry year. Due to the higher water soil content under elevated pCO$_2$, the microbial activity was consistently greater under elevated pCO$_2$ (Rice et al., 1994). Rice et al. (1994) have suggested that due to CO$_2$-induced increase in microbial N, plant
production may become limited by N availability. It seems, however, unlikely that CO$_2$-induced increase in N immobilization is a long-term and the only process involved in the reduction of N availability. This is supported by the fact that the total number of heterotrophic bacteria was only slightly affected by CO$_2$ fumigation in this field experiment (Schortemeyer et al., 1996).

The reduction in soil-N availability under elevated pCO$_2$ may be evoked by a CO$_2$-induced increase in denitrification. This is likely, because conditions promoting denitrification activity are favored under elevated pCO$_2$. This favorisation is due to a greater soil moisture as a result of a lower water consumption by plants (Morison, 1985; Goudriaan and Unsworth, 1990) and a lower oxygen partial pressure induced by increased microbial activity and root biomass (Jongen et al., 1995; Hebeisen et al., 1997). In the infrequently defoliated L. perenne monoculture, elevated pCO$_2$ appeared to increase the output of N$_2$O by 27%, thought to be the consequence of enhanced root-derived C available in the soil, the energy source for denitrification (Ineson et al., 1997). The recorded flux rates of N$_2$O were the highest ever reported for soils. It was estimated that the amount of N lost through denitrification would be approximately 149 kg ha$^{-1}$ a$^{-1}$ for ambient pCO$_2$ and 189 kg ha$^{-1}$ a$^{-1}$ for elevated pCO$_2$ (Ineson et al., 1997). Even though these denitrification measurements presents preliminary data of a short time period, they illustrate impressively the importance of N loss through denitrification in grassland ecosystems. Moreover, it implies that this form of N loss may become increasingly important in a CO$_2$-rich world.

Decomposition rate has been shown to decline under elevated pCO$_2$ as a result of the enhanced C:N ratio in plant material produced under elevated pCO$_2$ (Cotrufo et al., 1994; Cotrufo and Ineson, 1995; Gorissen et al., 1995). Hence, the strong CO$_2$-induced reduction in the N concentration of L. perenne (Table VI) would result in a delay in the N release from the dead grass material. Such a process would accentuate the previously suggested enhanced N limitation under elevated pCO$_2$. In contrast to the grass, the N concentration of T. repens was only marginally affected by elevated pCO$_2$ (Table IV) due to the increase in symbiotic N$_2$ fixation. Thus, the decay of clover material may not be particularly affected by elevated pCO$_2$. This is an important aspect contributing to an increased introduction of N into the system. The CO$_2$-induced enhanced clover proportion in the mixed sward would ultimately enhance the amount of N available in the soil medium through the decomposition of more dead clover plant material and/or more clover losses during harvest on a square meter.
basis. Results from the tallgrass prairie showed strong species-specific differences (C₃ and C₄ plants) in characteristics of leaf litter decomposition. It was concluded that despite the lack of a direct CO₂ effect on the plant's initial chemical composition and its subsequent rate of decay, indirect effect of elevated pCO₂ on decomposition and nutrient cycling could occur if elevated pCO₂ induces changes in the relative above-ground biomass of the prairie species (Kemp et al., 1994). The litter material (dead and green foliage) for this specific study was collected at the end of a wet growing season. In wet growing seasons, elevated pCO₂ has been reported to have minor or no effect on the ecosystem productivity (Owensby et al., 1993a) and on N concentration of the investigated plant material (Owensby et al., 1993b). Therefore, the impact of elevated pCO₂ on the quality and the decay of litter produced in a wet growing season may be also less pronounced than on that in a dry year. It would be important to repeat this study with litter which has been produced during a growing season characterized by a strong CO₂ effect.

1.5 CO₂ effect on forage quality

A very commonly observed, secondary effect of elevated pCO₂ is the alteration in plant tissue composition, related mostly to a reduced N but also to increased fiber content in forage (Owensby et al., 1996). Such changes, reflecting an altered C and N metabolism under elevated pCO₂, may affect the feeding behavior of any herbivores (insects, wild animals and cattle). It has been shown that insects compensate for lower N concentrations by increasing their consumption rate by 20% to 80%. However, this did not improve insect performance on plants exposed to elevated pCO₂ (reviewed by Bazzaz, 1990). In contrast to insects, ruminants do not respond with a compensatory intake to reduced forage quality but reduce their intake of low quality forage (Owensby et al., 1996). Hence, milk and meat productivity of ruminants may be negatively influenced by the CO₂-induced reduction in forage intake and digestion. Even though this lower forage quality could be partly alleviated by dietary supplements, such additions would, however, have important economical consequences for the farmer.

Based on the species-specific CO₂ response in respect to N concentration in the above-ground plant material of this experiment (Tables IV and VI; Zanetti et al., 1996; Zanetti et al., 1997), it is suggested that forage quality, i.e. crude protein, of the grass under elevated pCO₂ will be reduced to a greater extent
than that of clover. However, a potential positive effect of elevated pCO₂ on forage quality may be related to an increase in the content of highly digestible non-structural carbohydrates. Crude protein content is generally high in *T. repens* monocultures, even under elevated pCO₂ (Frehner et al., 1997). For this reason, Frehner et al. (1997) suggested that the slight CO₂-induced reduction in the crude protein in the clover monoculture is of minor importance for the forage quality. Moreover, a potential negative effect of elevated pCO₂ on protein concentration of a mixed sward will probably be counterbalanced by the CO₂-induced increase in yield proportion of clover (Hebeisen et al., 1997). However, these CO₂-induced shifts in species proportion within mixed plant communities may affect the cyanide intake by ruminants, even though cyanide concentration in clover is not affected by elevated pCO₂ *per se* (Frehner et al., 1997).

2 IS IT POSSIBLE TO EXTRAPOLATE THE DATA FROM THE GRASSLAND MODEL ECOSYSTEM REPORTED HERE TO OTHER ECOSYSTEMS?

The impacts of increased pCO₂ on plant performance are diverse. The complexity of the CO₂ effects increases with the number of investigated plant species and implied experimental factors. *L. perenne* and *T. repens* are predominant species in managed grassland, an important vegetation type in middle Europe. Investigating these well characterized species in a model ecosystem is an adequate approach to identifying key processes involved in the CO₂ response of a fertile grassland under field conditions. The investigated plant material originates from commercially produced seed of highly productive varieties. Commercially produced seed has a smaller genetic variability which may help to diminish the variability of the plant's response to experimental factors. This reflects a possible experimental advantage. However, the mentioned decrease in variability will never be too great due to the cross-pollination in these pasture species.

In contrast to wild plant species (e.g. plants of the arctic tundra or of alpine grassland) cultivated plant species generally have a high potential to respond to elevated pCO₂ (Poorter, 1993). Thus, from the genetic background of the plant material used in this experiment, a strong CO₂ response of both plant species was expected. However, fast growing plants require increased
amounts of nutrients. This implies that N availability plays a very important role in the CO₂ response. The data of *L. perenne* illustrate that too little N was available to keep pace with increasing C fixation, even under very high N application (Chapter V).

One might argue that the investigated plant material is not representative of the large genetic variability of a natural pasture. However, in a related experiment, different genotypes of the two investigated plant species, which originated from native grassland, showed the same qualitative CO₂-response pattern (Lüscher et al., 1996) as bred cultivars (Hebeisen et al., 1997). Moreover, the wide range of tested grassland species demonstrated that *L. perenne* and *T. repens* are both valuable representatives of their functional groups (Lüscher et al., 1996). These results and the fact that elevated pCO₂ similarly affected all genotypes within a specific plant species (no CO₂ x genotype interactions) (Lüscher et al., 1996) suggest that the CO₂ response of *L. perenne* and *T. repens* can be qualitatively extrapolated to other plant species in fertile grassland ecosystems within their functional groups.

### 2.1 Possible impacts of elevated pCO₂ on biological N₂ fixation in other ecosystems

It has been shown that symbiotic N₂ fixation is an important factor in the strong CO₂ response of legumes under fertile grassland conditions (Chapter IV). N introduction by asymbiotic N₂ fixation was estimated to be 1 to 2 kg ha⁻¹ a⁻¹ in grassland (e.g. Woodmansee et al., 1981). In comparison to symbiotic N₂ fixation (100-200 kg ha⁻¹ a⁻¹; Robson et al., 1989), the contribution of asymbiotic N₂ fixation may, therefore, be of little importance in a fertile grassland ecosystem. In contrast to fertile grassland, asymbiotic N₂ fixation may introduce greater amounts of N into ecosystems such as deserts and moist, tropical environments (Stevenson, 1986; Sprent and Sprent, 1990).

Under elevated pCO₂, the activity of organisms which fix N asymbiotically may increase as a result of CO₂-induced stimulation of rhizodeposition (van Veen et al., 1991). In contrast, asymbiotic N₂ fixation was not affected by elevated pCO₂, as concluded from nitrogenase activity, unaffected by elevated pCO₂, in the rhizosphere of four forage non-leguminous plant species in early stage of vegetative growth (Crush, 1994). It has been pointed out, however, that the results may have underestimated the potential effects of elevated pCO₂. During this early growth stage the amount of rhizodeposition which is
positively related to the nitrogenase activity may be still small and probably not strongly affected by elevated pCO$_2$.

From the previously mentioned CO$_2$-induced stimulation in symbiotic N$_2$ fixation it would be expected that Alnus spp would also be more responsive to elevated pCO$_2$ than other non-N$_2$-fixing trees. This is confirmed by results from a related study carried out in this FACE experiment (Bucher, personal communication). An induced alteration in the community structure, in favor of Alnus, may however be counteracted by the continuously increasing amount of dry and wet deposition of nitrogenous components (Vitousek, 1994), which would ultimately enhance the N availability to non N$_2$-fixing tree species.

Another important aspect to be considered in the issue of symbiotic N$_2$ fixation is that the macrosymbiont has a wider ecological range than the symbiotic association itself (reviewed by Hartwig and Nösberger, 1996). Stressful events, such as extreme temperatures, soil-water conditions, nutrient limitations, etc., are known to have strong impacts on symbiotic N$_2$ fixation. Any of these extreme conditions may inhibit N$_2$ fixation or affect nodule formation and, therefore, be of major importance in considering the potential CO$_2$ response of N$_2$ fixation in other ecosystems.

Legume species in a calcareous grassland did not respond to elevated pCO$_2$ (Leadley and Körner, 1996; Leadley and Stöcklin, 1996). This strong contrast to our data may be due to non-responding symbiotic N$_2$ fixation. Low P and K availability in the calcareous grassland may have restricted a potential stimulation of symbiotic N$_2$ fixation and, thus, resulted in a weak CO$_2$ response of legumes. This suggestion is consistent with other studies reporting that a low P or K supply reduced or prevented symbiotic N$_2$ fixation (Cadisch et al., 1993; Sangakkara et al., 1996a;b). The suggested potential restriction of the CO$_2$ response due to a low P supply is supported by another experiment: different model ecosystems (with or without legumes) were cultivated in boxes containing calcareous grassland soil, with or without additional P. A CO$_2$-induced biomass stimulation was observed only in the model ecosystem with legumes and additional P (significant CO$_2$ x model ecosystem x P interactions) (Stöcklin, personal communication). These results support the previously made statement that the ecological range of the legumes is larger than that of the symbiotic association. This implies that conditions which ensure a high performance of symbiotic N$_2$ fixation are a prerequisite for the CO$_2$-induced stimulation of symbiotic N$_2$ fixation reported here (Figs. 3 and 4; Zanetti et al., 1996; Zanetti and Hartwig, 1997).
2.2 Possible impacts of elevated pCO₂ on another association: the mycorrhiza-plant interaction

As well as the *Rhizobium*-legumes association, another symbiotic plant-microorganism interaction is common in grassland: the arbuscular mycorrhizal symbiosis. This symbiosis is very common in terrestrial ecosystems. The movement of a large proportion of assimilates from the host plant to the fungus and the transfer by the fungus of phosphate and other minerals from the soil to plant tissue suggest that this plant-fungus interaction also plays an important role in the functioning of natural ecosystems (Sanders, 1996). Thus, this association may be of particular importance for ecosystems with low P availability (e.g. the calcareous grassland) and probably of increasing importance for ecosystems exposed to elevated pCO₂. However, it was found in the calcareous grassland that the detrimental effect of elevated pCO₂ on the growth of *Prunella vulgaris* coincided with an increased proportion of root length colonized by mycorrhizal fungi. In contrast, a significant decrease in mycorrhizal colonization was observed in a co-existing species (*Prunella grandiflora*) which showed no change in yield due to elevated pCO₂ (Sanders, 1996). These observations have given rise to speculations that, under elevated pCO₂, the mycorrhizal symbiotic interactions may remain stable or become either increasingly or decreasingly mutualistic (Sanders, 1996). Such CO₂-induced modifications of the plant-fungus interactions probably contribute to changes in the structure and functioning of the community.

2.3 Environmental factors and sward structure influencing the CO₂ response of the two grassland species

Distinct seasonal growth pattern: Elevated pCO₂ improved the competitive ability of *T. repens* cultivated in mixed swards under field conditions and, thus, indirectly altered the community composition (Lüscher et al., 1996; Hebeisen et al., 1997). This result is consistent with other experiments conducted under field-like or controlled conditions (Newton et al., 1994; Schenk et al., 1995; Soussana and Hartwig, 1996). The expression of competitive ability is strongly determined by prevailing environmental conditions such as temperature (Campbell and Hart, 1996). This implies that the changing yield proportion of the various species is a consequence of the seasonal temperatures. In mixed swards of *T. repens* and *L. perenne* such fluctuations are accentuated by the complementary seasonal growth patterns of
the two species. L. perenne is dominant early and late in the growing season, whereas T. repens becomes more competitive with increasing temperature (late spring and summer) (e.g. Harris, 1990). These species-specific differences in growth optimum may also be related to the CO₂ response, which is reported to be temperature-dependent (e.g. Bowes, 1993). The warm season growth of T. repens coincides with the period of, in theory, high potential stimulation by elevated pCO₂. This would result in a generally stronger CO₂ responsiveness of T. repens (Newton et al., 1994). In this field experiment, however, the relative CO₂ response in the dry-matter production did not show a pronounced seasonal growth pattern (Hebeisen et al., unpublished data). Therefore, these data suggest that the CO₂ responsiveness of T. repens was observed over the entire growing season and not especially accentuated by increasing temperature as suggested by other authors. Despite an observed CO₂ response of clover in the early growing season clover remained a weak competitor in this period. This favors a cool season niche for L. perenne especially under conditions of elevated pCO₂ (Newton et al., 1994). However, the response of L. perenne to the CO₂ enrichment was also small in this early cooler season (Hebeisen et al., unpublished data).

**Availability of water:** In general, warm temperatures are coincided with low water availability. The effects of water and temperature on sward development are difficult to separate. Water limitation seemed to be the predominant factor in the CO₂ response of a tallgrass prairie. In growing seasons with substantial water stress the improved water use efficiency allowed the C₄ plants to increase productivity under elevated pCO₂ (Owensby et al., 1993b). This could lead to important year-to-year fluctuations in the CO₂ response (Mooney et al., 1991) and points out the importance of a long-term experiment to identify the impact of such climatic interactions. The CO₂-induced decline in transpiration, however, may also restrict the transport of nutrients within the plants and may contribute to the lower N concentration in the plant material (Conroy and Hocking, 1993). In general, no major seasonal pattern in the relative CO₂ effect was observed in the N concentration of the plant material or in the IN of the swards (data not shown).

**Sward structure:** Elevated pCO₂ has been reported to affect the sward structure (Stadelmann, 1993; Sæbø and Mortensen, 1995). Already after a few weeks of CO₂ fumigation, frequently defoliated L. perenne cultivated in monocultures showed an increased tiller number under elevated pCO₂ (Stadelmann, 1993). The very dense sward structure observed in the CO₂-
fumigated *L. perenne* monocultures was persistent and, thus, may have prevented a further increase in tiller number, an important yield-determining parameter. The enhanced sward density was accompanied by a postrated growth habit of *L. perenne* cultivated under elevated pCO$_2$ (Stadelmann, 1993). A CO$_2$-induced reduction in plant height was also reported for *Phleum pratense* and *L. perenne* (Sæbø and Mortensen, 1995). The result of the latter experiment was considered to be due to low temperature. However, the strong CO$_2$ response of the *Trifolium* species (*T. repens* and *T. pratense*) contradict this assumption due to the growth sensibility of *Trifolium* species to low temperature. Postrated growth habit however is generally induced by low nutrient supply and may have been accentuated by the suggested CO$_2$-induced reduction in N availability (Zanetti et al., 1997). Indeed, the extent of the postrated growth was most pronounced in the CO$_2$-fumigated *L. perenne* of the low N treatment (Stadelmann, 1993).

A consequence of the postrated growth habit was that the leaf area below cutting height and, thus, remaining after defoliation was greater under elevated pCO$_2$. It was expected that the enhanced area of photosynthetically active plant tissue in combination with an increase of accumulated non-structural carbohydrate (Fischer et al., 1997) would result in accelerated regrowth (Nijs et al., 1988) and a faster replenishment of carbohydrates after defoliation under elevated pCO$_2$. Moreover, leaf gas exchange measurements have shown a significant increase in net C assimilation (20-60%) of *L. perenne* leaves (Long, personal communication). In contrast to all expectations, no evidence was found for a faster regrowth of *L. perenne* exposed to elevated pCO$_2$ (Stadelmann, 1993; Fischer et al., 1997). Faster development of the canopy under elevated pCO$_2$ would, however, result in an earlier self-shading of the plant resulting in decline of the growth stimulation of elevated pCO$_2$ (Nijs et al., 1989; Ryle et al., 1992a). This is in line with results showing that the CO$_2$ response becomes weaker with increasing plant density (Lüscher et al., 1996). Specific leaf area and carbohydrates suggest that *L. perenne* was increasingly sink-limited under elevated pCO$_2$ (Stadelmann, 1993; Fischer et al., 1997). Such sink limitation may have been induced in part by an imbalance of the C and N nutrition as was suggested to occur under elevated pCO$_2$ (Zanetti et al., 1997).

*T. repens* has important morphological characteristics which may attribute to the strong CO$_2$ responsiveness. First, *T. repens* has stolons as storage organs. These may act as C sinks for the additionally available C assimilated
under elevated pCO₂. Second, root nodules represent a large sink for assimilates (Gordon et al., 1987) and the regrowth of *T. repens* is very fast. *T. repens* was shown to reach its critical leaf area index already 10 to 15 days after defoliation (Stadelmann, 1993); this ensured an early, optimal capture of the incident radiation. Moreover, petiole elongation permits *T. repens* to position new leaves in the top canopy layers or in sunflecks of lower canopy layers (Dennis and Woledge, 1982; Boller and Nösberger, 1985). This ensures that very active leaves are exposed to optimal light conditions. This is considered to be an important advantage for *T. repens* over the grass, especially under elevated pCO₂ when competition becomes more intense. Infrequently defoliated swards, the relative contribution of petioles to the total above-ground yield was higher under elevated pCO₂ (Hebeisen et al., unpublished data). Such changes in sward structure may also contribute to the observed reduction in N concentration in the above-ground plant material. Based on carbohydrate concentration, it was concluded that, under elevated pCO₂, a consistent sink strength must be present in *T. repens* during the entire regrowth period (Fischer et al., unpublished data). *T. repens* responded very strongly to elevated pCO₂. This suggests that, under fertile grassland conditions, *T. repens* is limited mainly by the CO₂ supply and not by N which can be attributed in part to its association with the N₂-fixing bacteria.

It is, however, important to mention that the impact of elevated pCO₂ on grass productivity is less pronounced than the effects of other management factors (defoliation and N fertilization). This suggests that an adjustment of the sward management may counterbalance at least partially the potential CO₂ effects on sward productivity and composition.

The aspects mentioned in the previous chapters (summarized in Figs. 10a and 10b) show that the data from the grassland ecosystem may, to a certain extent, be extrapolated to other ecosystems. It has also been pointed out that other factors may interact with the CO₂ response and play a predominant role. In addition, it has to be considered that the increase in atmospheric pCO₂ is known to be paralleled by an increase in temperature and in N deposition (Vitousek, 1994). The response pattern of different ecosystems to the combined increase in temperature, in N deposition and atmospheric pCO₂ becomes even more complex.
3 CONCLUSION:
N SUPPLY CO-DETERMINES THE EXTENT OF A PLANT'S CO₂ RESPONSE AND, THUS, SYMBIOTIC N₂ FIXATION IS AN IMPORTANT N-INTRODUCING PROCESS FOR ECOSYSTEMS UNDER ELEVATED pCO₂

The reported and suggested effects of elevated pCO₂ on a fertile grassland model ecosystem (Zanetti et al., 1996; Hebeisen et al., 1997; Ineson et al., 1997; Zanetti et al., 1997) are summarized in Figs. 10a and 10b. It has been shown that symbiotic N₂ fixation is not changed by elevated pCO₂ per se (Chapter VI). It is suggested that the effect of elevated pCO₂ is an indirect one by stimulating the growth and thus by altering the demand of an individual plant for symbiotically fixed N. Under field condition the amount of N available in the soil could not cover the CO₂-induced enhanced N demand of the clover resulting in an increased symbiotic N₂ fixation. As a consequence the competitive ability of clover was improved under elevated pCO₂ and the species composition shifted in favor of clover. The higher yield proportion may have resulted in a higher amount of clover litter. The clover litter with a relatively low C/N ratio (even under elevated pCO₂) is fast rechanneled in the nutrient cycling and results in an increased amount of N transfer. In contrast to the clover, elevated pCO₂ resulted in a strong reduction in the N concentration of the grass. This may induce a decline in the decomposition rate of the grass material leading to a litter accumulation retaining nutrients from cycling. A temporal N immobilization may be accentuated by the CO₂-induced increase in microbiomass (Díaz et al., 1993).

It is suggested that due to the increased C supply N becomes the predominant growth restricting factor of the ecosystem. This induces an increased N demand of the entire system resulting in a reduced amount of N leached out under elevated pCO₂ (Soussana et al., 1996). Enhanced N losses through denitrification may further accentuate the CO₂-induced amplification of the N limitation. This illustrates the tight linkage between the C and N cycling and emphasizes the role of the symbiotic N₂ fixation: N₂ fixation is tuned according to the plant's N demand for symbiotically fixed N and, thus, indirectly by the N availability in the rooting zone. Under low N availability the performance of N₂ fixation increases and induces shifts in plant composition. This ultimately leads to higher N introduction into the system and, hence, symbiotic N₂ fixation helps to maintain the C:N balance within a system.
Figure 10a. Scheme of some important processes in a mixed and fertile grassland ecosystem under ambient pCO$_2$. Arrows indicate qualitative fluxes. Scheme is not drawn to scale.
Figure 10b. Occurring and suggested qualitative changes indicated by altered thickness and shade of arrows in a mixed sward exposed to elevated pCO₂. Scheme is not drawn to scale.
4 OUTLOOK

Based on the reported stimulation of symbiotic N\textsubscript{2} fixation under elevated pCO\textsubscript{2} (Chapter IV) and the suggested enhanced N limitation under elevated pCO\textsubscript{2} (Chapters IV and V), it would be interesting to investigate the following topics:

It was reported that the number of Rhizobium bacteria in the rhizosphere of T. repens increased under elevated pCO\textsubscript{2} (Schortemeyer et al., 1996). It is important to determine whether or not shifts in the community structure of Rhizobium leguminosarum bv. trifolii populations occurred under elevated pCO\textsubscript{2}. If so, it is necessary to identify the factors influencing the competitive ability of the different bacterial strains and how a potentially more competitive bacterial strains interact with the host plants (nodulation and N\textsubscript{2}-fixing capacity).

Symbiotic N\textsubscript{2} fixation was suggested to be adapted to the plant's demand for symbiotically fixed N. There is increasing evidence that assimilates are not restricting the symbiotic N\textsubscript{2} fixation (e.g. Weisbach et al., 1996). It would be important to gain a physiological understanding how the suggested 'N-demand orientated' regulation of the symbiotic N\textsubscript{2} fixation is functioning.

It was suggested that increased denitrification activity under elevated pCO\textsubscript{2} is a major factor contributing to a reduction in amount of soil N available to the plants. It is essential to measure the N\textsubscript{2}O efflux under field conditions (Ineson et al., 1997) to obtain more detailed information about the potential role of denitrification over time under elevated pCO\textsubscript{2}.

Decomposition is a crucial process linking above- and below-ground components of the nutrient cycle. In addition, decomposition is known to be affected by any disturbance in litter quality (C:N ratio; lignin content). The reduced N availability under elevated pCO\textsubscript{2} was suggested to be accentuated by reduced decomposition rate of the plant material produced under elevated pCO\textsubscript{2} resulting from an enhanced C:N ratio (Cotrufo et al., 1994; Cotrufo and Ineson, 1995; Gorissen et al., 1995). In contrast, an investigation in the field showed large interspecific differences in leaf litter decomposition which were, however, only marginally affected by elevated pCO\textsubscript{2} (Kemp et al., 1994). It is necessary to elucidate these seemingly contradictory data. Decomposition of plant material produced under elevated pCO\textsubscript{2} should be studied in soil which was exposed to elevated pCO\textsubscript{2} over a long period of time. Further investigations on decomposer population should be carried out. These aspects
should be studied with monocultures and mixed swards. The effect of elevated pCO$_2$ on the C:N ratio was species specific. This may further influence decomposition processes in mixed swards, known to alter their species composition under elevated pCO$_2$. The study of decomposition should be accompanied by an investigation on processes of senescence.

In addition, CO$_2$ research should determine the role of other nutrients in the CO$_2$ response of ecosystems. A key element may be phosphorus. Phosphorus is known to be important for symbiotic N$_2$ fixation and to be increasingly available for plants colonized by arbuscular mycorrhizal fungus. Both associations are involved in the functioning of ecosystem, especially in a world of increasing CO$_2$.

It was shown that, as well as the symbiotic N$_2$ fixation (Zanetti et al., 1996; Zanetti and Hartwig, 1997), the interaction of other plant microorganisms may play an important role in the CO$_2$ response (Sanders, 1996). It would be useful to identify the prerequisites and reasons for shifts in this fungus-plant interaction (mycorrhiza, endophytes, fungal pathogens). Such knowledge may help in predicting the CO$_2$ response of different ecosystems and in understanding the processes involved in the functioning of the ecosystem.
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DANK


Die Arbeit wurde durch einen Forschungskredit des NEFF und der ETH ermöglicht.

VOILÀ

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