The effects of nitrogen uptake and nitrogen fixation on trees grown in elevated [CO_2]: *Alnus glutinosa* and *Pinus sylvestris*

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Abstract

The aim of this thesis is to investigate the influence of nutrient uptake and nitrogen fixation on two temperate tree species grown in elevated carbon dioxide concentration: *Alnus glutinosa* and *Pinus sylvestris*. The former is a nitrogen-fixing deciduous species, the latter a mycorrhizal conifer. One short-term experiment (15 weeks) and one long-term experiment (three years) were set up, both using seedlings grown in open top chambers (OTCs). In the short-term experiment, *c.* 90 one-year-old *Alnus* seedlings (common alder) and 85 one-year-old *Pinus* seedlings (Scots pine) in pots were exposed to either ambient or elevated [CO_2] for fifteen weeks. They were supplied with either a high nitrogen (N) fertiliser designed to allow maximum growth, or a low nitrogen fertiliser. Changes in growth, physiology and leaf biochemistry were measured in both experiments. Elevated [CO_2] affected the conifer and the deciduous nitrogen-fixer differently. Growth was enhanced in both species, although common alder trees grown in elevated [CO_2] were taller and responded more than Scots pine. The latter showed more signs of photosynthetic acclimation than common alder, unlike in the long-term experiment. Stem basal diameter of Scots pine was more stimulated by elevated [CO_2] then total biomass, especially in those trees receiving full nutrient fertiliser.

In the long-term experiment, *c.*600 one-year-old common alder and Scots pine seedlings were planted directly into soil in OTCs in either ambient or elevated [CO_2]. Half the trees were supplied with a full nutrient solution designed to permit maximum growth rate. The remaining trees received no fertiliser. In July 1994 a spike addition of ^{15}N-labelled fertiliser was added to half the trees. The allocation of labelled N to different tissues was followed from September 1994 to April 1996, and nitrogen fixation was measured in alder. Both species, especially common alder, grew bigger in elevated [CO_2] although leaf area remained the same and increased growth rate only occurred during the first year of growth. Net assimilation rate was enhanced in alder but not in pine and leaf area ratio was reduced in both species. There was a larger mass of more efficient nitrogen-fixing nodules on elevated [CO_2] alder roots in 1995, but this effect seemed to disappear in 1996. Signs of photosynthetic acclimation

appeared around August 1995, particularly in alder which was more sink-limited than Scots pine. By summer 1996 starch had accumulated significantly in the leaves of alder, there was a slight reduction in photosynthetic proteins and photosynthetic acclimation was found. This is a surprising result since deciduous nitrogen fixing trees are expected to be less sink-limited than conifers. I attribute this reduction in growth stimulation after two growing seasons in alder to the occurrence of canopy closure and root restriction, as well as to the existence of an upper ceiling of nitrogen fixation. A nutrient effect on variables was usually not found, possibly because fertiliser addition and nitrogen fixation both produced similar growth rates. In Scots pine, in contrast, growth enhancement was larger in the fertiliser treatment than in the non-fertilised plots. The only effect of elevated [CO_2] on allocation of labelled N to different tissues was increased allocation to the stem and leaves in common alder. Labelled N for new leaf growth was derived from the previous year's needles in pine, whereas in alder it seems to come from the stems.

Declaration

This thesis has been composed by myself from the results of my own work, except where otherwise stated, and has not been submitted in any other application for a degree.

Vicky Temperton
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Symbols and Abbreviations

Symbol	Description	Units
#	number	-
[ ]	concentration	mg g^{-1}
A	net photosynthetic rate	µmol m^{-2} s^{-1}
A	spectrophotometric absorbance (ln $[I_0/I]$)	nm
C_a	atmospheric $[CO_2]$	µmol mol^{-1}
C_i	intracellular $[CO_2]$	µmol mol^{-1}
F	variance value in ANOVA	-
$\underline{G}$	specific growth rate	g g^{-1} day^{-1}
I	irradiance	W m^{-2}
I_0	unattenuated reference value	W m^{-2}
L	leaf mass	g
L / S	leaf to shoot ratio	
M_t	total dry mass	g
M_f	fresh mass	g
M_d	dry mass	g
N	nitrogen	
n	treatment sample size	-
P	inorganic phosphorus	
p	level of probability	-
R	root mass	g
r^2	level of confidence in a linear regression	-
R / S	root to shoot ratio	
R / L	root to leaf ratio	
S	shoot mass (stem and branches without leaves)	g

Abbreviation	Description
A	ambient [CO_2] treatment
A-N	ambient [CO_2] treatment without fertiliser
A+N	ambient [CO_2] treatment with fertiliser
a / b	ratio between chlorophyll *a* and chlorophyll *b*
ATP	adenosine triphosphate
C	control treatment (no OTC)
CI	95 % confidence interval for a mean
C-N	control plot without fertiliser
C+N	control plot with fertiliser
chl	chlorophyll
df	degrees of freedom
DMF	dimethyl formamide
FACE	free air carbon dioxide enrichment
E	elevated [CO_2] treatment
E-N	elevated [CO_2] minus fertiliser treatment
E+N	elevated [CO_2] plus fertiliser treatment
G	gigatonne
H	high nitrogen supply
L	low nitrogen supply
LAI	leaf area index
LAR	leaf area ratio
LMR	leaf mass ratio
LSU	large sub-unit of an enzyme
NAR	net assimilation rate
ns	not statistically significant
NUE	nitrogen use efficiency
OTC	open top chamber
PPFD	photosynthetic photon flux density
r	area of leaf disc core
rbcS	small sub-unit gene of Rubisco
Rubisco	ribulose 1,5-bisphosphate carboxylase-oxygenase

RuBP	ribulose 1,5-bisphosphate
SE	standard error of a mean
SBD	stem basal diameter
SBA	stem basal area
SGR	specific growth rate (see also $\underline{G}$)
SLA	specific leaf area
SSU	small sub-unit of an enzyme

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CHAPTER 1
Introduction

1.1 Introduction

1.1.1 The Greenhouse Effect and Rising Atmospheric Carbon Dioxide

Amidst the controversy surrounding the greenhouse effect one core of evidence is difficult to refute. Atmospheric carbon dioxide concentrations, [CO_2], have been rising from the mid 18th century to the present (Eamus and Jarvis 1989, Bazzaz and Fajer 1992). Fuel combustion, cement manufacture and land use changes, especially tropical deforestation, are altering the earth's atmosphere via the production of 'greenhouse gases' such as carbon dioxide, methane, nitrous oxides, ozone and clorofluorocarbons (CFCs). Water vapour is another important greenhouse gas and there are large uncertainties in the role it plays in global warming. Greenhouse gases are transparent to incoming short-wave radiation from the sun but absorb long-wave radiation emitted from the earth's surface, thus warming the earth's atmosphere and keeping it at an overall mean temperature of 15 °C. Carbon dioxide contributed 50 % to the greenhouse effect in the 1980s (Boyle and Ardill 1989). [CO_2] in the earth's atmosphere has fluctuated throughout geological time, but the present increase in [CO_2] is occurring at a rate hitherto unseen in historical records (Bowes 1993). [CO_2] is currently increasing at a rate of *c.* 1.8 μmol mol^{-1} (0.5 %) per year, and is predicted to double its current concentration between 2050 and 2100 if the current trend continues (Boyle and Ardill 1989, Evans 1994).

In 1896 the Swedish scientist Arrhenius was the first to predict that increased atmospheric [CO_2] caused by industrial activities could, over a period of centuries, double the amount of carbon dioxide in the atmosphere and cause global warming (Boyle and Ardill 1989, Rodhe *et al.* 1997). Until 1957 most climatologists dismissed Arrhenius' warnings but the breakthrough came with the first report to put global warming on the scientific agenda produced by the Scripps Institution of Oceanography in California. In 1958 the first atmospheric measurements of [CO_2] were made at Mauna Loa in Hawaii and at the South Pole, and atmospheric [CO_2]

has been recorded ever since. There is now a global consensus amongst scientists that [CO_2] has increased from *c.* 280 μmol mol^{-1} [CO_2] in pre-industrial times to *c.* 363 μmol mol^{-1} [CO_2] in 1996 (Rodhe *et al.* 1997). Ice-core data from Vostok in Antarctica have shown that periods of high atmospheric [CO_2] occurred at the same time as increases in temperature (Boyle and Ardill 1989, Jouzel *et al.* 1993). Despite general agreement in the scientific community that increasing [CO_2] will engender some form of climate change, the extent of such a change is still hotly debated. Until recently temperature measurements made from space with satellites indicated a cooling of the troposphere in contrast to measurements at meteorological stations and to climate models which reported a warming of the atmopshere (Hansen *et al.* 1998). Wentz and Schnabel's (1998) correction to the satellite data seems to have removed the inconsistency between the satellite data and surface measurments (Gaffen 1998, Hansen *et al.* 1998). The new calculations of atmospheric temperatures measured by satellites, take into account the decline in orbital height (altitude) of the satellites (Gaffen 1998). Rising temperatures will affect global evaporation, precipitation and cloud formation patterns and there are still many uncertainties regarding negative and positive feedbacks on the greenhouse effect. General circulation models (GCM) predict that a doubling of atmospheric [CO_2] some time in the next century will cause mean annual temperatures to increase between 1.5 and 4.5 °C (Boyle and Ardill 1989, Melillo *et al.* 1993) depending, amongst other things, on whether increased water vapour in the atmosphere will be a positive or a negative feedback (Pearce 1997). Predictions so far of carbon dioxide-induced temperature rises for this century exceed the actually observed warming of *c.* 0.5 °C. Despite all the above-named ambiguities with regard to the extent of the greenhouse effect, increasing [CO_2] will have direct effects on plants since CO_2 is crucial to photosynthesis in all terrestrial higher plants (Bowes 1991, Long 1991, Lawlor 1993). Inevitably, increasing [CO_2] has prompted a multitude of research in the field of plant responses to such a change.

Estimates of the global carbon budget indicate that *c.* 7.5 gigatonnes (G) of carbon are released into the atmosphere from fossil fuel burning and deforestation (sources) annually. Approximately 5 to 6 G of carbon dioxide have been accounted for in the oceans and atmosphere leaving us with a 'missing' sink of c. 1-2 G of carbon (Sedjo

1992). Although assessment of the global carbon cycle is based mostly on estimates and is still little understood, recent findings suggest northern forest ecosystems as the missing sink (Sedjo 1992, Jarvis 1995).

1.1.2 Effects of Increasing [CO_2] on Forests

Carbon enters the biosphere primarily through the photosynthetic process in plants. The global uptake of CO_2 in photosynthesis is *c.*120 G annually and virtually all the carbon is assimilated through one enzyme, ribulose bisphosphate carboxylase / oxygenase (Rubisco). Rubisco initiates both the photosynthetic carbon reduction (PCO) cycle and the photorespiratory carbon oxidation (PCR) cycle. CO_2 acts as both activator and substrate at once, and CO_2 and oxygen compete for the active site of the enzyme. At the current atmospheric [CO_2] photosynthesis is limited by CO_2 and therefore any increases in the atmospheric [CO_2] / [O_2] ratio is expected both to stimulate photosynthesis and to diminish photorespiration. An increase in photosynthesis is in turn expected to increase plant growth. Horticulturalists have long known of the positive effects of increased [CO_2] in greenhouses on plant productivity (Besford 1990). In general C_3 plants respond more to growth in elevated [CO_2] than C_4 plants (Bowes 1991, 1993, Poorter 1993), because the latter type of photosynthesis already involves CO_2 concentrating mechanisms. Recent studies on intact prairie communities and some field experiments have found no such difference or the reverse to be true (reviewed in Körner 1995) so that caution is required in making generalisations. C_3 plants constitute *c.* 95 % of all plants, however, and the large extent of forests, all C3, world-wide render impact studies of elevated [CO_2] on forest structure and growth of vital importance.

Forests cover a large area of the land surface of this planet, with estimates ranging from one fifth (Mousseau and Saugier 1992) to more than a third of land cover (Linder *et al.* 1996). 80 to 90 % of global biomass is found in forests (Körner 1995, Linder *et al.* 1996) compared with only 1 % in agrosystems (Körner 1995) despite lower annual productivity in the forests compared with crops. Forests are thus a major form of current and future land use and play a crucial role in the global carbon balance. Forests form long-lived communities so that increasing atmospheric [CO_2]

and any concomitant temperature rise will have significant effects on the forests of both the present and the future (Eamus and Jarvis 1989, Linder *et al.* 1996).

Forests may be a net sink or a net source of atmospheric carbon dioxide (Dixon *et al.* 1994). Terrestrial higher plants assimilate *c.* 15 % of the atmospheric carbon pool each year and a similar amount is returned to the atmosphere via respiration and decomposition of soil organic matter and plant litter (Amthor 1995). A global change in carbon metabolism could thus influence atmospheric [CO_2] over a period of years to decades, especially since the CO_2 efflux from terrestrial plant and soil metabolism is larger in magnitude than that from fossil fuel burning (Amthor 1995). Model predictions indicate that forests may be substantial sinks for CO_2, despite tropical deforestation, since the northern temperate and boreal forests are expanding and increased carbon uptake by trees in a changing climate has been predicted (Mellilo *et al.* 1993). There is still considerable uncertainty as to the present and future role of forests as carbon sinks or sources, however. In addition to the above considerations, an understanding of how forests will respond to rising [CO_2] is important for the management of forest systems in terms of future establishment, growth and timber quality (Conroy *et al.* 1990b) as well as for recreational, biodiversity and conservation purposes.

1.1.3 The Special Case of Trees and Experimental Approaches

The main characteristics of trees as compared with other plants are their size, longevity and their complexity (Ceulemans and Mousseau 1994, Norby *et al.* 1996). This makes them challenging to study in global climate change impact studies, since one cannot set up experiments which last for the whole life-cycle of the trees. The sheer size of trees means that most experiments are limited to using juveniles (reviewed in Eamus and Jarvis 1989) or only exposing branches of mature trees to elevated [CO_2] using branch bags (Barton *et al.* 1993). Juvenile trees are more sensitive to their environment than mature trees, often showing responses of stomatal conductance, photosynthesis and water use efficiency that are different to adult trees (Ceulemans and Mousseau 1994) so that it is difficult to know how mature trees, let alone whole forests will respond to climate change from their responses.

Only in the last decade or two has the emphasis on research in agricultural crops (Kimball *et al.* 1993) extended into the realm of forestry and tree responses to rising [CO_2]. There are many published reviews of terrestrial vegetation and ecosystem responses to elevated [CO_2] (e.g. Eamus and Jarvis 1989, Jarvis 1989, Bazzaz 1990, Field *et al.* 1992, Mousseau and Saugier 1992, Bowes 1993, Kimball et al. 1993, Poorter 1993, Ceulemans and Mousseau 1994, Amthor 1995). Most of the experiments have studied the response of agricultural species so that there is a need to improve our knowledge of tree reponses to climate change (Ceulemans and Mousseau 1994, Norby *et al.* 1996). Nevertheless, studies on trees and climate change are increasing, not only on American species which formed the focus of most of the original tree experiments, but also on European species (see the Ecocraft Report, Jarvis 1998) and to a lesser extent Australian species (see Duff *et al.* 1994).

Until 1992 most studies were undertaken for short time spans on young trees growing in controlled or semi-controlled environments, many without regard for either the nutritional status of the trees (Eamus and Jarvis 1989) or their rooting volume with plants grown in pots (McConnaughay *et al.* 1993). While experiments in controlled environments are crucial to understanding some of the mechanisms behind plant reponses to elevated [CO_2], it is difficult to extrapolate from them to the real world where plants do not grow in isolation or in pots (Morison 1990, Körner 1995). Despite the fact that numerous short-term experiments have shown a growth and photosynthetic stimulation in elevated [CO_2] in trees, there are still few experiments extending beyond two growing seasons in length (Ceulemans and Mousseau 1994, Lewis *et al.* 1996, Johnson *et al.* 1996, Rey 1997). In field experiments open top chambers (OTCs) are most commonly used (Leadley and Drake 1993) since they are better approximations to natural conditions than growth cabinets where small replication is an additional drawback. Until recently impact studies on intact ecosystems were very rare (reviewed in Körner 1995). So far there have been very few ecosystem experiments on trees using the free air, CO_2 enrichment system (FACE) which is the only realistic way of assessing the impact of climate change on whole forest ecosystems (Ceulemans and Mousseau 1994). Experiments at natural CO_2 springs can also provide realistic results although the number of locations is

limited, and adequate replication and definition of conditions are proablematic. All methods of exposing plants to elevated [CO_2] have their advantages and disadvantages depending on the main aims of the study, which should be explicitly defined (Ceulemans and Mousseau 1994). The above points highlight the need for additional tools such as computer simulation models to explore responses and to make predictions for whole ecosystems at forest regional and global scales. These large scale process models need to be based on mechanistically sound sub-models, so that there will always be a need for basic empirical research such as the experiments presented in this thesis. Consequently, there is a clear necessity for further, more realistic experiments on trees in the field in appropriately controlled conditions (Körner 1995).

1.1.4 Interactions With Other Environmental Variables

It is now clear that many environmental variables such as nutrient and light availability (Sage *et al.* 1990), drought (Townend 1993) and temperature (Ziska and Bunce 1994) influence the response of plants to [CO_2]. This is becoming increasingly clear as the results of studies at different locations and times show a whole spectrum of growth and physiological responses, sometimes by the same species, without adequate explanation.

Nutrient Availability

Most natural terrestrial ecosystems, especially forests grow on soils that are nutrient limited (Linder and McDonald 1993, Körner 1995, Norby *et al.* 1996) so that impact studies with a luxurious mineral nutrient supply to the trees are not very relevant to natural vegetation. Woodward *et al.* (1991) and Körner (1995) point out that a wide range of the [CO_2] reponses found in the literature can be 'produced' by altering mineral nutrition. Linder and McDonald (1993) also emphasise the importance of maintaining good control of mineral nutrition (and other environmental variables) in order to avoid attributing growth reponses to [CO_2] instead of the interaction of [CO_2] and mineral nutrition. Experiments with free mineral supply are nonetheless of considerable use in agricultural and agroforestry systems (Körner 1995). Several authors have found that limitation by nutrients may have a larger effect on tree

reponses than elevated [CO_2] (see Eamus and Jarvis 1989, Mooney *et al.* in press), although a number of field experiments have shown positive responses to [CO_2] by trees with low nutrient supply (see Kimball *et al.* 1993, Norby *et al.* 1996). The picture is made more complex becasue even poor natural soils when dug up may undergo enhanced mineralisation for some time afterwards thus improving nutrition (Körner 1995). It may take longer than the usual funding period for such soils to 'settle'. Other factors which could come into play in plant responses to elevated [CO_2] are atmopsheric nitrogen (N) deposition from atmospheric pollutants (up to 50 kg ha-1 per annum in Europe) and enhanced mycorrhizal growth. Atmospheric nitrogen deposition from air pollution is another driving factor in climate change, along with rising [CO_2], temperature, tropospheric ozone and increased UVB (see Mooney *et al.* in press). It is as yet unclear whether atmospheric N deposition alone enhances growth or if the response is a positive interaction between rising [CO_2] and N deposition. There are reports of higher yield classes in old forest stands compared with the past (see Mooney *et al.* in press). The concept of 'critical load' of pollutants needs to be taken into consideration, however, since living systems have thresholds beyond which damage occurs (Rosen *et al.* 1992).

Temperature

Rising atmospheric [CO_2] will in all likelihood lead to an increase in mean atmospheric temperatures. Both factors are likely to affect photosynthesis and stomatal conductance in complex ways. Rising [CO_2] will reduce photorespiratory carbon loss whereas rising temperatures will increase respiration. The specificity of Rubisco and the solubility of CO_2 decrease as temperature rises favouring oxygenation (Long 1991). Overall, however, photosynthesis is more sensitive to [CO_2] at higher temperatures than at lower temperatures. Elevated [CO_2] also affects photosynthetic response to temperature, often raising the temperature optimum (Long 1991). Plants growing in warmer climates have generally shown a larger response to elevated [CO_2] than those in colder climates (see Drake *et al.* 1997).

Water Availability

A common response of plants to elevated [CO_2] is an increase in water use efficiency (see Drake *et al.* 1997, Mooney *et al.* in press) either via increased photosynthesis or decreased stomatal conductance, although a number of more recent studies on trees have found that stomatal conductance is often not reduced very much in elevated [CO_2] (reviewed in Mooney *et al.* in press). There is a need for more experimentation and elucidation of mechanisms in this area.

Plant-Plant Interactions

Trees do not normally grow in isolation but interact with neighbouring plants. Responses of isolated trees in OTCs or controlled environment chambers to elevated [CO_2] have been shown to be larger than when trees were grown close together (Körner 1995, Norby *et al.* 1996, Drake *et al.* 1997). Canopy closure reduced or removed the positive growth response, indicating that trees may grow faster at the seedling stage in a changing climate but once canopy closure occurs the difference my disappear (see Morison 1990).

1.2 The Issues

1.2.1 Growth and Biomass

An almost universal response to short-term exposure to doubling [CO_2] is a stimulation of photosynthesis of between 25 and 75 % compared with photosynthesis in ambient [CO_2] (Stitt 1991). Increased carbon assimilation in elevated [CO_2] often leads to increased biomass production in the plant (Eamus and Jarvis 1989, Morison 1990, Ceulemans and Mousseau 1994) although not always (Körner 1995). In C_3 crop plants and trees this increase varies from 30 % to 70 % (Idso and Kimball 1991, Kimball *et al.* 1993, Poorter 1993, Ceulemans and Mousseau 1994). According to the review of Ceulemans and Mousseau (1994) deciduous trees show mean biomass increments of + 63 % compared with + 38 % in conifers. Often the largest enhancement of biomass was observed in nitrogen-fixing trees, where carbon-expensive nitrogen fixation becomes more efficient (Norby 1987, Mousseau and Saugier 1992). C_4 plants have generally been found to respond less to elevated [CO_2] than C_3 plants (Bowes 1991) although recent experiments on more natural systems

indicate that this tenet does not always hold true (Mooney *et al.* in press). Differential species reponses according to functional types (i.e. C_3 versus C_4 or nitrogen fixers versus non-fixers) suggest that competitive interactions between plants could change in a changing climate, leading to new constellations of species and successional dynamics (Bazzaz 1990).

Other observed effects of exposure to elevated [CO_2] are increased mycorrhizal infection of tree roots (Rey 1997), the inducement of earlier flowering as well as shortening of the life cycle because of earlier senescence (Mousseau and Saugier 1992) and decreased respiration (Morison 1990, Ziska and Bunce 1993) and stomatal conductance although the last two responses are not always found (Mooney *et al.* in press).

1.2.2 Biomass Allocation

Biomass increases in elevated [CO_2] result from increases in carbon assimilates which are allocated to different plant structures, often changing the overall morphology of plants and in particular root to shoot ratios (Ceulemans and Mousseau 1994) In experiments with fertilised trees three different scenarios have been found with regard to the effect of elevated [CO_2] : a) root biomass increases proportionally more than shoot biomass, b) root biomass decreases proportionally to shoot biomass, or c) the ratio remains unchanged (Mousseau and Saugier 1992). In unfertilised soils the root to shoot ratio usually increases substantially in elevated [CO_2] (Rogers *et al.* 1992 and 1994) and may ensure better acquisition of mineral nutrients. As a tree ages its pattern of biomass allocation changes, partitioning more carbon to woody tissues with time and less to assimilatory material. A general plant response to elevated [CO_2] is a speeding up of growth and development and a concomitant shortening of the growing season. Often interpretation of differences in biomass allocation found in elevated [CO_2] impact studies has not considered such ontogenetic differences (Morison 1990).

1.2.3 Short-Term and Long-Term Responses

In the short-term, photosynthesis is stimulated in elevated [CO_2] in C_3 plants by increasing rates of carboxylation of ribulose-1,5-bisphosphate (RuBP) and decreasing photorespiration rates. It has been predicted that photosynthesis will increase in both light-saturated and light-limiting conditions (Long 1991). Whether plants will be able to sustain increased rates of carbon assimilation over long periods of time and whether they will have large enough sinks for the extra carbon is one of the most important questions addressed in this thesis and in more recent impact studies in general.

Frequently observed initial increases in photosynthesis in short-term studies have been shown not to be sustained in the long-term where several scenarios have been observed. Either a) relative rates of photosynthesis show an overall decrease in assimilation, b) a readjustment in assimilation rates occurs so that the ambient [CO_2] trees 'catch up' with the elevated [CO_2] trees, or c) assimilation rates fluctuate over the season (Mousseau and Saugier 1992). These results are consistent with the idea that some limiting factor or feedback mechanism comes into play which slows down the newly stepped-up production. This negative feedback is manifest as a reduction in photosynthetic rate induced by either so-called *down-regulation* ('fine control') or *acclimation* ('coarse control'). The former involves a reduction in photosynthetic rate without a change in leaf composition, whereas in the latter case photosynthetic capacity is reduced via diminished photosynthetic components such as Rubisco, chlorophyll or other enzymes (Delgado *et al.* 1994). Photosynthetic acclimation has been associated with accumulation of carbohydrates because of low sink demand and / or reduction in Rubisco amount or activity (Stitt 1991). The exact mechanisms of acclimation are not yet known, but could act via limited availability of NADPH or ATP. For every molecule of RuBP regenerated in the carbon reduction cycle one molecule of carbon is used up. An increase in the activity of the cycle necessitates increased production of NADPH and ATP from the light reactions of photosynthesis (Lawlor 1993). Alternatively, limitation by phosphorus and / or protein regeneration because of reduced triose phosphate metabolism has been put forward as a potential, if short-term mechanism of photosynthetic acclimation (Stitt 1991).

The source-sink balance of a plant is a finely tuned web of processes which behaves in a homeostatic manner. Thus some plants grown in elevated [CO_2] accumulate carbohydrates in the chloroplasts or export them as structural or non-structural carbohydrates to other parts of the plant. In the event of limited sink availability, the carbon is often exported to the rhizosphere (Mooney *et al.* in press). Alternatively the accumulation of starch has been seen to cause chloroplast disruption. Why it is that some plants restore the balance within their cells by sending the extra carbon to the rhizosphere whereas others let the carbohydrates accumulate until the chloroplast is disrupted, is unsure. Both could be strategies for coping with the extra assimilate, but the latter might be a consequence of the inability of the chloroplast to balance the carbon sink with the carbon source. Work by Van Oosten *et al.* (1994) provides a potential molecular model in which photosynthesis-associated genes (PAG) in the nucleus, but not those coded for in the chloroplast are down-regulated in elevated [CO_2]. This could be the reason why disruption occurs in the chloroplast but not in the rest of the plant cell. Furthermore, Van Oosten *et al.* (1994a) found that the action of sugars mimics the regulation of genes thus acting like hormones.

1.2.4 Can Nutrition Keep Up With Increased Carbon Assimilation?

One of the most generally observed reponses of plant growth in elevated [CO_2] is an increased C : N ratio (Mousseau and Saugier 1992, Poorter 1993, Ceulemans and Mousseau 1994). A dilution of nitrogen almost always occurs as more carbon gets incorporated into the plant. This dilution occurs either because nutrient uptake does not increase along with carbon fixation, or because of a protein 'dilution' effect (i.e. less nitrogen per unit plant mass than carbon per unit plant mass) or remobilisation of nitrogen from Rubisco to processes other than carbon fixation.

Thus the carbon cycle seems to be closely linked to and dependent on the nitrogen (and phosphorus) cycle. Gifford (1994) disputes this, stating that the nitrogen cycle may actually be following the carbon cycle. The frequently observed phenomenon of increased root : shoot ratio in elevated [CO_2] as well as increased carbon exudation from the roots could be, with the help of increased temperatures, providing readily

metabolised carbon in the soil. Greater mineralisation and thus increased soil nitrogen would ensue, which would lead to a re-equilibrium of the C : N ratio. Other factors which could help re-balance C : N ratios are nitrogen deposition from atmospheric pollutants (considerable in the case of Europe) and enhanced mycorrhizal growth.

Drake, Gonzalez-Meler and Long (1997) dispute the commonly-held view that photosynthetic stimulation will be strongly limited in the long run by nutrient availability, providing evidence for increased nitrogen use efficiency in elevated [CO_2]. Evidence for this come from several long-term experiments in which plants grown with low nitrogen supply were as stimulated in growth as those given high nitrogen supply. In addition, photosynthetic acclimation occurring after long periods of exposure rarely completely compensates for stimulation of the rate of photosynthesis by elevated [CO_2].

1.2.5 Biochemical Responses to [CO2]

The most common responses of leaves to longer-term growth in elevated [CO_2] are reduced nutrient concentrations especially [N] and decreased Rubisco, soluble protein and chlorophyll concentrations (Ceulemans and Mousseau 1994, Drake *et al.* 1997). Accumulation of soluble carbohydrates and starch in the leaves is also very frequently found (see Stitt 1991). There is some debate in the literature concerning the relative roles of carbohydrate accumulation (i.e. low sink strength) and decreases in photosynthetically important proteins in photosynthetic acclimation. For more details of this discussion and of possible mechanisms behind photosynthetic acclimation see Chapter 5.

1.2.6 Nitrogen Fixation

Nitrogen-fixing plants are universally predicted to be more responsive to elevated [CO_2] than other plants. Trees generally do not usually increase their biomass production as much as herbaceous crop plants in elevated [CO_2], although some of the largest effects have been recorded in nitrogen-fixing trees (Norby 1987, Mousseau and Saugier 1992, Poorter 1993). However, more recent experiments on natural or semi-natural grassland swards do not confirm that growth of nitrogen-fixers is

necessarily strongly enhanced (see Körner 1995). Furthermore, studies involving complex assemblages of nitrogen-fixing plants exposed to elevated [CO_2] have also shown inconsistencies in this respect (Mooney *et al.* in press). Previous short-term studies of the effects of elevated [CO_2] on alder seedlings suggested that nodulated plants had higher whole plant photosynthesis, nitrogenase activity, nitrogen content and total biomass compared with nodulated plants grown in ambient carbon dioxide, or compared to non-nodulated plants grown in both ambient and elevated carbon dioxide concentrations (Norby 1987, Arnone and Gordon 1990). One possibility is that nitrogen-fixing trees grown in elevated carbon dioxide possess larger sinks than other trees and should be able to export extra photosynthate to increase nodulation of roots and so be more independent of external nitrogen additions.

Nitrogen and carbon are nevertheless not the only important variables for nitrogen-fixation. An important aspect of nitrogen fixation is phosphorus (P), which is very important for nodulation particularly in the nodule development stage (Sprent and Sprent 1990). Alder nodules were found to be larger when given a plentiful phosphorus supply. This suggests that nitrogen-fixing plants have a larger P requirement than other species, although Sprent and Sprent (1990) concluded that once the nodule system has been set up, an extra demand for phosphorus need not be the case *per se*. Any advantage over other trees in growth stimulation of nitrogen-fixing trees grown in elevated [CO_2], therefore, is also dependent on phosphorus availability and uptake. There is, however, a three-way symbiosis in nature which can alleviate this problem. Some studies have shown that nodulated plants (legume and non-legume, annual and perennial) are sometimes infected with vesicular-arbuscular (VA) mycorrhizas, and that the latter can greatly improve phosphorus uptake (see Sprent and Sprent 1990). Thus the two endosymbionts may interact to produce an effect which is larger than the sum of its parts. Since mycorrhizas, like nodules, are a carbon cost to the host plant growth in elevated [CO_2] could alleviate this extra cost.

1.2.7 Nitrogen Allocation to Different Tissues

Internal nitrogen cycling plays an ecologically important role in tree physiology and growth, in that it uncouples growth from nutrient uptake in the spring (Millard and Neilson 1989). It can provide up to 90 % of N required for leaf growth in both evergreen and deciduous species (Proe and Millard 1994, Millard 1995). Such cycling processes typically involve seasonal storage of N in the autumn followed by remobilisation during periods of growth or senescence (Millard 1995). One of the main forms of N storage is the enzyme Rubisco (Huffaker and Miller 1978, Camm 1993, Millard 1996) although bark storage proteins have also been recently isolated (reviewed in Millard 1996). Since it is present at such high concentrations in leaves, often at higher levels than required for maximum photosynthesis (Long 1991, Stitt 1991), Rubisco constitutes the largest sink for nitrogen in the photosynthetic system. Thus any changes in Rubisco concentration in leaves exposed to elevated [CO_2] could be correlated with N translocation to other parts of the plant as storage. To my knowledge, there is no experimental evidence to date on the reponse of internal N allocation in trees to elevated [CO_2]. There is a need for experiments investigating the effects of elevated [CO_2] on internal N cycling within plants if the responses within leaves and between tissues are to be better understood.

1.2.8 Common alder and Scots pine

Common alder (sometimes known as 'black alder'), *Alnus glutinosa* (L.) Gaertn., belongs to the deciduous *Betulaceae* family and is closely related to the birches (*Betula*). It is native to Europe, Western Asia, and North Africa. Common alder grows to a mature height of between 20 and 30 metres, with a trunk of up to 4 metres in girth. It derives its Latin name from its glutinous young shoots (Bean 1973). Alder trees have been known for centuries to be well suited to wet sites often growing near rivers (Johns 1912). A prominent characteristic of alder being a nitrogen-fixing pioneer species is its ability to grow where many other tree species cannot, particularly on nutrient-poor soils (although not at low pH), in time rendering these soils more nutrient-rich. Alder species are thus often used on reclaimed sites such as disused open-cast coal spoils or derelict land reclaimed for forestry, where they, along

with conifers improve the site and tree nutrition (Hood 1993). *Alnus* spp fix nitrogen via a symbiotic relationship with *Frankia* spp of bacteria which form nodules on the roots (see the Introduction to Chapter 4 for further details). Alder species are sometimes used in mixed species plantations to make nitrogen available for the companion species. One particular example of this is the planting of inter-rows of alder and a coniferous species which improves the nutritional status of the conifer, although care must be taken since the alder species has a tendency to out-compete the conifer in the first years (Moffat *et al.* 1992). Although common alder is not a major plantation tree species, its use in the reclamation of disused industrial sites and its nitrogen-fixing capability make its use in climate change experiments very relevant.

Scots pine, *Pinus sylvestris* (L.), belongs to the economically important and extensive *Pinaceae* family and has a range which extends over northern Eurasia, from Scotland through Scandinavia, the Baltic region and Russia. It reaches a mature height of between 30 and 40 metres, with a trunk of up to 6 metres in girth. It can survive in the most inclement of conditions, from the Arctic circle to the borders of the steppe (Bean 1973). Scots pine grows best on sandy and light soils in conjunction with heather (*Calluna vulgaris* and *Erica* spp) and bilberry (*Vaccinium myrtillus*). It is wind-firm, light demanding, very frost-hardy and will grow under varying conditions, often regenerating naturally. In Britain, although now only native to Scotland, it is widely used in plantation forestry, despite competition from faster-growing species such as *Pinus contorta* Dougl. var. *contorta* (Lodgepole pine) or *Pinus nigra* var. *maritima* (Ait.) Melville (Corsican pine). Most importantly, it forms the dominant biomass in many areas of Boreal forest, rendering any study of its response to its changing environment very pertinent.

Both species of tree have in common a capacity to grow on sites with low nutrient availability through the help of symbioses with other organisms, the one via nitrogen-fixation, the other via mycorrhizal soil exploration.

1.3 Aims of the Study

The main hypothesis in this research is that inadequate nutrient acquisition (particularly nitrogen) and the ensuing C : N imbalance in Scots pine grown in elevated [CO_2] without fertiliser will produce a more marked down-regulation of photosynthetic capacity than those trees grown in elevated [CO_2] with fertiliser. Common alder being able to fix nitrogen (particularly in conditions of low soil nitrogen availability) will be able to benefit more from the extra carbon availability by coupling it with additional nitrogen fixation. The increased availability of carbon, a rate-limiting step in nitrogen fixation, could result in enhanced growth in the nitrogen-fixing trees. It is hypothesised that alder grown in elevated carbon dioxide should be able to export extra starch and sugars to increase nodulation of roots and so be more independent of external nitrogen additions. Inadequate nitrogen acquisition should not be the cause of down-regulation in the alder grown in elevated [CO_2] without fertiliser. In the longer-run a lack of adequate phosphate supplies for ATP or NADPH could cause a reduction in photosynthetic and growth stimulation (acclimation). An experiment was therefore started using a comparison between a nitrogen fixer (Alnus) and a non-fixer (Pinus). These species constitute a comparison between deciduous and coniferous and between nitrogen-fixing and mycorrhizal trees.

The objective of this study was to determine the interactive effects of the environmental variables carbon dioxide and nitrogen on growth, physiology and biochemistry of the two species over three growing seasons, with particular reference to any changes in photosynthetic components and the source / sink balance of fixed carbon as well as nitrogen allocation to different tissues.

To investigate both the short-term and long-term response of the two distinct tree species to growth in elevated [CO_2] and fertiliser addition two experiments were set up in 1994. The short-term experiment at the Institute of Terrestrial Ecology lasted for fifteen weeks with seedlings grown in pots in OTCs, whereas the long-term experiment at Glendevon involved trees grown directly in the ground in OTCs for three consecutive years.

1.3.1 Specific Aims

Specific aims of both the short-term experiment and long-term experiments were to elucidate whether:

1) exposure to elevated [CO_2] leads to significant increases in growth and plant biomass of both Scots pine and common alder;
2) nutrient addition would affect the growth and physiological response to elevated [CO_2];
3) there would be interactive effects between elevated [CO_2] and nutrient addition on the growth, physiological and biochemical response of both species;
4) biomass allocation would be altered in common alder and Scots pine grown in elevated [CO_2];
5) starch and soluble carbohydrate accumulation would occur in the leaves of common alder and Scots pine grown in elevated [CO_2];
6) changes in leaf chlorophyll concentration would occur in elevated [CO_2];
7) there would be species-specific responses to growth in elevated [CO_2], particularly in common alder where inorganic nitrogen could be fixed from the atmosphere and the trees thus be less dependent on soil nitrogen sources?

Aims specific to the long-term experiment were as follows:

8) to test whether long-term exposure to elevated [CO_2] also leads to increases in plant growth and biomass in both species;
9) to test whether the growth stimulation often observed in short-term studies can be maintained over several growing seasons in the field or if photosynthetic acclimation occurs;
10) to determine if nitrogen fixation was occurring in the alder and if it would increase or be more efficient in elevated[CO_2];
11) to study whether elevated [CO_2] would affect allocation of nitrogen to different tissues;
12) to determine in both species where nitrogen was allocated over the winter and from which tissues nitrogen for new growth in the spring was derived;

13) to identify the main long-term changes in biochemical leaf composition in both species; and

14) to determine whether nitrogen-fixing alder would exhibit less of a source-sink imbalance than Scots pine, since nitrogen-fixing nodules could act as a large sink for any extra carbon fixed?

These hypotheses were tested using an array of growth, physiological and biochemical measurements on samples from trees grown both in soil in OTCs and in pots. The trees were sampled as well as destructively harvested at periodic intervals. A particular emphasis of the research was how leaf Rubisco concentration and nitrogen allocation were affected by [CO_2] and fertiliser addition.

1.4 Outline of the Thesis

The thesis is organised into six chapters that cover responses to elevated [CO_2] at different temporal and spatial scales.

Chapter 1: Introduction

This chapter introduces the background and sets the context for this thesis. It includes a general overview of the greenhouse effect and CO_2 experiments, the main issues in this study and the main aims of the experiments.

Chapter 2: Materials and Methods

This chapter presents the field sites, experimental design and set-up of both experiments in more detail than is provided in the methods sections of the results chapters. In addition OTC descriptions and performance are included.

Chapter 3: Growth, biomass and biochemistry of *Alnus glutinosa* and *Pinus sylvestris* grown in elevated carbon dioxide concentration - a fifteen-week experiment at the Institute of Terrestrial Ecology

This chapter reports the short-term response of both species to growth in elevated [CO_2] with different nutrient addition, at both the whole tree-scale and the leaf scale. It focuses on biomass changes and allocation, leaf morphology and leaf biochemical composition.

Chapter 4: Growth and biomass, nutrient concentration and internal nitrogen cycling *Alnus glutinosa* and *Pinus sylvestris* grown in elevated carbon dioxide concentration - a three-year experiment at the Glendevon field site

This chapter covers long-term growth and internal nitrogen responses to elevated [CO_2] in both species of tree from 1994 to 1996. In particular the effects of elevated [CO_2] on allocation of labelled-nitrogen to different tissues over the winter and spring 1994 / 1995 is reported.

Chapter 5: Biochemistry of *Alnus glutinosa* and *Pinus sylvestris* grown in elevated carbon dioxide concentration - a three-year experiment at the Glendevon field site

In this chapter the long-term biochemical responses to elevated [CO_2] at leaf scale are presented. These include the responses of chlorophyll, proteins, Rubisco concentration, soluble sugars and starch.

Chapter 6: Synthesis and Conclusions

This chapter brings together the most relevant conclusions from this study and includes a general discussion of the aims outlined in Chapter 1.

CHAPTER 2
Materials and Methods

2.1 Introduction

One of the main characteristic of field experiments is an increased incidence of experimental error compared with controlled environment studies, since environmental variables cannot be easily controlled in the field. This engenders a larger variability in environmental variables present in the field. In open top chamber (OTC) experiments such as the two outlined in this research, not only did the $[CO_2]$ and nutrient treatments affect the results of the experiment but also the presence of the glass chambers (Leadley and Drake 1993). Because of this inevitable chamber effect on the environment of the trees and hence on physiological processes, an un-chambered control plot was included in the experimental design. This enabled the effect of the chambers to be assessed separately from the effect of the $[CO_2]$ or nutrient treatments. A second source of error in these experiments was the inherent variability of the seedlings, especially the alder. The seedlings were not clones and as such natural variation was high (sometimes visible morphologically). A third source of variability came from the lack of uniformity of the field site.

All three sources of variability could have caused only large treatment effects to be statistically significant and many effects to be termed tendencies towards an effect. It was attempted to take these factors into account in the design of both experiments by using a randomised plot design in which the location of elevated or ambient $[CO_2]$ chambers and fertilised or unfertilised quadrants were determined randomly and blocks were assigned each containing one treatment. The effect of lack of uniformity in the field site was also reduced by testing for the effect of differences between each individual chamber on the results. Whenever this effect was significant it was pointed out in the results sections of the chapters. Because of logistical constraints, the control plots were not assigned randomly amongst the chambers but were separate at both sites. Despite all these considerations the experimental error is considered to be relatively small and the number of replicates reduces this error, although financial

considerations involved in this kind of experimental set-up limited the replication that could be used.

The variables described in this chapter relate mostly to the long-term field site at Glendevon since the construction of OTCs and CO_2 supply to the OTCs was very similar in both experiments. For details of most materials and methods see the relevant chapters. Outlined below are the experimental design and field site details including data on variables such as soil profile, soil pH, soil water, soil nutrient content and climate, which require more detail than could be added to each individual chapter. In addition, the performance of CO_2 fumigation and soil fertiliser is shown below (section 2.4) as well as a detailed description of statistical considerations.

2.2 Experimental Set-Up and Design

For details of the Institute of Terrestrial Ecology and Glendevon field locations see the Methods sections in the appropriate chapters. A detailed description of experimental design and field conditions is given below for the Glendevon field site. For details of the ITE experimental design see the Methods section of Chapter 3.

2.2.1 The OTC Set-Up

The Chambers

The chambers were constructed in an octagonal form, from lightweight aluminium frames containing 3 mm horticultural glass, and had a diameter of 3 m and a height of 3.5 m. The chambers and control plots were set up in a multi-factorial manner, randomised with respect to carbon dioxide, nutrition, species and aspect (see Figure 2.1), although the control plots were located non-randomly away from the chambers. Each chamber was supplied with either ambient (350 μmol mol^{-1}) or elevated (ambient + 350 μmol mol^{-1}) carbon dioxide concentrations, and each chamber (or control plot) was separated into four quadrants separated from each other by a plastic barrier which reached 40 cm below the soil and 20 cm above soil level (see Photo 2.5). Half the quadrants were planted with common alder, the other half with Scots pine. The alder trees were situated on the same side as the door in the chamber, the pine on the opposite side (see Figure 2.5).

Two quadrants per chamber received full strength nutrient solution (70 kg nitrogen ha^{-1} per season) provided at rates calculated previously from growth rate of the Sitka spruce (see below) added via a computer-controlled drip fertigation system (the fertiliser treatment) and one without any additional nutrition (the non-fertiliser treatment). An irrigation system with one outlet per seedling supplied the trees in each chamber daily with water for ten minutes (to field capacity).

The Glendevon fertigation system was based on the Ingestad theory of plant nutrition (see Ingestad 1979, 1980 and 1981). This theory states that in order to maintain steady internal nutrient concentrations and hence optimum growth rate in a plant, the relative nutrient uptake rate must equal both the relative growth rate and the relative addition rate of nutrients. Every plant species has its own optimum growth rate which also changes according to climatic conditions such as air and soil temperature, water availability, photosynthetically active radiation and atmospheric [CO_2]. Relationships between nutrition and growth are characteristically linear in the sub-optimum range of the relationship between growth rate and relative addition rate. In the supraoptimum range this relationship is no longer controlled by the relative addition rate but by the external nutrient concentration, resulting in reduced growth (due to saturated nutrient utilization). In order to apply Ingestad nutrition correctly one must know the following parameters: firstly, the starting values for all variables (such as dry mass of plants and internal nutrient concentrations) and secondly, the rate of growth with 'free access' to nutrients enabling the calculation of optimum growth rate and nutrient concentrations needed for sub-optimal treatments earlier in the growing season. It is also helpful to track internal nutrient contents over the season to determine the extent of nutrient uptake.

With these ideas in mind a computer-controlled system of Ingestad nutrition was set up at the Glendevon field site which supplied nutrients at different rates over the growing season (according to the optimum growth of Sitka spruce, the first species grown on site) to all three tree species growing at the site. This formed the main limitation of this system in that only one nutrient tank was available for the three species, and every tree species has a species- and climate-dependent optimum growth

rate and growth curve. In addition the optimal growth rate of trees in elevated [CO_2] versus trees in ambient [CO_2] is very likely to be different, necessitating the calculation of separate optima for the two treatments. One of the main assumptions behind the Ingestad approach is that the nutrient concentrations of plant tissues do not change over time or in different treatments. In conclusion, applying true Ingestad nutrition in a field experiment necessitates a pilot study to determine the optimal growth rate at the site in question and in the different treatments, which was not deemed useful in the case of Glendevon since three tree species were supplied with one nutrient solution. The Glendevon nutrition system nevertheless provided one of the most sophisticated Ingestad nutrition systems in use in field experiments. In looking at the proportions of macro- and micro-nutrients calculated for Sitka spruce and applied to the whole site (compared with the requirements of Scots pine and common alder) it seems that common alder was supplied with slightly sub-optimum proportions of phosphorus, magnesium and calcium and Scots pine with slightly supra-optimum proportions of potassium (see Appendix 3 and Ingestad 1979 and 1980). The former could have had an effect on the nitrogen fixing capacity of fertilised alder trees, since phosphorus and magnesium are particularly important in nitrogen fixation. This would not have affected the non-fertilised plots, however.

CO_2 Control System

Liquid carbon dioxide was delivered under pressure as enriched gaseous carbon dioxide (at a concentration of 350 μmol mol^{-1} above background, i.e. on average 700 μmol mol^{-1}) via a mass flow system to the elevated [CO_2] chambers (numbers 2, 3, 7 and 12) (Figure 2.2). Since atmospheric [CO_2] varies diurnally, a constant amount of carbon dioxide was added to the elevated [CO_2] chambers allowing the concentration in these chambers to track the ambient atmospheric [CO_2].

Photo 21 Aerial view of the Glendevon Open-Top Chamber site, Perthshire (near Yetts O' Muckhart)

Figure 2.1 Map of the Glendevon field site showing the experimental design with four chambers per [CO_2] treatment (ambient [CO_2], elevated [CO_2], or the control plots in top right hand corner; the nutrient treatments (H = with fertiliser, L = without fertiliser) were nested within each chamber or control unit.

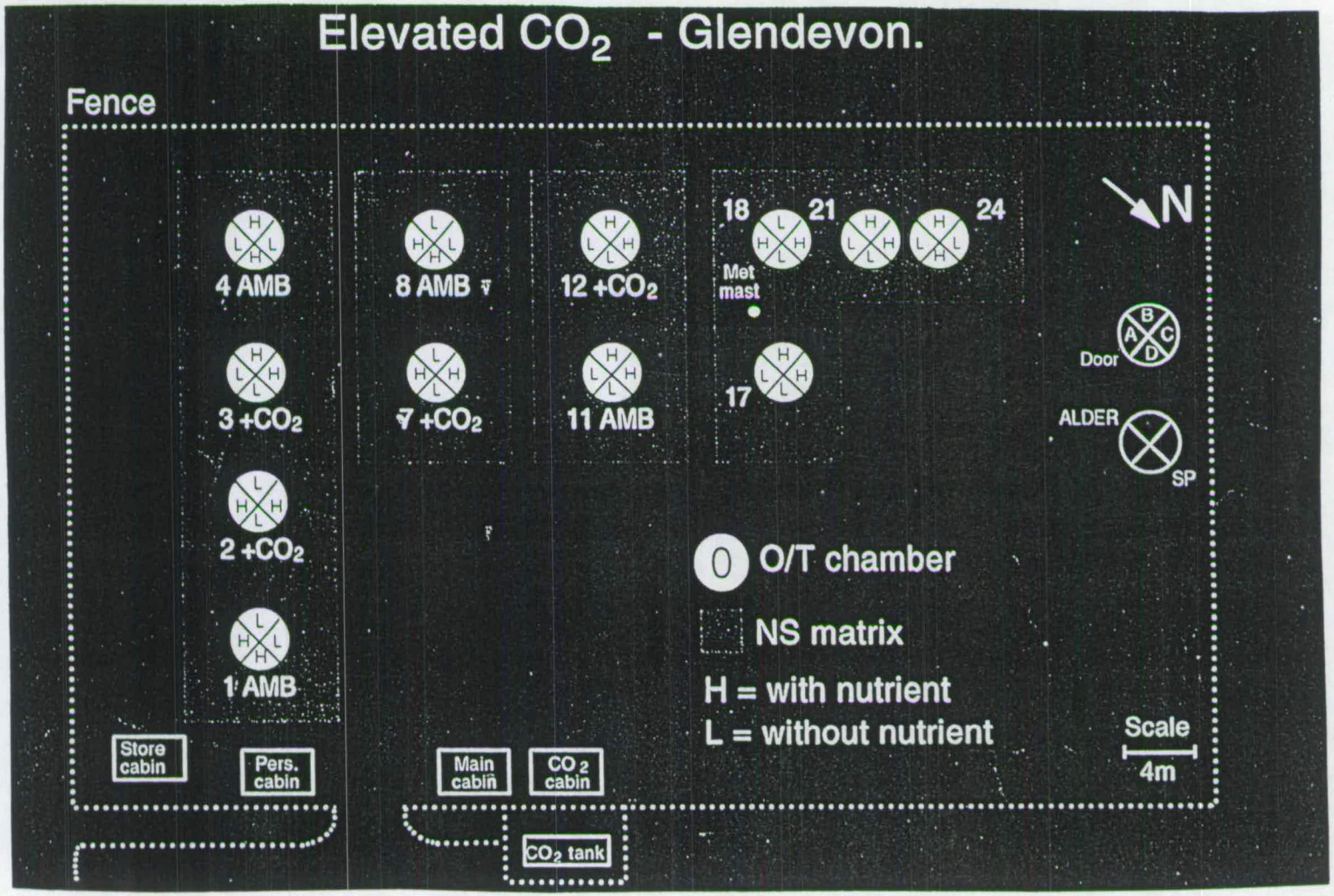

Figure 2.2 Excerpt from the Forestry Commission's Research Information Note 238: "A long-term carbon dioxide enrichment experiment examining the interaction with nutrition in Sitka spruce" (Durrant *et al.* 1993). Photo: Aerial view of an OTC containing one Sitka spruce tree per quadrant. The CO_2 monitoring and control system is depicted and described in detail at the bottom of the page.

Figure 2: View through the top of a chamber, showing barriers and removable walkways

The CO_2 monitoring and control system (Figure 3) consists of sample and injection sub-units controlled by a personal computer via an interface card (Barton *et al.*, 1993). A diaphragm pump draws air continuously from all the chambers through nylon sample lines. To allow for different flow resistances in the sample lines, each is fitted with a needle valve flow meter, enabling flow rates through the lines to be balanced. Each sample line contains a three-way solenoid valve which, when activated, diverts the air stream to the infra-red gas analyser (IRGA), through which it is drawn by the IRGA's internal pump. On switching, the IRGA rapidly stabilises (20 seconds) on a new sample line. The solenoids are controlled by the computer via a multiplexer and relays. The control program cycles through the chambers, spending one minute on each sample line. The first 35 seconds are used to flush the IRGA and to allow it to settle on the new reading. During the remaining 25 seconds a reading is taken every two seconds, and the average is logged to the hard disk and stored in the memory for graphical display. The elevated chambers have a natural diurnal cycle which is achieved, using the precision needle valves, by assuming the mass flow of air through the chamber is constant and requires a constant fixed addition of CO_2 to the airstream.

The CO_2 is supplied from a 16 tonne tank (Distillers M.G., U.K.) through a pressure reducing valve, and then through a fail-safe solenoid valve (in case of power failure). A data logger is used to record both environmental data and CO_2 concentration, and the information is transmitted via a modem to the Forestry Commission's research station at Alice Holt Lodge, Surrey.

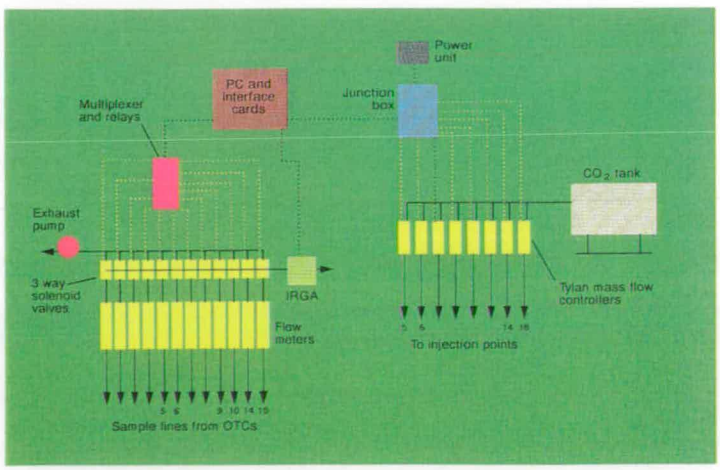

Figure 3: CO_2 monitoring and control system

Photo 2.2 View of common alder seedlings inside an OTC in May 1994 not long after the seedlings had been transplanted into the soil. Note the fertigation system.

Photo 2.3 View of Scots pine seedlings inside an OTC in May 1994 not long after the seedlings had been transplanted into the soil.

Photo 2.4 Common alder growing in July 1995 within an OTC, showing the polythene 'doughnut' through which CO_2 fumigated the chamber.

Photo 2.5 Digging up an alder root in December 1995 in a control plot. Note the plastic barrier separating the two species as well as the two nutrient treatments.

Photo 2.6 View of Glendevon open top chambers (OTC) with surrounding Sitka spruce to simulate natural forest conditions and to reduce incident infra-red light on the seedlings in the chambers or control plot.

The [CO_2] was monitored by needle valves in a control shed. One of the two valves per chamber operated at night, both during the day. If adjustment of the carbon dioxide level was required, the valves were turned to increase the [CO_2] (one turn corresponded to *c.*50 mg dm^{-3}). A frustum and a glass shelf situated 0.5 m below the frustum were incorporated at the top of the chamber to reduce external air incursion into the chamber. Air (either at ambient or elevated [CO_2]) was introduced into the chamber via a perforated polythene 'doughnut' (see Photos 2.4 and 2.6) that encircled the inside frame at a height of *c.* 1.5 m. [CO_2] in chambers was monitored using an infra-red gas analyser (IRGA) connected to a computer system (see Figure 2.2 for more details of the CO_2 monitoring and control system designed by Craig Barton, University of Edinburgh).

Nutrient Treatments

A full-strength nutrient solution (75 kg ha^{-1} N) was supplied to half the plants (a total area of 85 m2) via an irrigation / fertiliser controller (Biotronic Cultivation Systems, Uppsala, Sweden) once a day (around 5 pm to 5.30 pm) (see Appendix 3 for details of solution). Computer-control of this system allowed the programming of the first day and last day of irrigation in each growing season, the initial injection of fertiliser, and an estimation of the timing of optimum growth (using Ingestad data on optimum growth rates of Sitka spruce, see section 2.2.1) was controlled via computer. Although there were three different species of tree growing on the site only one nutrient solution, that for Sitka spruce, could be used for all three species growing at the site (see section 2.2.1 *The Chambers* for more details). The irrigation / fertiliser passed through piping and was added to the ground using irrigation nozzles (one per tree), which were checked for malfunctioning at the beginning of each season. In winter the [CO_2] supply and fertiliser system were shut off.

2.2.2 Statistical Considerations

All data were checked for homogeneity of variance (using an F-test if the replicate numbers were equal or Levene's test if not) and normality. Any data which did not meet these assumptions were log transformed. Unless otherwise specified all statistical analyses were carried out with the SAS 6.1 general linear model (glm) and the probabilities for the F values at which significance was established are referred to as * *p*

< 0.05, ** $p < 0.01$, *** $p < 0.001$. Trends towards statistical significance were reported for probabilities of $p < 0.1$ as (*).

SAS 6.1 programming for the Glendevon site

A SAS 6.11 (SAS Institute Inc., Cary, NC 27513, USA) general linear model (glm) programme was written for analysing all variables measured (see appednix 8 for details of the model used). The model used a randomised, split-plot multi-factorial (a 2*3 factorial layout) analysis of variance (SAS Sum of Squares Type III) with [CO_2] as the main plot factor. The nutrient treatments were sub-plot factors nested inside each chamber or control plot, e.g. with or without fertiliser. Control plots were incorporated into the design so as to assess the effect of the open top chambers (chamber effect) on the measurements. In addition the variance attributable to each individual unit was assessed (unit effect) This effect was only mentioned in the results section if it was significant. As mentioned in the Introduction to this chapter the experimental design was completely randomised with respect to OTCs, [CO_2], and fertiliser treatment within each OTC or control plot (see Figure 2.1). In order to take account of any gradients across the field site and since the Glendevon site was on a slope, blocks were designated at random each containing one of the four replicated chambers (each in turn containing two nested nutrient treatments within each block) (Bailey 1981). The blocking procedure was the same at both field sites used in this research (ITE and Glendevon).

Source of variation due to CO_2 treatment, nutrient treatment, [CO_2] and nutrient interaction, chamber effect and unit (i.e. chamber number) was calculated by the SAS programme. Calculation of error terms for [CO_2] and nutrient was made complicated by the nested character of the experiment. One error term related to the residuals from the comparison between [CO_2] treatments, the other related to the fertiliser treatments. With this experimental design one can either assign two error bars to each combined [CO_2] and nutrient treatment, or use the overall mean square as an error term (giving the same pooled overall variance for each treatment) for every treatment. Alternatively, one can indicate when the treatments are significantly different in the legend and give a standard error as an indication of the spread of the data. The latter choice was made in this study. Thus, although most of the tabulated data have been presented with one standard error

or 95 % confidence intervals, these should be treated with caution since they only show the variation of the combined treatment data sets (e.g. elevated [CO_2] and fertiliser) but do not show whether different treatments are significantly different. For this reason refer to ANOVA *p* values for statistical significance.

2.3 Characteristics of the Glendevon Field Site

2.3.1 Meteorological Data

The climate at the Glendevon field site at an elevation of 600 m is harsher than the surrounding lowlands of Perthshire. In this experiment the growing seasons extended from early April until early October in 1994. In 1995 common alder did not lose their leaves until December because of a mild autumn. It is important to point out that 1995 was a drier summer than 1994, with little rain throughout the growing season and high temperatures, particularly in August (see Figure 2.3b). Annual rainfall was 1586.6 mm in 1994 compared to 1073.2 mm in 1995. The unusually dry 1995 growing season probably caused the fungal infection (*Taphrina tosquinetti*) in June on the common alder leaves. Leaves were wilted by August and some leaves were lost. The following graphs, Figures 2.3a - 2.3c, give an indication of the variability in rainfall across the three years of the study.

2.3.2 Soil Water

Soil water in every quadrant in every chamber and control plot was measured by Gail Jackson and Diarmuid O'Neill in April 1996 before the fertigation system was activated, using the wet and dry mass of a soil core. Percentage moisture was calculated by dividing the difference between the wet and dry mass by the wet mass and multiplying by a hundred. There was a significant difference between percent soil water in the [CO_2] treatments and the control plots with an average of 36.72 % in the ambient [CO_2] chambers, 36.20 % in the elevated [CO_2] and 38.78 % in the control plot. The difference between the means was very significant when analysed by one-way ANOVA (p = 0.0070). There was also a significant chamber effect on soil water content when analysed for each species (p = 0.029 for Scots pine and p = 0.0043 for common alder). This highlights the key problem in trying to keep the chamber effect on the micro-environment to a minimum, namely that control of one environmental variable is often in conflict with

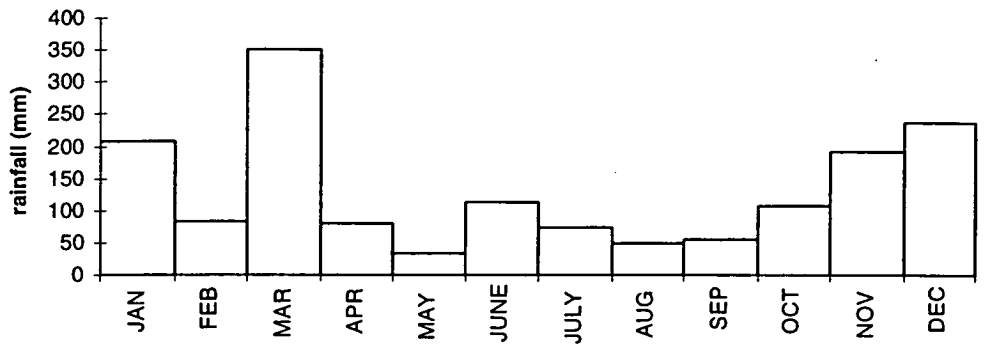

Figure 2.3a Total monthly rainfall (mm) in 1994 at the Glendevon field site. Daily measurements were made outside of the chambers.

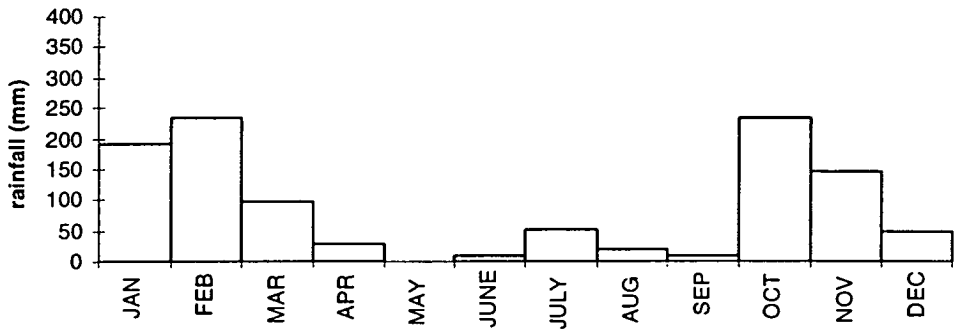

Figure 2.3b Total monthly rainfall (mm) at the Glendevon field site in 1995.

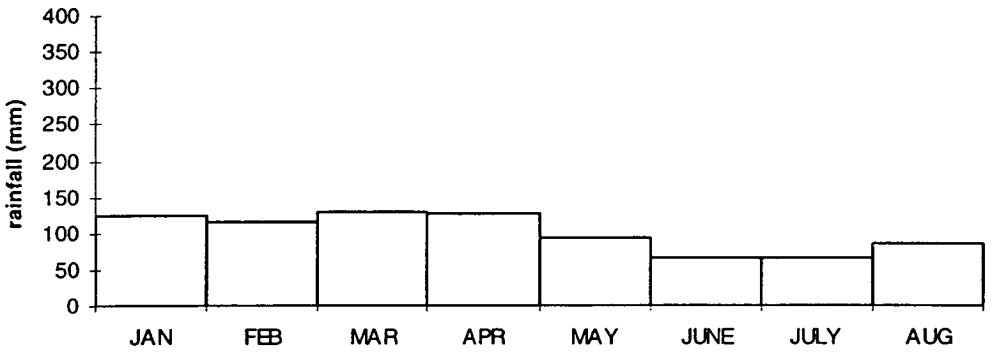

Figure 2.3c Total monthly rainfall (mm) at the Glendevon field site from January to August 1996.

controlling another (Leadley and Drake 1993). The presence of a frustum at the top of the chamber to reduce incursion of ambient air into the chamber had the disadvantage that it reduced the availability of rainfall into the chamber. The smaller stature of alder seedlings growing farthest from the middle of the chamber and nearest to the perforated

plastic 'donut' through which CO_2 entered the chamber could have been a result of less direct rainfall availability as well as lack of space.

2.3.3 Soil Profile

The soil at Glendevon is a cultivated shallow brown earth, locally podzolised. Soils of the brown earth group are divided into those of low base status and those of high base status. The Glendevon soils are of the former category with a tendency to acidity throughout the profile. The following is a detailed soil profile for the site (c /o Forestry Commission):

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0 - 18 cm	humose (7.5 YR2/2) unmottled *silt loam* ;
Ap	common small and medium subangular stones, strongly developed subangular blocky and coarse granular structure ; abundant and very fine fibrous roots ; smooth, nearly abrupt boundary.
18 - 29 cm	unmottled (7.5 YR4/4) *sandy silt loam ;*
Eb	common small and medium subangular stones; well developed medium and fine subangular blocky structure; many fine and very fine fibrous roots; smooth abrupt boundary
29 - 39 cm	unmottled (7.5 YR4/4) *silty clay loam ;*
R	common, small and medium subangular stones; well developed medium and fine subangular blocky structure with 7.5 YR3/2 ped faces; common fine and very fine fibrous roots; sharp boundary.
At 39 cm	Rock
R	

Legend -

Ap	ploughed layer of cultivated soils
Eb	brown (paler when dry) friable weakly structured horizon depleted of clay

humose	a soil containing between 7.5 and 15 % organic carbon; the prefix is given together with the textural class of the mineral fraction e.g. humose sandy loam.
small stones	1-5 cm
medium stones	5-10 cm
large stones	10-20 cm
7.5 YR 2/2	the colour of a moist soil fragment is compared with the Munsell Soil Color Charts; each colour is considered the result of *three variables* : a) the *hue* (e.g. 7.5 YR brown) indicating its relationship to the spectral colours yellow, red or blue b) the *value* - its lightness or darkness c) the chroma - the strength or departure from a neutral colour of the same value. The three variables are combined to give a notation such as 10 YR 5/6.

2.3.4 pH of the Soil

The Glendevon site has loamy, acidic soil. Table 2.1 indicates the small variation in soil pH. across the chambers used in this research. In a one-way ANOVA no statistically significant difference in soil pH was found between the [CO2] treatments (p = 0.6, sample size = 13).

Table 2.1 Summary of pH (analysed in water) within each OTC in April 1996. Values are the means of four measurements, one from each quadrant within an OTC (numbers 1-12) or control plot (numbers 17, 18, 21 and 24).

OTC number	mean pH per OTC
1	4.7
2	4.8
3	4.6
4	4.6
7	4.8
8	4.6
11	4.7
12	4.7
17	4.5
18	4.5
21	5.0
24	4.7
overall mean	**4.7**

2.4 Performance

2.4.1 CO_2 Performance

[CO_2] Performance at Glendevon

Figure 2.4 shows a representative time course of [CO_2] recorded continuously over three days in an ambient chamber (base line) and one elevated [CO_2] chamber (number 12 in Figure 2.1). [CO_2] concentrations were highest during the night time and there was considerable noise during this period in the elevated [CO_2] OTCs. [CO_2] from February to August 1995 was on average 356.0 (0.058 SE) in the ambient [CO_2] chambers. Concentrations in the elevated [CO_2] chambers were on average 688.55 μmol mol^{-1} (0.53 SE) in OTC number 2; 686.54 μmol mol^{-1} (0.62 SE) in OTC number 3; 688.61 μmol mol^{-1} (0.58 SE) in OTC number 7; and 652.59 μmol mol^{-1} (0.68 SE) in OTC number 12. On average, 70 % of the time, the [CO_2] was kept at the target value of 700 μmol $mol^{-1}CO_2$ ± 100 μmol mol^{-1}.

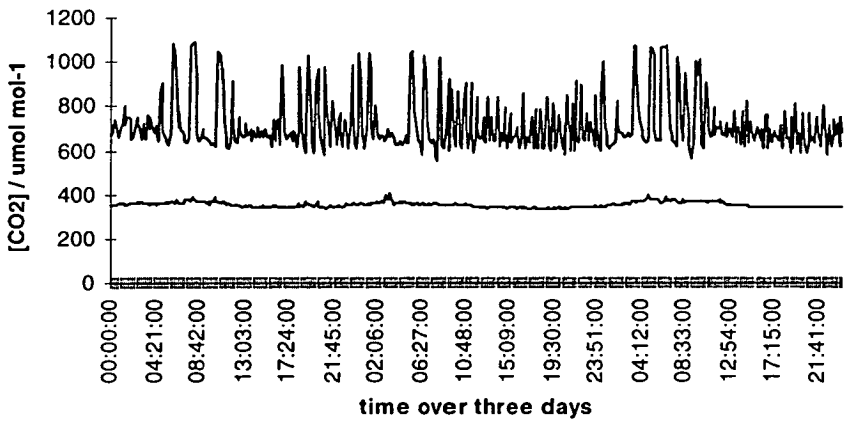


Figure2.4 Typical [CO_2] recorded over three days in July 1995 in the ambient and elevated [CO_2] chambers.

[CO_2] Performance at ITE

In summer 1994 the [CO_2] inside the elevated CO_2 chambers varied around the target concentration of 700 μmol $mol^{-1}CO_2$ by ± 80 μmol mol^{-1}.

2.4.2 Fertiliser Performance

The concentration of nitrogen in fertiliser coming through the irrigation nozzles located at each plant was monitored in June 1995 by collecting a sample of liquid overnight and

analysing for nutrient concentration in the lab (see nutrient analysis in Methods sections of Chapters 3 or 4 for details). Fertiliser samples from chambers 1, 2, 4, 8 and 12 had nitrogen concentrations of 8.82, 5.96, 3.55, 2.06 and 6.28 mg per dm^3. The variation in nitrogen concentrations could be a result of the collection beaker over-flowing or some of the fertigation nozzles may have been slightly blocked (due to an accumulation of residues and dirt, as well as disturbance within the chambers when sampling, measuring, harvesting etc.). All the nozzles were thoroughly checked for blockage at the beginning of the growing season. It is apparent that for a more even distribution of nutrients to the plants, one would have to check the nozzles for blockage more than once during a season. This was not possible in this experiment for logistical reasons. In conclusion, one can only be sure that all the nutrients diluted into the nutrient tank did end up in the appropriate quadrant, even if some parts of the quadrant (i.e. certain trees next to un-blocked nozzles) may have received slightly more than others.

Soil Nitrate and Ammonia at Glendevon

In early April 1996 Gail Jackson and Diarmuid O'Neill took two soil samples per quadrant per chamber for the determination of soil nitrate and ammonia variation within and between chambers. NO_3 Epsilon, the sum of nitrate and nitrite, was determined in water by flow-injection analysis using a colorimetricc procedure (see Application note number ASN 62-03 / 84, Perstorp Analytical, Maidenhead, Berkshire, UK). NH_4 was determined in water on a flow-injection analyser using a gas diffusion method (see Application note number ASN 50-03 / 84, Perstorp Analytical, Maidenhead, Berkshire, UK).

Results were expressed as mg / 100g of soil and analysed using the SAS glm for two-way ANOVA. There was no significant [CO_2], nutrient or interaction effect between the two on either soil ammonia or nitrate for both species ($p > 0.05$). Mean ammonia (mg / 100 g soil) in quadrants without fertiliser addition during the two previous growing seasons was 2.28 versus 2.63 mg / 100 g of soil in the quadrants supplied with fertiliser. Nitrate concentrations were 5.42 mg / 100 g of soil in non-fertilised quadrants versus 4.87 mg in fertilised plots. Since the soil samples were taken before the fertiliser system was activated at the start of the growing season, these results could be showing that after

the 1995 growing season most of the available fertiliser was either taken up into the plants or was leached from the soil during the winter.

2.4.3 Temperature and Light Performance

Glendevon

Air temperature and humidity within the OTCs was measured at a height of 1.5 m from the ground using an Ancom Combined Humidity and Temperature Probe. Outside air temperature was measured using a platinum resistance thermometer (PT 385 Delta-T devices Ltd, Burwell, Cambridge). Light was measured inside the OTCs using a tube solarimeter (Delta-T, Burwell, Cambridge) at a height of *c.* 1.5 m. Air temperature within the OTCs was generally higher than outside. The temperature inside and outside the OTCs in 1995 is shown in Figure 2.5 and the deviation from ambient temperature in 1995 is shown in Figure 2.6. On average the temperature increase in the chambers compared with the outside was 2.8 °C in 1995. Other authors have found similar increases in temperature. Although such an increase in temperature will probably affect photosynthesis, respiration and water relations and may shift the temperature optimum of photosynthesis (Long 1991), the expected increase in global air temperature as a result of rising atmospheric [CO_2] is similar to the increase caused by the chambers.

Averaged over the whole of 1995 relative humidity within the OTCs was 18 % lower than outside the OTCs. It was not possible to assess light attenuation since the outside solarimeter was overgrown by the surrounding Sitka spruce giving lower values than those in the chambers. Light attenuation is usually 10 % in OTCs (see Leadley and Drake 1993).

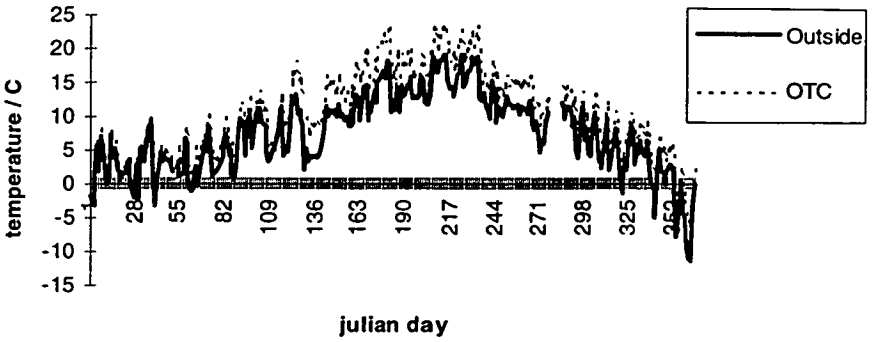


Figure 2.5 Temperature (C) inside and outside the OTCs in 1995. Each value is the mean temperature in all the chambers or outside over 24 hours.

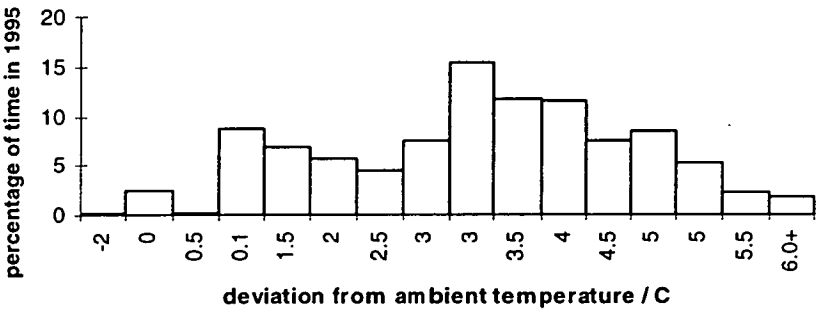


Figure 2.6 Frequency histogram of deviation from ambient temperature in OTCs from January to December 1995. Values are based on 24-hour temperature means.

ITE

Air temperature was measured inside the OTCs at a height of *c.* 1.5 m from the ground using a T351-PX sensor. Outside air temperature was measured using a platinum resistance thermometer (Delta-T devices Ltd, Burwell, Cambridge) in the vicinity of the OTCs. On average the temperature increase in the chambers compared with the outside was *c.* 2.0 °C in the summer of 1994.

2.5 Methods Not Described in Detail in Chapters

2.5.1 Grinding of Plant Samples

All sub-samples of tree tissues (mostly leaves and needles) used for biomass determination, labelled nitrogen analysis and biochemical analysis (except for Rubisco, soluble protein content and chlorophyll samples) were ground using different forms of

grinder. In order to obtain very evenly and finely ground samples for subsequent analysis, a ball-mill was used (Ball Mix, UK) with two steel ball bearings. This mill was used primarily for the ^{15}N stable isotope analysis, since this required very even and fine sub-samples. A centrifugal grinder (Retsch, Glen Creston, Stanmore, UK; 220 V, 50 Hz; mesh size 0.5 mm) was used for samples which were already relatively fine (leaves and small twigs). A large industrial grinding machine (C & N Laboratory Mill Size 8 ″, Christy and Norris Ltd., Chelmsford, England) was used for larger tissues such as whole branches and stems, and the sample subsequently re-ground in the centrifuge machine. A coffee grinder (Braun Automatic) was also occasionally used to break down larger tissues into manageable sizes.

2.5.2 Pest Incidence and Control

Green-fly were a continuous presence on alder leaves during the growing seasons, although their presence did not seem to reduce plant vigour. This cannot be said for the fungus *Taphrina tosquinetti*, a form of leaf curl (Alexopoulos 1952) which was a problem at Glendevon in 1995. In June to September 1995 it infected common alder plants in all treatments and quite a number of leaves were lost because of it. Wind dispersal across the chamber compartments and away from the control plots did not enable the measurement of lost phytomass. In order to alleviate the problem one alder tree per quadrant was removed in June 1995, and the plants were sprayed. The biomass of the removed trees was incorporated into the cumulative biomass of all trees calculated at the end of the experiment.

Pest control on the site was effected using 'Fightagrub' which contained the microscopic *Heterohabditis* nematode against vine weevil and sciarid fly, 'pp captan 80 wg' against apple, pear scab, and for the prevention of *Gloeosporium* and *Phytophthora* storage rots, or 'Murphy Mortegg' (sprayed on the alder in April 1995).

CHAPTER 3

Growth, biomass and biochemistry of *Alnus glutinosa* and *Pinus sylvestris* grown in elevated carbon dioxide concentration - a fifteen-week experiment at the Institute of Terrestrial Ecology

3.1. Introduction

In response to the convincing evidence of rising atmospheric CO_2 concentrations, $[CO_2]$, in the early nineteen eighties, the main focus in the scientific community fell on the effects of elevated $[CO_2]$ on major crop plants (Cure and Acock 1986, Kimball *et al.* 1993). Woody plants have generally been under-represented in the large number of publications on this topic until recently (Eamus and Jarvis 1989, Mousseau and Saugier 1992, Ceulemans and Mousseau 1994). Over the past twenty years experiments with both herbaceous crop and woody tree species have shown varied photosynthetic and growth responses to carbon dioxide enrichment (Eamus and Jarvis 1989, Mousseau and Saugier 1992, Kimball *et al.* 1993, Poorter 1993, Ceulemans and Mousseau 1994). Many studies have shown a capacity for increased growth, biomass production and photosynthesis as well as differing allocation of carbon and nitrogen in plants grown in elevated $[CO_2]$ conditions (Mousseau and Saugier 1992, Kimball *et al.* 1993), whereas in other experiments significant stimulation of photosynthesis, growth or biomass production was not found (Mousseau and Enoch 1989, Kimball *et al.* 1993). Often the impact of elevated $[CO_2]$ on plants is closely inter-linked with the availability of other environmental variables, such as nitrogen, phosphorus, photosynthetic photon flux density (PPFD), temperature or water. The intricacies of these interactions need to be elucidated in order to have a better grasp of the mechanisms involved in response to elevated $[CO_2]$. Another complicating factor is that responses often appear to be species-specific, with some species exhibiting a large response and others barely any. Different species seem to have different limits to their plasticity such as size constraints which may be defined by their genetic makeup (determining the way in which they can grow) and by the environmental conditions.

The question arises whether species that grow relatively quickly under optimum conditions respond more strongly than those with lower maximum relative growth

rates (Poorter 1993)? This question will be addressed in this study which is a comparison between two native trees with quite different developmental and growth patterns. Common alder (*Alnus glutinosa* (L.) Gaertn) is a deciduous pioneer species which can fix nitrogen from the atmosphere with the help of *Frankia* actinomycetes within root nodules. It is fast-growing and hardy but not a dominant forest species, and its shape and size are greatly affected by its habitat (Fife 1994). The alder has the capacity for indeterminate growth. This can be seen in its plasticity of growth in varying habitats. It can survive on wasteland or heathland with nutrient limited soils, as well as thriving on river banks often submerged in water. It is more restricted in its range than its cousin the birch (*Betula* spp), however and does not grow over as wide a range of altitudes and latitudes as Scots pine. Common alder is contrasted with a slower-growing, mycorrhizal conifer, Scots pine (*Pinus sylvestris* L.), a dominant forest species. Scots pine has a fibrous, ectomycorrhizal root system enabling it to tap nutrients very well and it can tolerate a range of soils (acid and alkaline). It has determinate growth, but can have second flushes of (lammas) growth in new needles in the second half of the growing season.

Previous short-term studies of the effects of elevated [CO_2] on alder seedlings suggested that nodulated plants had larger whole plant photosynthesis, nitrogenase activity, nitrogen content and total biomass compared to nodulated plants grown in ambient [CO_2], or compared to non-nodulated plants grown in both carbon dioxide concentrations (Norby 1987, Arnone and Gordon 1990). One possibility is that nitrogen-fixing trees grown in elevated carbon dioxide are able to export extra photosynthate (sugars) to increase nodulation of roots and so be more independent of external nitrogen additions. While the Scots pine will not have this advantage, being evergreen, it can continue to fix carbon throughout the year when environmental conditions are suitable.

Intraspecific variation has also been shown in some studies (Poorter 1993). This can sometimes be explained by the use of different seed provenances or clones, as well as different aged trees, ranging from seedling to mature (Lee and Jarvis 1996).The extent to which this is a product of varied methodologies (i.e. some plants grown for

longer or shorter periods than others, some grown in inadequate rooting medium or constricting pots) needs to be better understood. Although this is not directly relevant to this research, it is a point to be kept in mind when drawing conclusions from different authors' work scaling up to whole populations and stands.

It is becoming increasingly clear that any particular response to elevated [CO_2] in a species is often time-dependent, with negative feedback inducing a reduction in photosynthetic rate after a certain time period, by mechanisms that remain to be found (Mousseau and Saugier 1992). Photosynthetic acclimation has been associated with accumulation of carbohydrates because of low sink demand and / or reduction in Rubisco amount or activity (Stitt 1991). This longer-term acclimation effect of carbon dioxide enrichment varies in its time of onset in different plant species and in different clones. In addition, McConnaughay *et al.* (1993) found that the response to carbon dioxide can be further affected by growth in small pots. Although pots could not be avoided in this fifteen-week study, the longer term experiment used seedlings directly planted into the ground. The following short-term experiment had as an overall aim to investigate how elevated [CO_2] and nitrogen supply affects the growth, physiology and biochemistry of *Alnus glutinosa* and *Pinus sylvestris.*

3.1.1 Hypotheses and Particular Aims

The hypothesis is that common alder, by fixing nitrogen will be able to take advantage of the extra carbon assimilated in elevated [CO_2] conditions and increase its growth, biomass and photosynthetic efficiency. In addition, nitrogen limitation and nitrogen dilution resulting from the extra carbon is not expected to occur.

The Scots pine, on the other hand, after an initial increase in growth in elevated [CO_2] may develop a source-sink imbalance in which excess starch and other carbohydrates induce a dilution of the nitrogen content leading to negative feedback with the result that particularly those trees without fertiliser addition will show no significant differences in growth, physiology and biochemistry compared to those grown in ambient [CO_2]. An accumulation of starch and soluble sugars is likely to be visible during the peak growth period, but will taper off towards the end of the fifteen weeks.

The particular aims of this experiment were to elucidate :

1) whether one-year old Scots pine and common alder seedlings grown in elevated [CO_2] over a fifteen-week time span would respond by increased growth and biomass, or changes to their photosynthetic system compared to those grown in ambient [CO_2];
2) whether the allocation of carbon would differ between the treatments, since trees grown in elevated carbon dioxide have been found to be ontogenetically more advanced than those grown in ambient conditions;
3) whether there would be any interactive effects of the environmental variables carbon dioxide and nitrogen on growth, physiology and biochemistry of the two species; and
4) whether the nitrogen-fixing alder and the pine would respond differently to increased availability of carbon and possible concomitant dilution of nitrogen (creating a source-sink imbalance).

3.2 Materials and Methods

3.2.1 Site Description

The open top chamber (OTC) site at the Institute of Terrestrial Ecology, Bush Estate, Penicuik, near Edinburgh, previously used for pollution studies, was set up for elevated carbon dioxide research. The site is situated at a latitude of 55 ° 51' N, an altitude of 198 m and is surrounded by the Pentland Hills. Average yearly rainfall is 1000 mm.

3.2.2 Experimental Design

The study was a fifteen-week experiment from 11 April 1994 until the end of July 1994. Eighty-eight one-year-old common alder seedlings and eighty-five Scots pine seedlings were grown for a year in either elevated carbon dioxide concentration (ambient + 350 μmol mol^{-1}) or ambient carbon dioxide concentration greenhouses (*c*.350 μmol mol^{-1} CO_2) at the Forestry Commission's Southern Research Station (SRS) in Alice Holt, Farnham, Surrey. The trees were transplanted into 4 dm^3 pots : the alder in a mixture of peat, perlite and bark in a 1 : 1 : 1 ratio by volume; the pine in a mixture of peat, perlite and sand (2 : 1 : 1 ratio by volume), and grown in eight

open-top chambers (OTC), four in elevated (ambient [CO_2] + 350 μmol mol^{-1}) and four in ambient (*c.*350 μmol mol^{-1}) [CO_2] and an outside plot. Since carbon dioxide concentrations vary diurnally, a constant amount of carbon dioxide was added to the CO_2-enriched chambers allowing the concentration in the elevated [CO_2] chambers to track that in the ambient chambers and control plot. For details of the CO_2 control system and performance see Chapter 2 Materials and Methods. The chambers were constructed in an octagonal form, from lightweight aluminium frames containing 3 mm horticultural glass with an open top, and had a ground area of 7 m^2, a height of 2.3 m and a sliding door for access. There were seven or eight individuals of each species in each OTC (see Photo 3.1 and Photo 3.2). An irrigation system with a nozzle for each individual plant was set up in each OTC at the beginning of the season.

The experiment was a multi-factorial experiment (2 × 3 × 2) with four chamber replicates, two species, three carbon dioxide-chamber treatments (elevated [CO_2] chambers, ambient [CO_2] chambers and a control plot), and two nitrogen treatments (low nitrogen and high nitrogen). This gives an experiment with two environmental factors, the first being 'CO_2' with two 'levels', the second 'fertiliser' with two 'levels'. Each species was analysed separately (statistically) but impacts of carbon dioxide and nutrition were compared between species as well as between treatments (see section 3.2.4 on Statistical Analysis).

3.2.3 Experimental Procedure

General Overview

All seedling were regularly supplied with water and fed weekly with nutrients (see sub-section on Nutrient Addition below). The saplings were placed inside the chambers along with trees from other experiments, namely three-year old beech (*Fagus sylvatica*) and two-year old Sitka spruce (*Picea sitchensis*). This could potentially have affected the experiment since the other saplings were larger, and thus slightly overshadowed the species in this experiment (see Photo 3.2 showing *Alnus glutinosa* situated next to *Picea sitchensis*).

Photo 3.1 ***Pinus sylvestris*** **seedlings inside an OTC at the start of the fifteen-week experiment. The seedlings were approximately 10 cm in height.**

Photo 3.2 ***Alnus glutinosa*** **seedlings inside an OTC at the start of the fifteen-week experiment. The seedlings were approximately 30 cm in height.**

Nutrient Addition

A nutrient solution developed for silver birch was considered satisfactory for the common alder, since common alder and silver birch have similar growth characteristics (Ingestad 1981). Half of the trees in each chamber were fed weekly with a full nutrient solution (macronutrients and micronutrients) (Ingestad and Lund, 1986), see Appendix 1. The other half of the seedlings (low N) were given the full nutrient treatment except for nitrogen: ammonium nitrate, the main nitrogen-contributing compound, was omitted from the solution (see Appendix 1). The Scots pine seedlings received the same nutrient solutions.

Harvests, Growth and Sampling

Trees were harvested at the beginning (initial harvest) and end of the experiment (final harvest) for growth and biochemical analyses. In the initial harvest (using trees which had not been inside chambers) the height, basal diameter, leaf number, branch number, and fresh mass (M_f) and dry mass (M_d) were measured of 12 pine (six grown in elevated and six in ambient [CO_2], see Section 3.2.2) and 12 alder (six in elevated, six in ambient [CO_2]). At the final harvest the 72 pines and 74 alder were destructively harvested; in addition to the measurements made at the initial harvest, biomass was separated into root, stem, leaves, and branches, oven dried at 80 °C and dry mass and total leaf area were measured. At both harvests sub-samples of the oven-dried leaves and current-year needles were collected for nutrient analysis and ground in a centrifugal grinder (Retsch, Glen Creston, Stanmore, UK; mesh size 0.5 mm). For leaf nutrient analysis in alder at the final harvest four samples per [CO_2] treatment per OTC and two per nutrient treatment per OTC were taken, although no samples from the control trees were taken. No Scots pine needles were analysed at the final harvest because the samples were mouldy. Heights of all trees were measured weekly throughout the experiment. Leaf area was measured using a leaf area meter (Li-300, Li-Cor Inc., Lincoln, Nebraska, USA). Measuring Scots pine needle area with the leaf area meter proved rather inaccurate, therefore the pine leaf area data and their derivatives need to be seen primarily as a comparison and not as absolute data.

During week eight all the trees were sampled for chlorophyll, Rubisco and soluble

protein analysis. Six trees per [CO_2] treatment per OTC and three per nutrient treatment per OTC per species were sampled by removing three leaf discs 0.42 cm in diameter from the 2nd leaf from the top in alder, and three current-year needles *c.* 5 cm from the top of the leader in pine as close to midday as possible.

Biochemical Analyses

Sampling at the initial harvest involved taking enough leaves *c.* 2 cm from the top of the seedlings to fill a 25 cm^3 Universal sampling vial (for carbohydrates) or three leaf discs or needles (for chlorophyll, Rubisco and soluble protein content) giving six [CO_2] treatment samples per OTC per species and three per nutrient treatment per OTC. At subsequent sampling or at the final harvest two samples from each OTC per [CO_2] treatment and one sample per nutrient treatment per OTC were sampled per species, giving a total of 24 trees per species per analysis. An exception to this was the soluble carbohydrate analysis for which two samples were pooled to make one, because of lack of sufficient plant material.

Soluble carbohydrates were measured by anion exchange chromatography with pulsed amperometric detection (Dionex Corp., Sunnyvale, CA 94088, USA) using a Carbopak PA carbohydrate column (see Appendix 5 for details of integration parameters used to calculate peak area). 50 mg (± 1 mg) of freeze-dried and ground leaves in 10 cm^3 of double distilled water were extracted once by shaking and placing in a 30 °C water bath for 15 minutes. After centrifuging at 4500 rpm for 15 minutes, the supernatant was removed, and filtered (0.2 µm nitro-cellulose membrane filters, Maidstone, England. The retrieved solution was diluted 1 :10 and then analysed by ion chromatography using a Carbopak PA carbohydrate column (Ion Chromatography System hplc, DX500; see Appendix 5 for details of integration parameters used to calculate peak area). Each sample was run for one hour through the Dionex machine according to the following protocol: prior to sample injection the machine was washed with double distilled water, then 200 mmol dm^{-3} NaOH (100 % of the eluent) was injected into the system for the first 15 minutes of the 1 hour run (per sample), followed by 16 mmol dm^{-3} NaOH (i.e. 8 % of the eluent) and double distilled water (92 % of eluent) for the last 45 minutes. This method was used on all the samples

analysed. A set of ten pure sugars and polyols (inositol, manitol, fucose, rhamnose, arabinose, galactose, glucose, xylose, fructose and sucrose) was run as a standard with each set of samples.

Starch was analysed in perchloric acid extracts by the iodine method (Allen 1989) or in ethanol extracts by the enzyme method using amyloglucosidase (AGS) which splits the starch into D-glucose, followed by glucose phosphorylation and then the production of NADPH upon reaction with NADP (measured colorimetrically using a spectrophotometer) (Böhringer Mannheim 1994). Chlorophyll was analysed spectrophotometrically (Series 2, Cecil Grating, Cambridge, UK) in dimethyl formamide (DMF) according to the equations and extinction coefficients of Porra *et al.* (1989). Nutrient samples from the initial harvest (root and leaf samples) were analysed by the Forestry Commission at Alice Holt, using the same methods as outlined below for the samples analysed in Edinburgh.

Nutrient concentrations were determined using an acid digest (Allen, 1974). Prior to analysis, an acid oxidation was necessary in order to oxidise the organic matter. The samples were first oven-dried at 80 °C for 48 hours, ground and then kept in desiccators until analysis. 2 cm^3 of 18 µmol mol^{-1} boiling sulphuric acid were added to 100 (±10) mg of the ground sample, and 100 vol (2 × 0.75 cm^3) hydrogen peroxide added carefully and shaken. The sample tubes were then heated at 320 °C for six hours, cooled and transferred to 100 cm^3 volumetric flasks. 0.5 cm3 of 10 % lanthanum solution was added and flasks adjusted to correct volume. Nitrogen concentration was determined by a gas diffusion method using a flow injection analyser (Fiastar, Tecator Ltd, Wilsonville, OR, USA). Phosphorus concentration was determined using a molybdenum blue method, using a flow injection analyser (Fiastar, Tecator Ltd, Wilsonville, OR, USA). Potassium, calcium and magnesium were determined by flame emission spectroscopy using an atomic absorption spectrometer (Unicam 919, Cambridge, UK). The values obtained for the samples were compared with standards of known concentration (of 20, 40, 60 mg dm^{-3} Ca ; 5, 10, 15 mg dm^{-3} Mg ; 10, 20, 40 mg dm^{-3} K). Each sample was measured three times, and a relative standard deviation calculated to trace large inaccuracies. Based on 100 mg of dried

plant material made up to a fixed volume of 100 cm^3, the concentration of nutrients (mg g^{-1}) was calculated as follows:

$$c\ v\ /\ m \qquad (3.1)$$

where c = measured concentration of sample (mg dm^{-3}),
v = volume used (0.05 dm^3), and
m = mass of sample (0.1 g).

Rubisco and soluble protein content analysis was not possible for this short-term study because of a freezer shutdown which destroyed the samples. See Chapter 5 for Rubisco and soluble protein concentration results of the long-term experiment.

Hypothesis Testing

The methods outlined above were used to test the hypotheses in Section 3.1.2. Stem height, stem basal diameter, leaf area and biomass data were used to test the hypothesis that those seedlings grown in elevated atmospheric [CO2] will grow bigger than those in ambient conditions and show differing patterns of carbon allocation. Chlorophyll contents were used to assess any changes in the photosynthetic capacity of the leaves or needles. Nutrient content data were used to assess whether a nitrogen dilution effect took place, and carbohydrate contents (starch and other carbohydrates) were determined to see if a source-sink imbalance occurred.

3.2.4 Statistical Analysis

Data were analysed either using a Student's t-test (for data from the initial harvest where no nutrient treatments only carbon dioxide had been applied) or a two-way analysis of variance (ANOVA) using the general linear model (glm) in SAS 6.11 (SAS Institute Inc., Cary, NC 27513, USA). Unless otherwise specified all statistical probabilities relate to two-way ANOVA . Factors in this analysis were carbon dioxide concentration (350 µmol mol^{-1} CO_2 versus 700 µmol mol^{-1} CO_2), nutrient availability (no-fertiliser versus fertiliser) and chamber presence (no chamber versus chamber). The number of chamber replicates was four (n = 4). For further statistical details see

section 2.2.2 or Appendix 8.

3.3 Growth and Biomass Results

3.3.1 Stem Height, Growth Rate per Day and Stem Basal Diameter

Stem Height

Scots pine and common alder stem heights responded slightly differently to elevated [CO_2]. In the Scots pine exposure to elevated [CO_2] did not significantly increase stem height ($p > 0.05$, sample size = 30) (see Figure 3.1a). Differences in mean height in the nitrogen treatments although not statistically significant ($p > 0.05$, sample size = 15) show fluctuating means across the season with a general tendency towards the ambient / low nitrogen (A - N) and the elevated / low nitrogen (E - N) treatment trees growing taller than the rest, (see Figure 3.1b).

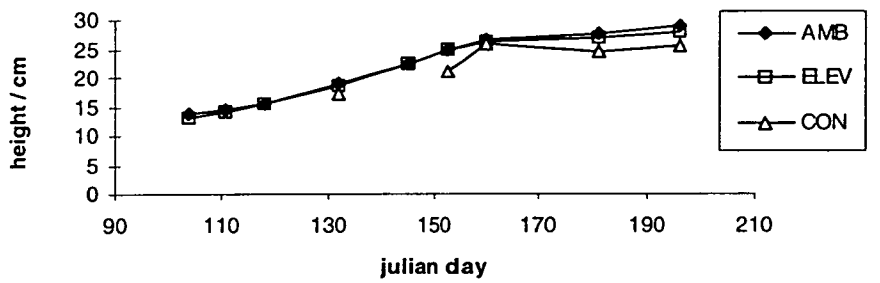


Figure 3.1a Seasonal course of mean stem height in Scots pine over the fifteen-week experiment. Values are the means of 30 measurements. AMB = ambient [CO_2], ELEV = elevated [CO_2], CON = control plot. There were no significant differences between treatments ($p > 0.1$).

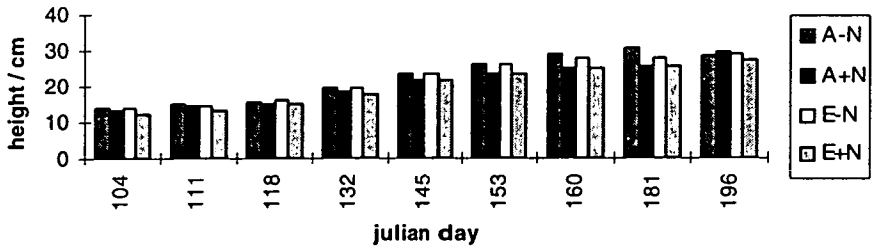


Figure 3.1b Seasonal course of mean stem height in Scots pine over the fifteen-week experiment (showing all treatments except control). Values are the means of 15 measurements.
A-N = ambient [CO_2] low N, A+N = ambient high N; E-N = elevated [CO_2] low N, E+N = elevated high N. There were no statistically significant nutrient or [CO_2] effects ($p > 0.1$).

The common alder on the other hand showed an initial CO_2 effect, albeit in taller stem heights in the ambient not elevated [CO_2] seedlings in the first four to five weeks but which then seems to have cancelled out in the last ten weeks of the study (see Appendix 5 for full ANOVA details, and Figure 3.2a). A nutrient effect was only found in the common alder in weeks one and three (see Appendix 3). There is a general trend towards the 'ambient plus nitrogen' (A + N) alder seedlings reaching the tallest height of all the treatments, followed by A-N and then the elevated nitrogen treatments (see Figure 3.2b). Thus the hypothesis that common alder seedlings grown in elevated [CO_2] conditions without fertiliser (i.e. stimulating nitrogen fixation) would grow taller than those in other treatments was rejected.

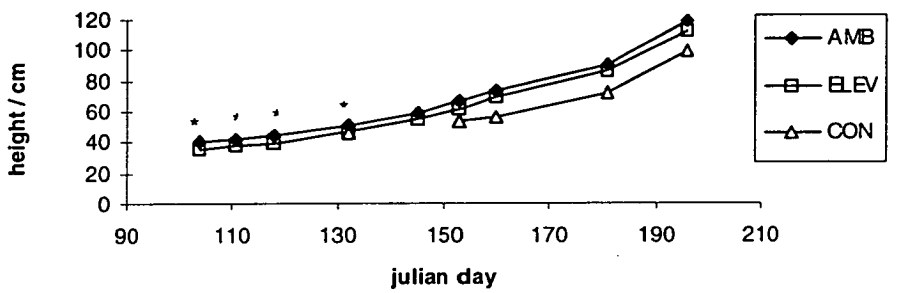


Figure 3.2a Seasonal course of mean stem height in common alder over the fifteen-week experiment. Values are the means of 30 replicates. AMB = ambient [CO_2] low and high N, ELEV = elevated [CO_2] low and high N. AMB means were significantly higher than elevated in the first five weeks (* $p < 0.05$).

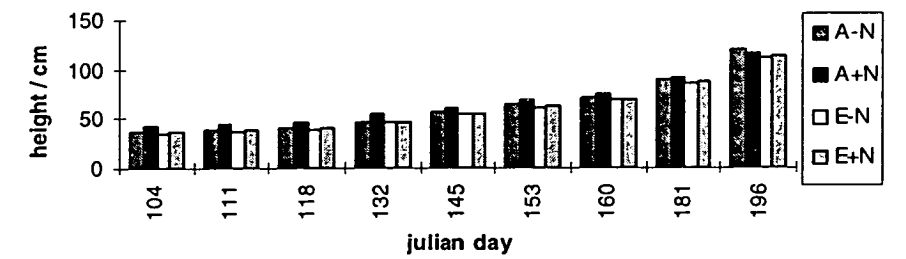


Figure 3.2b Seasonal course of mean stem height in common alder over the fifteen-week experiment (showing all treatments except control). Values are the means of 15 replicates. A-N = ambient [CO_2] low N, A+N = ambient high N; E-N = elevated [CO_2] low N, E+N = elevated high N. A significant nutrient effect was found on julian days 104 and 118 ($p < 0.05$).

An increasing chamber effect in the alder occurred throughout the experiment ($0.05 > p > 0.001$) (see Appendix 3). Although the chamber effect was not statistically significant in the pine ($p > 0.05$), one can see in Figure 3.1 that such an

effect was clearly also occurring. Thus any differences in stem height in this short-term experiment were primarily between the control plot trees of both species and those grown in chambers.

Growth Rate Per Day

The growth rate data for both common alder and Scots pine (Table 3.1) refute the hypothesis that the elevated-grown alder would have a larger advantage over the ambient-grown seedlings than the elevated [CO_2] pine trees.

Table 3.1 Height growth rate per day (cm) of both common alder and Scots pine over the fifteen-week experiment, measured by calculating the difference in mean stem height (cm) between the start and the end of the experiment and dividing by the number of days in which this stem increase occurred.

Treatment	common alder cm day^{-1}	Scots pine cm day^{-1}
Ambient	0.821	0.161
Elevated	0.815	0.159
Outside	0.583	0.090

Stem Basal Diameter (SBD)

In the common alder exposure to elevated [CO_2] did not significantly increase SBD either at the beginning or at the end of the experiment, with initial harvest SBD means in ambient [CO_2] of 8 mm ± 1.71 95 % confidence interval (CI) and of 7.36 mm ± 0.84 CI in elevated [CO_2] (t-test, $p > 0.05$, $n = 6$). The elevated [CO_2] seedlings had higher mean SBD values than the ambient [CO_2] at the final harvest ($p = 0.07$) (see Table 3.2). The difference was not significant at $p < 0.05$ but was at $p < 0.1$ which indicates a trend towards larger SBD in the elevated [CO_2] treatment. Over the 95 days of the experiment, the ambient [CO_2] seedlings increased in mean basal diameter by 0.074 mm day^{-1}, whereas the elevated [CO_2] seedlings increased by 0.100 mm day^{-2}.

Table 3.2 Alder mean stem basal diameters (plus 95 % confidence intervals) at the final harvest showing all treatment combinations. Means were derived from 25 measures for the ambient versus elevated [CO_2] comparison and 11 measures for the CO_2 plus nutrient treatments. Each measure consisted of the mean of two measurements at the base of the stem 2 cm above the lateral roots.

Treatment (mm)	Mean	(95 % confidence interval)
Ambient	15.33	(1.45)
Elevated	17.28	(1.54)
Outside	14.19	(0.79)
A-N	16.94	(1.87)
A+N	14.08	(2.07)
E-N	17.10	(2.63)
E+N	17.45	(1.96)
C-N	14.25	(1.39)
C+N	14.10	(1.01)

In the Scots pine stem basal diameter was significantly different in ambient and elevated [CO_2] seedlings at the initial harvest with an ambient [CO_2] mean of 3.71 ± 0.55 95 % CI and an elevated [CO_2] mean of 4.64 ± 0.25 95 % CI (t-test, $p < 0.01$, sample size = 6). Differences in stem basal diameter were also significant at the end of the period ($p = 0.01$, sample size = 24)(see Table 3.3). There was no nutrient effect ($p > 0.05$). Overall, these data do not confirm the hypothesis that 'elevated [CO_2] plus nitrogen' pine seedlings grow bigger than seedlings in other treatments at first, followed by acclimation to elevated [CO_2]. The difference in SBD between seedlings in ambient and elevated [CO_2] seems to have been significant throughout the experiment not just at the beginning.

Table 3.3 Pine mean stem basal diameters (SBD) at the final harvest showing all treatment combinations. Means were derived from 25 measures for the ambient versus elevated [CO_2] comparison and 11 measures for the [CO_2] plus nutrient treatments. Each measure consisted of the mean of two measurements at the base of the stem.

Treatment	Mean (mm)	(95 % confidence interval)
Ambient	5.14	(0.43)
Elevated	6.49	(0.86)
Control	6.29	(0.58)
A-N	5.12	(0.66)
A+N	5.15	(0.65)
E-N	7.17	(1.45)
E+N	5.75	(0.90)
C-N	6.03	(0.79)
C+N	6.57	(0.97)

A chamber effect was present in both species at the final harvest. Seedlings of both species grown in the control plot had significantly lower SBD values than those grown inside the chambers (common alder $p = 0.017$; Scots pine $p < 0.05$). This indicates that not only did the concentration of CO_2 increase the basal diameter of the stems in both species, but also the presence of the glass chamber.

3.3.2 Total Biomass

There was no significant difference in total biomass of either Scots pine or common alder between ambient and elevated [CO_2] seedlings either at the beginning or at the end of the fifteen-week study (t-test, $p > 0.1$ for initial harvest ; $p < 0.1$ for final harvest data). In the alder, despite the lack of statistical significance, there was a 20 % increase in total phytomass in elevated [CO_2] compared to ambient [CO_2]. There was no nutrient effect ($p < 0.05$). The A-N treatment mean was very similar to the elevated [CO_2] treatment means and this may explain the lack of statistical

significance between the nitrogen treatments.

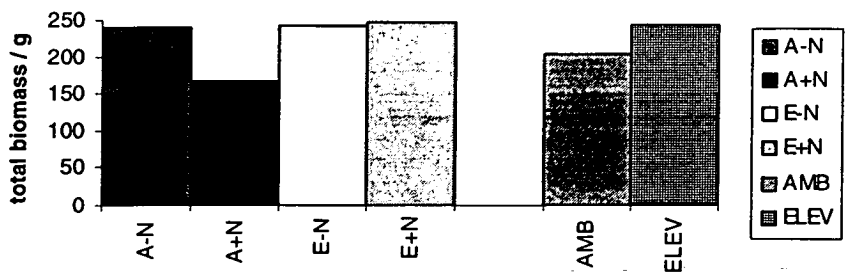


Figure 3.3 Mean total biomass of common alder at the final harvest after fifteen-week exposure to elevated [CO_2]. AMB = ambient [CO_2] low and high N, ELEV = elevated [CO_2] low and high N, n = 16; A-N = ambient low N, A+N = ambient high N, E-N = elevated low N, E+N = elevated high N, n = 8. There were no significant differences between treatments ($p > 0.1$).

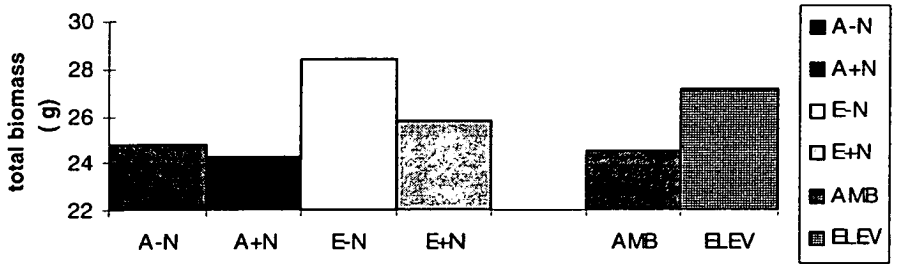


Figure 3.4 Mean total biomass of Scots pine at the final harvest after fifteen week exposure to elevated [CO_2]. AMB = ambient [CO_2] low and high N, ELEV = elevated [CO_2] low and high N, n = 16; n = 8 for combined [CO_2] and nutrient treatments. There were no significant differences between treatments ($p > 0.1$).

In the case of Scots pine, the increase in total biomass of elevated [CO_2] seedlings compared with ambient [CO_2] seedlings was 10.5 %. There was no significant [CO_2] or nutrient effect ($p > 0.05$) (see Figure 3.4). Mean biomass of the E-N treatment was the largest, and this does not support the hypothesis that Scots pine seedlings growing in elevated plus fertiliser would grow best (see Section 3.1.2). Since nutrients were carefully measured and added by hand, the possibility that the fertilised plants did not have access to the nutrients is unlikely.

There was no significant chamber effect in the Scots pine ($p > 0.05$) despite there being 20 % more biomass in elevated [CO_2] compared with control seedlings. In contrast the increase from control to elevated [CO_2] trees in common alder was a

considerable 77 % and was significant at $p < 0.06$ (ANOVA on log-transformed data, chamber effect, $p = 0.058$).

3.3.3 Allocation

Biomass Allocation

At the final harvest most of the biomass in common alder was allocated to the leaves (*c.* 40 %), with c. 20 % in the branches, 30 % in the stem and 10 % in the roots. This was true for all treatments since allocation was not affected by elevated [CO_2] ($p > 0.1$) (see Table 3.4). There was a slight tendency however, towards a [CO_2] effect on leaf fraction (LMR) at the final harvest ($p = 0.11$). Nutrient addition had a significant effect on LMR at the final harvest ($p = 0.0032$). For root and leaf fractions there was an interaction effect between [CO_2] and nutrients in alder ($p = 0.019$ and 0.079, respectively).

Looking at the raw data behind these ratios (dry mass per tissue, see Appendix 4) there is a trend towards leaves and roots in elevated [CO_2] growing larger than those in ambient [CO_2] ($p < 0.1$). Since there were only two harvests it was not possible in this study to look at ontogenetic changes in dry mass allocation in trees of the same mass compared to the above analysis using trees harvested at the same time (see Chapter 4 for details of this type of analysis of ontogenetic changes and their effects on specific growth rate).

Allocation of dry mass in Scots pine was also not affected by elevated [CO_2] ($p > 0.1$) except in the roots ($p = 0.060$) where there was a tendency towards more mass being allocated below ground (see Table 3.5). There was a significant nutrient effect only on needle fraction (or leaf mass ratio, LMR) ($p = 0.011$).There was also an interaction effect between [CO_2] and nutrients on needle allocation ($p = 0.031$) but not on any other tissues. Nutrient addition thus had a positive effect on needle fractions, with high N seedlings allocating more carbon to needles then low N seedlings. Data of dry mass per tissue (see Appendix 4) show that pine followed a similar pattern to common alder, with a tendency towards seedlings in elevated [CO_2] having more mass in the leaves and the roots than in ambient [CO_2]. In common alder there was no chamber

effect on any tissues, whereas in Scots pine there was a chamber effect on leaf mass (p = 0.0023) only.

Table 3.4 Biomass allocation at the final harvest in common alder, shown as plant tissue mass to total biomass ratios. Total plant biomass is also shown (g). Data are the means of 14 measurements for [CO_2] treatments and 7 measurements for nutrient and [CO_2] treatments.

Treatment	Total Mass	Leaf Fraction	Branch Fraction	Stem Fraction	Root Fraction
Ambient	206.01	0.39	0.18	0.32	0.11
Elevated	245.31	0.41	0.16	0.30	0.13
Outside	139.63	0.41	0.17	0.31	0.12
A-N	239.29	0.41	0.19	0.31	0.94
A+N	167.97	0.37	0.16	0.34	0.14
E-N	240.74	0.39	0.17	0.30	0.14
E+N	250.53	0.44	0.15	0.29	0.12
C-N	153.48	0.41	0.18	0.30	0.12
C+N	121.83	0.41	0.16	0.32	0.12

Table 3.5 Biomass allocation at the final harvest in Scots pine, shown as plant tissue mass to total biomass ratios. Total plant biomass is also shown (g). Data are the means of 14 measurements for [CO_2] treatments and 7 measurements for nutrient and [CO_2] treatments; * = [CO_2] effect significant at p < 0.05; (*) = [CO_2] effect significant at p < 0.1.

Treatment	Total Mass	Needle Fraction	Branch Fraction	Stem Fraction	Root Fraction
Ambient	24.52	0.54	0.11	0.24	0.10
Elevated	26.81	0.53	0.11	0.22	0.14 (*)
Outside	22.76	0.49	0.13	0.27	0.12
A-N	24.76	0.52	0.12	0.26	0.10
A+N	24.27	0.57	0.11	0.23	0.10
E-N	28.43	0.52	0.10	0.23	0.14
E+N	25.18	0.54	0.12	0.21	0.13
C-N	20.00	0.49	0.13	0.26	0.12
C+N	25.52	0.48	0.12	0.28	0.12

Root to Shoot Mass Ratio(R / S)

At the initial harvest the root to shoot mass ratios (*R / S*) of ambient and elevated [CO_2]-grown seedlings were not significantly different (t-test, $p > 0.05$) in either common alder or Scots pine. The mean alder *R / S* ratios (and confidence intervals in brackets) of 0.71 (0.21) in ambient and 0.70 (0.127) in elevated [CO_2] seedlings show that the one-year-old seedlings were investing most of their carbon into their roots. The *R / S* ratios in Scots pine on the other hand, in ambient [CO_2] with a mean of 0.30 (0.086) and an elevated mean of 0.31 (0.091) show that approximately one third of the resources were being invested into their roots in both treatments. This difference between common alder and Scots pine highlights their very different initial allocation and growth strategies. After 15 weeks there was no statistically significant effect of [CO_2] on alder *R / S* ratio (Table 3.6). An interaction effect of [CO_2] and nutrients was almost significant however. The combined effect of nutrients and [CO_2] on the allocation of carbon to roots in this case is difficult to interpret (see Table 3.5) since there was no clear nutrient or carbon dioxide effect (i.e. the A + N and the E - N seedlings had the largest allocations of resources to the roots). For root to leaf ratio (*R / L*) see Chapter 4 Glendevon data.

In the Scots pine at the final harvest the *R / S* ratio of the ambient and elevated [CO_2] trees were significantly different at $p < 0.06$ if not at $p < 0.05$ (see Table 3.7). Otherwise there were no significant differences in *R / S* ratios at the final harvest (see Table 3.7 below for details).

Table 3.6 Common alder and Scots pine mean root to shoot mass ratios at the final harvest. Data are means of seven measurements for the alder CO_2 and nutrient treatments, and means of 11 measurements for the pine. Ambient [CO_2] , elevated [CO_2] and control treatment data are the means of 14 for the alder and 22 for the pine.

Species Alder	Mean ratio	95 % confidence interval
Ambient	0.13	0.028
Elevated	0.15	0.020
Outside	0.13	0.012
A-N	0.10	0.022
A+N	0.16	0.046
E-N	0.16	0.024
E+N	0.067	0.037
C-N	0.13	0.010
C+N	0.13	0.026
Pine	**Mean**	**95 % confidence interval**
Ambient	0.11	0.012
Elevated	0.17	0.046
Outside	0.15	0.030
A-N	0.11	0.017
A+N	0.11	0.020
E-N	0.18	0.074
E+N	0.15	0.043
C-N	0.14	0.049
C+N	0.14	0.035

Table 3.7 Probability values (p) from two-way ANOVA on root to shoot mass ratio of common alder and Scots pine exposed to fifteen weeks of differing carbon dioxide and nutrient regimes. The data, from the final harvest, were log-transformed before ANOVA analysis and the p values shown relate to each treatment effect on R / S mass ratio; sample size = 7 per [CO_2] and nutrient treatment for alder, and sample size = 11 for the pine; p value significant at $p < 0.05$ * , significant at $p < 0.1$ (*).

Species	CO_2	Nutrients	CO_2 × Nutr. Interaction	Chamber Effect
Alder	0.15	0.28	0.017 (*)	0.59
Pine	0.056 (*)	0.80	0.97	0.25

3.3.4 Leaf Area, Specific Leaf Area (SLA) and Leaf Area Ratio (LAR)

Leaf Area

Leaf area in both species of tree was not significantly affected by carbon dioxide or fertiliser (see Figure 3.5a and 3.5 b) at both the initial and final harvest ($p > 0.1$), although in Scots pine there was a tendency towards a nutrient effect at the final harvest ($p = 0.10$).

The presence of chambers round the ambient and elevated alder trees had a positive effect on their leaf area (ANOVA on non-log transformed data, $p = 0.02$; log-transformed data showed no significant chamber effect).

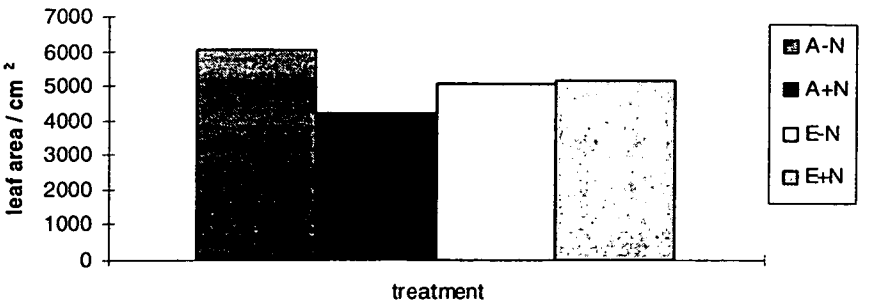


Figure 3.5a Effect of [CO2] and nutrients on leaf area in common alder at the final harvest. A+N and A-N = ambient plus or minus nutrients; E+N and E-N = elevated plus or minus nutrients. Data are the means of 12 or 13 measurements. There were no significant differences at $p < 0.05$.

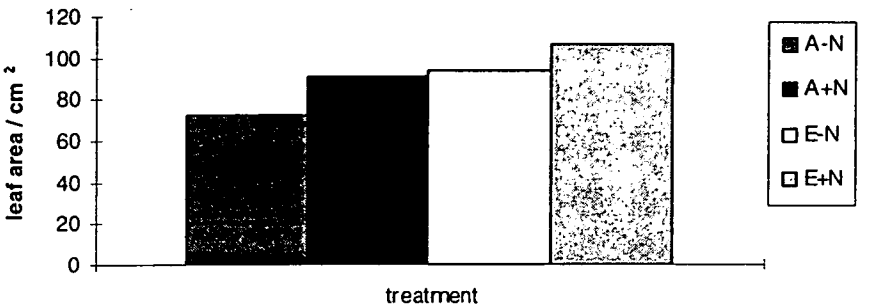


Figure 3.5b Effect of [CO2] and nutrients on leaf area in Scots pine at the final harvest. A+N and A-N = ambient plus or minus nutrients; E+N and E-N = elevated plus or minus nutrients. Data are the means of 12 or 13 measurements. There were no significant differences at $p < 0.05$.

Specific Leaf Area

Table 3.8 shows the specific leaf areas of the alder and the pine seedlings at the final harvest. Specific leaf area in the common alder was significantly different between the elevated and the ambient [CO_2] chambers ($p = 0.017$) at the final harvest. There was no nutrient or carbon dioxide and nutrient interaction effect ($p > 0.05$). In the Scots pine, on the other hand, there were no significant differences between CO_2 treatments or nutrient treatments. It is possible that the lower SLA in elevated alder seedlings is an indication of either an increase in leaf thickness through an accumulation of starch.

There was no chamber effect on SLA in common alder or in Scots pine ($p > 0.05$).

Table 3.8 Mean specific leaf area of alder and pine (m^2 kg^{-1}) at the end of the fifteen-week exposure to elevated [CO_2]. Data are the means and 95 % confidence intervals of 12 measurements per [CO_2] and nutrient treatment (i.e. 24 measurements for each [CO_2] treatment).

	Alder		Pine	
Ambient	6.18	(0.27)	0.60	(0.09)
Elevated	5.45	(0.31)	0.69	(0.10)
Outside	6.18	(0.22)	0.67	(0.07)
A-N	6.15	(0.30)	0.59	(0.11)
A+N	6.16	(0.46)	0.61	(0.16)
E-N	5.66	(0.26)	0.63	(0.13)
E+N	5.20	(0.62)	0.75	(0.18)
C-N	6.10	(0.35)	0.61	(0.07)
C+N	6.30	(0.32)	0.73	(0.13)

Leaf Area Ratio (LAR)

Leaf area ratio in the alder was not significantly affected by [CO_2] in this short-term experiment. There was no interaction effect of nutrients and [CO_2] on LAR ($p > 0.1$). Thus, exposure to elevated [CO_2] and the two different nutrient regimes did not significantly affect the ratio of assimilatory area to total plant mass i.e. LAR, (see Table 3.9).

Table 3.9 Mean leaf area ratio (LAR), $m^2\ kg^{-1}$, of common alder and Scots pine at the final harvest. Values are the means of 14 measurements for the carbon dioxide treatments and the means of seven measurements for the [CO_2] and nutrient treatments. 95 % confidence intervals are in brackets after the means.

Treatment	alder	pine
Ambient	2.45 (0.34)	3.39 (0.53)
Elevated	2.37 (0.22)	3.94 (0.69)
Control	2.73 (0.21)	3.47 (0.41)
A-N	2.82 (0.27)	3.35 (0.79)
A+N	2.32 (0.66)	3.42 (0.86)
E-N	2.51 (0.19)	3.85 (0.93)
E+N	2.24 (0.43)	4.03 (1.20)
C-N	2.92 (0.23)	3.50 (0.52)
C+N	2.55 (0.35)	3.43 (0.73)

In Scots pine there was no significant [CO_2] effect on LAR (p = 0.94) at the final harvest (see Table 3.9). There was also no nutrient effect or interaction effect between [CO_2] and nutrients on LAR ($p > 0.1$). Exposure to elevated [CO_2] did not therefore significantly affect the ratio of assimilatory area to total plant mass i.e. LAR.

There was no chamber effect on LAR in common alder ($p > 0.1$) and a tendency towards a chamber effect in Scots pine ($p = 0.94$).

3.3.5 Specific Growth Rate

Specific growth rate is also more commonly known as 'relative growth rate' or RGR, although the former is more in line with modern nomenclature. Elevated [CO_2] had no significant effect on specific growth rate (SGR) between the initial harvest and the final harvest in either species (Table 3.10). Any stimulation in SGR must have occurred prior to the experimental period, therefore, since total biomass increased in

elevated [CO_2]. The hypothesis that the SGR of common alder in elevated [CO_2] would be higher than in ambient [CO_2] is not clearly supported. The lack of significantly different SGRs in the Scots pine, on the other hand, could confirm that a negative feedback process was taking place, not enabling those seedlings grown in elevated [CO_2] to take full advantage of the extra carbon dioxide available.

Table 3.10 Mean SGR (mg g^{-1} day^{-1}) of common alder and Scots pine over the course of the fifteen-week experiment; calculated as (ln M_2 - ln M_1) / t where M_2 is mean total biomass of 30 seedlings at the final harvest and M_1 is mean total biomass of six seedlings at the initial harvest.

Species	Ambient	Elevated
Alder	39.60	39.80
Pine	26.60	25.40

3.4 Biochemical Leaf Composition

3.4.1 Nutrient Concentration

Nutrient concentrations in the common alder seedlings were not significantly different at the start (see Table 3.11) or at the end of the study ($p > 0.1$, see Table 3.12), despite exposure to elevated [CO_2] during the first year of growth as well as during the experimental period. This result has not usually been found in elevated [CO_2] experiments. Mousseau and Saugier (1992) state in their review that *all* tree species grown in elevated carbon dioxide were found to have lower concentrations of nitrogen than the control trees. This does not seem to be the case with common alder in this study. There was no nutrient or interaction effect of nutrients and [CO2] on nutrient concentration in common alder ($p > 0.1$).

Table 3.11 Concentrations (mg g^{-1}) of nitrogen, phosphorus, potassium, magnesium and calcium of leaves and roots at the initial harvest. Data are means of six measurements for each treatment. * = significant difference between elevated and ambient [CO_2] at $p < 0.05$ (t-test); (*) = trend towards significant difference between treatments at $p > 0.1$ (t-test).

Species alder	N	P	K	Mg	Ca
Leaves					
Ambient	25.6	3.3	8.6	1.9	4.0
Elevated	26.7	3.3	9.0	1.9	4.0
Roots					
Ambient	19.6	4.0	7.5	2.4	3.3
Elevated	20.7	4.2	7.4	2.3	3.1

Scots pine	N	P	K	Mg	Ca
Needles					
Ambient	17.6	2.0 *	7.5 *	1.2 (*)	3.0
Elevated	16.4	1.8 *	6.9 *	1.0 (*)	2.6
Roots					
Ambient	11.2	1.8	6.2	1.0	1.2
Elevated	12.9	1.8	6.2	1.1	1.3

Nutrient concentration data for Scots pine from the final harvest were not available, but at the initial harvest the nitrogen concentration was lower in the elevated [CO_2] treatment needles (although not significant, t-test, $p > 0.05$) but not in the roots, where nutrient concentrations were higher than or similar to ambient [CO_2]. This pattern occurred with all the elements analysed (see Table 3.11). The only statistically significant differences in nutrient concentration were in phosphorus and potassium

concentration in the needles ($p < 0.05$). There was a trend towards a significant effect of elevated [CO_2] on nitrogen concentration in the pine roots ($p = 0.086$), as well as on magnesium concentration in the needles ($p = 0.067$). These results, unlike the alder data, are in agreement with the statement by Mousseau and Saugier (1992) that all trees grown in elevated [CO_2] have lower nitrogen concentrations than in ambient [CO_2].

Table 3.12 Concentrations of nitrogen, phosphorus, potassium, magnesium and calcium (mg g^{-1}) in alder leaves in the final harvest. Data are means of five samples for each [CO_2] and nutrient treatment. No control samples were analysed.

Treatment	N	P	K	Mg	Ca
Ambient	17.2	1.0	4.8	6.2	16.2
Elevated	18.1	1.3	4.8	5.6	15.9
A-N	16.7	0.9	4.5	6.0	16.7
A+N	17.8	1.2	5.0	6.3	15.7
E-N	21.0	1.2	4.5	5.5	16.0
E+N	15.1	1.4	5.0	5.8	15.8

3.4.2 Chlorophyll Concentration

At the initial harvest there was no significant difference in chlorophyll *a* and *b* concentration in common alder leaves, either on a dry mass or on an area basis (t-test, $p > 0.05$). In Scots pine, on the other hand, there were statistically different chlorophyll *a* and *b* concentrations on a dry mass basis but not on an area basis at the initial harvest (t-test, $p = 0.03$).

Table 3.13 Alder chlorophyll concentration on an area basis (mg m^{-2}) of leaves sampled on the 30th May 1994 (week nine of experiment) and on the 10th June 1994. Values are means of 25 samples for the [CO_2] treatments, and at least 12 samples for the [CO_2] and nutrient treatments.

Treatment	Chl. *a*	Chl. *b*	Chl. *a* + *b*	Ratio *a* / *b*
30 May 1994 (week nine)				
Ambient	1.94	0.80	2.74	2.43
Elevated	1.82	0.75	2.52	2.43
Control	1.91	0.77	2.68	2.48
A-N	1.87	0.74	2.61	2.53
A+N	1.97	0.85	2.82	2.32
E-N	1.75	0.71	2.46	2.46
E+N	1.88	0.80	2.68	2.35
C-N	1.98	0.79	2.77	2.51
C+N	1.83	0.75	2.58	2.44
10 June 1994 (week ten)				
Ambient	1.49	0.51	2.00	2.92
Elevated	1.47	0.53	2.00	2.77
A-N	1.29	0.53	1.81	2.43
A+N	1.73	0.49	2.22	3.53
E-N	1.58	0.54	2.12	2.92
E+N	1.36	0.51	1.87	2.67

Differences between treatments in chlorophyll *a*, *b*, *a* + *b*, and the ratio *a* / *b* were not significant on any of the sampling dates for common alder (Table 3.13) or at the final harvest for either Scots pine or common alder ($p < 0.05$) (Table 3.14). Although these data are not statistically significant ($p > 0.05$), there is a general trend in the alder (see Table 3.13) towards lower concentrations in elevated [CO_2] leaves than in ambient

[CO_2] leaves. This is in agreement with general observations that chlorophyll concentration is reduced in elevated [CO_2] (Mousseau and Saugier 1992). The Scots pine do not show this tendency, however (Table 3.15).

Table 3.14 Concentrations of chlorophyll in common alder leaves at the final harvest on a dry mass (mg kg^{-1}) and an area basis (mg m^{-2}). Values are means of 25 samples for the [CO_2] treatments, and at least 12 samples for the [CO_2] and nutrient treatments.

Dry mass basis Treatment	Chl *a*	Chl *b*	Chl *a* + *b*	Ratio *a* / *b*
Ambient	3.10	1.29	4.38	2.68
Elevated	3.06	1.26	4.33	2.48
A-N	2.79	1.08	3.88	2.60
A+N	3.33	1.45	4.78	2.75
E-N	3.25	1.34	4.59	2.58
E+N	2.86	1.18	4.04	2.37

Area basis Treatment	Chl *a*	Chl *b*	Chl *a* + *b*
Ambient	1.94	0.80	2.74
Elevated	1.82	0.75	2.57
A-N	1.90	0.75	2.65
A+N	1.97	0.85	2.82
E-N	1.85	0.75	2.59
E+N	1.79	0.75	2.54

Table 3.15 Concentration of chlorophyll in Scots pine needles at the final harvest on an area basis (mg m^{-2}). Values are means of 25 samples for the [CO_2] treatments, and at least 12 samples for the [CO2] and nutrient treatments.

Treatment	Chl. a	Chl. b	Chl. a + b	Ratio *a / b*
Ambient	0.24	0.097	0.33	2.47
Elevated	0.28	0.072	0.35	3.88
Outside	0.24	0.094	0.33	2.55
A-N	0.24	0.094	0.33	2.55
A+N	0.23	0.087	0.32	2.64
E-N	0.33	0.041	0.37	8.05
E+N	0.22	0.11	0.32	2.00
C-N	0.24	0.083	0.32	2.89
C+N	0.24	0.11	0.35	2.18

3.4.3 Soluble Carbohydrates and Starch

Sugars

Soluble carbohydrates in the Scots pine at the initial harvest were primarily inositol, glucose and fructose. Sucrose and xylose appeared in one in six of the samples only. There were no statistically significant differences between the treatments in percent inositol, glucose or fructose (t-test, $p > 0.1$) (see Table 3.16). At the final harvest total soluble sugar concentration (mg g^{-1}) in Scots pine was 180.50 for ambient [CO_2] needles and 180.95 for elevated [CO_2] needles (t-test, *ns* at $p < 0.05$). No calculation of the standard error of the means was possible here since two samples were pooled for each single 'sample' in order to have enough plant material for analysis. The alder samples were unfortunately lost in a freezer accident.

Table 3.16 Mean concentration of soluble sugars (mg g^{-1} dry mass) in Scots pine at the final harvest. Variance, and sample size for each treatment are given.

Treatment	Mean	Variance	Sample Size
Glucose			
Ambient	6.0	0.7	4
Elevated	8.4	1.1	8
Fructose			
Ambient	6.0	0.7	4
Elevated	7.8	1.0	8
Sucrose			
Ambient	5.6	1.6	4
Elevated	3.4	1.0	8
Total Sugars			
Ambient	35.0	5.4	4
Elevated	36.0	6.6	8

Starch

Scots pine needles grown in elevated [CO_2] showed a significantly higher starch concentration at the final harvest than the ambient [CO_2] needles (four replicates per treatment, $p = 0.01$) (see Table 3.17). There was no nutrient or interaction effect on starch in Scots pine ($p > 0.1$). No significant effect of elevated [CO_2] or nutrients was found in the alder (for all treatment effects $p > 0.1$), although caution must be used here since there were only two replicates analysed per [CO_2] and nutrient treatment.

Table 3.17 Starch concentration (mg g^{-1}) in Scots pine needles at the final harvest. Data are means of eight samples for the [CO_2] treatments, means of four samples for the CO2 and nutrient treatments. ** = significantly different at $p < 0.01$ in two-way ANOVA.

Treatment	Mean	95 % confidence interval
Ambient	11.47	5.01
Elevated	24.76 **	8.56
A-N	10.52	8.42
A+N	12.43	11.64
E-N	21.50	11.23
E+N	28.03	20.53

3.5 Discussion and Conclusions

Tables 3.18 and 3.19 are summaries of the main effects of elevated [CO_2] on common alder and Scots pine seedlings. They enable a quick overview of the main effects but do not include interactions between [CO_2] and nutrient addition. Any interaction effects are discussed in more detail in the subsequent sections.

Table 3.18 Summary of main effects of growth in elevated [CO_2] on common alder in the two nutrient treatments high supply, H and low supply, L, combined and separately . The table shows significant statistical differences and how a variable in elevated [CO_2] compared with the ambient [CO_2] control. ++ large increase, + increase, (+) slight increase, ~ no change, (--) slight decrease, -- decrease, --- large decrease, ? not measured. Stars * indicate that the result was statistically significant ($p < 0.05$) at least at some time during the experiment. (*) = trend towards significant difference between treatments ($p < 0.1$).

Variable	H+L together	H	L
stem height	-- *	-- *	-- *
growth rate per day	~	~	~
SBD	~	~	~
total biomass	+	+	~
leaf allocation	~	~	~
root allocation	~	~	(+)
branch allocation	~	~	(--)
stem allocation	~	(--)	~
R/S	(+)	(--)	(+)
leaf area	~	(+)	(--)
SLA	-- *	--	--
LAR	~	~	~
SGR	~	?	?
[N]	(+)	(+)	(+)
[P]	~	~	~
[K]	~	~	~
[Mg]	~	~	~
[Ca]	~	~	~
[chlorophyll]	(--)	(--)	(--)
[sugars]	?	?	?
[starch]	~	~	~

Table 3.19 Summary of main effects of growth in elevated [CO_2] on Scots pine in the two nutrient treatments high supply, H and low supply, L, combined and separately . The table shows significant statistical differences and how a variable in elevated [CO_2] compared with the ambient [CO_2] control. ++ large increase, + increase, (+) slight increase, ~ no change, (--) slight decrease, -- decrease, --- large decrease, ? not measured. Stars * indicate that the result was statistically significant ($p < 0.05$) at least at some time during the experiment. (*) = trend towards significant difference between treatments.

Variable	H+L together	H	L
stem height	~	~	~
growth rate per day	~	~	~
SBD	+ *	(+)	+
total biomass	+	+	++
needle allocation	-- *	-- *	~
root allocation	+ *	(+)	+
branch allocation	~	~	~
stem allocation	(--)	~	(--)
R / S	+ *	+	+
leaf area	~	(+)	(+)
SLA	~	+	~
LAR	(--)	(--)	(--)
SGR	~	?	?
[N]	(--)	?	?
[P]	-- *	?	?
[K]	-- *	?	?
[Mg]	-- (*)	?	?
[Ca]	(--)	?	?
[chlorophyll *a*]	(+)	~	+
[chlorophyll *b*]	(--)	(+)	(--)
[chlorophyll *a* + *b*]	(+)	~	+
chl *a* \ *b* ratio	+	--	++
[sugars]	~	~	~
[starch]	+ *	+	+

In both common alder and Scots pine specific growth rate (SGR) was not affected by growth in elevated [CO_2] during the fifteen weeks of this experiment, although total biomass increased, although not significantly. For this reason I suggest that the growth rate was stimulated during the first year of growth, before the experimental period. Reports of elevated [CO_2] stimulating total plant biomass are widespread (Poorter 1993, Kimball 1993, Ceulemans and Mousseau 1994, Saralabai *et al.* 1997, Tissue *et al.* 1997). This growth enhancement has often been shown to decline or completely disappear over time (Bazzaz 1990, Reining 1994). In contrast, other studies have found no growth enhancement (Oberbauer *et al.* 1986, Mousseau and Enoch 1989, Overdieck 1996). The results of this experiment agree with the majority of other studies in that total biomass was stimulated by growth in elevated [CO_2]. Deciduous species and pioneer species were found by Poorter in his comprehensive review (1993) to be stimulated more than coniferous or slower-growing species (*c.* 54 % for the former versus *c.* 23 % for the latter). This is in agreement with the increase in total biomass found in this study, although the growth stimulation was less pronounced here than Poorter's average stimulation. Norby and O'Neill (1991) on the other hand, found a similar increase in total biomass to that found in alder in this study, in yellow poplar (tulip tree), *Liriodendron tulipifera*, grown in 371, 493 and 787 $\mu mol\ mol^{-1}$ CO_2 (18 to 22 % stimulation).

Common alder

Common alder and Scots pine had quite different responses to elevated [CO_2] despite their total biomass being stimulated. In common alder stem height was significantly higher in ambient [CO_2] plants during the first weeks of the study, but this was cancelled out over the remaining weeks, indicating that the elevated [CO_2] seedlings were able to catch up with the ambient [CO_2] plants. Extra carbon was assimilated in alder seedlings in this experiment via an enhanced photosynthetic rate (although not measured directly, biomass accumulation in this experiment and measurement of photosynthetic enhancement in the long-term experiment confirm this) and stimulation of growth rate during the first year of exposure. Pettersson and McDonald (1992) found that slightly higher SGR values during the first few weeks of development in birch seedlings were sufficient to produce appreciable differences in biomass over a

period of several growing seasons.

Leaf area and LAR were not increased in elevated [CO_2] although alder leaves were thicker. This is contrary to the review of Mousseau and Saugier (1992) who found that LAR in many species of tree was significantly increased by elevated [CO_2]. Arnone and Gordon (1990) found that leaf area was larger in nitrogen-fixing *Alnus rubra* grown in pots in elevated [CO_2]. It is not clear whether this lack of enhancement of leaf area was an artefact of the experiment, namely pot growth and hence root restriction, or if it was a genuine [CO_2] effect. Lower SLA does not seem to be attributable to starch accumulation in the leaves since starch concentration was similar in the two [CO_2] treatments at the end of the experiment. The extra carbon seems to have been allocated to increased structural material in the leaf (perhaps as larger palisade cells and / or mesophyll cells) but also to new root growth. This is supported by the response of nitrogen fixation in alder in the long-term experiment (see Chapter 4) where elevated [CO_2] stimulated nodule growth and nitrogen fixation.

Contrary to expectations, alder seedlings grown without fertiliser (and therefore more likely to fix nitrogen particularly those in elevated [CO_2] chambers) did not grow bigger or allocate more biomass to leaves. This is not the case, however, with biomass allocation to the roots where the seedlings grown without fertiliser addition have bigger root systems, especially those grown in elevated [CO_2] without fertiliser. This result is to be expected since plants grown with a low nutrient supply usually allocate more resources to the roots. The response of the alder seedlings shows that the nutrient addition in this experiment was effective in that one treatment actually received low N and the other high N, with concomitant differences in root allocation.

This pattern of increased allocation to roots in the low N treatment could also be indicating an increased allocation of carbon to the site of nitrogen fixation in the root nodules. This is supported by measurements of nitrogenase activity in nodules of common alder in the long-term experiment, in which nodule mass was larger in elevated [CO_2] and nitrogen fixation seemed more efficient (see Chapter 4 section 4.10). This supports the idea that nitrogen-fixing trees grown in elevated [CO_2] may

be able to export extra photosynthate (sugars) to increase nodulation of roots and so be more independent of external nitrogen additions.

Nutrient concentrations were not affected by elevated [CO_2]. This is different to the findings of Arnone and Gordon (1990) or Norby (1987) where not only nitrogenase activity was enhanced by enriched carbon dioxide but also nitrogen concentration. Although elevated [CO_2] did not affect leaf nutrient concentrations, all alder leaves measured in this experiment started out with optimum nutrient amounts compared to those in the literature (Van den Burg 1985) but by the final harvest nitrogen concentration was intermediate to deficient and potassium and phosphorus were also intermediate, whereas other nutrients were still present in optimum amounts. Factors such as growth in pots and root restriction may have caused this reduction in leaf nitrogen. Field *et al.* (1992) point out that plants grown in elevated CO_2 often have decreased tissue nitrogen and biomass allocation to the leaves thus offsetting any extra respiratory costs caused by increased growth and maintenance. In this experiment biomass allocation to the leaves was reduced but leaf nitrogen was not.

Photosynthetic capacity in the form of chlorophyll was reduced in alder grown in elevated [CO_2], although not significantly. This agrees with the findings of other authors (El Kohen and Mousseau 1994, Mousseau and Saugier 1992) where chlorophyll content was reduced in elevated [CO_2]. This reduction in photosynthetic protein may have caused negative feedback which reduced the stimulation of photosynthesis and in turn the enhancement of growth rate. Common alder did not undergo acclimation to the same extent as Scots pine, however. This is in accordance with the idea that the nitrogen fixing alder is not limited by nitrogen availability in the soil and can increase its growth in conditions of elevated [CO_2].

Scots pine

In Scots pine the response to elevated [CO_2] was increased SBD and a tendency towards increased total biomass, as well as increased allocation of carbon to the roots away from needles and stems. Nutrient concentrations in the needles were reduced, chlorophyll *a* / *b* increased slightly, and starch accumulated in the needles in elevated

[CO_2]. Grulke *et al.* (1993) found no stimulation of either stem height or stem diameter in two full-sib ponderosa pine families, whereas total biomass was enhanced. The Scots pine growth response in this experiment was similar, although here SBD was significantly increased. Higginbotham *et al.* (1985) however, found an all-round positive response to elevated [CO_2] in lodgepole pine (*Pinus contorta*) grown in pots in growth chambers. Mean seedling height, leaf area, biomass of stems, secondary leaves and especially roots were all very stimulated by elevated [CO_2]. It seems therefore that different pine species react quite differently to elevated [CO_2]. On the other hand, it is unclear how much of the response was an artifact of growth in unnatural conditions and using different methodologies.

Norby *et al.* (1986) found increased root exudation, mycorrhizal density and allocation of photosynthate to fine roots in *Pinus echinata* grown in elevated [CO_2], although root exudation did not persist after the 41st week. They hypothesised that increased root exudation of carbon compounds could enhance nutrient availability for plants growing in elevated [CO_2]. The fact that nutrient concentrations were reduced, and starch accumulated in the needles in Scots pine in this study supports the idea that nutrient limitation occurred and the plants responded by allocating more carbon to the roots. Whether this carbon was then exuded from the roots into the soil or whether it was mainly used for increased fine root growth was beyond the scope of this short-term experiment.

The pine thus showed signs of having acclimated to elevated [CO_2] in the form of carbon export to the roots (including potential root exudation), decreased nutrient concentrations and starch accumulation in the needles. A decrease in nutrient content has often been found in plants grown in enriched carbon dioxide (Mousseau and Saugier 1992). Chlorophyll *a* and *b* concentrations were affected differently by elevated [CO_2]. Thus, an interesting result of this study was that there was a trend towards the ratio of chlorophyll *a* to *b* (*a* / *b*) changing in elevated [CO_2]. Growth in elevated [CO_2] changed the photosynthetic system slightly, and starch accumulation occurred.

This provides support for the hypothesis that a source-sink imbalance occurred in the Scots pine with starch accumulation in the leaves as the end result, as was predicted by Stitt (1991) in his review on rising CO_2 concentrations and their effects on carbon flow in photosynthetic cells. The exact mechanisms of this limitation can not be derived from this short-term experiment. Pettersson and McDonald (1992) hypothesised that decreases in SGR over time are mediated by decreases in NAR (net assimilation rate), explaining why growth enhancement stops. Increased nutrient demand decreases plant nutrient status leading to possible reduction in NAR and carbon allocation to the leaves. Exactly when this acclimation occurred is not clear, but the enhancement in growth rate seems to have only occurred during the first weeks of the study. Starch accumulation and possible chloroplast disruption in Scots pine needles may have induced a stronger negative feedback on the rate of photosynthesis or growth rate than in common alder (see Poorter 1993).

Comparison Between Species

As other authors have found (Kaushal *et al.*. 1989, Poorter 1993) there is a significant positive correlation between the potential growth rate of a plant and its reaction to elevated [CO_2]. The difference between fast-growing species and slow-growing species is the biggest of all the comparisons in Poorter's review (1993), although caution is needed when interpreting this result since no exact growth rate data for these species were available. He found in his experiment using ten species that fast-growing species had increased LAR compared with slow-growing species but NAR and the rate of photosynthesis were stimulated to the same extent. Thus any absolute gain in carbon per unit plant mass in the fast-growing species was because of larger leaf area per unit plant mass. This does not agree with the results in this experiment, where alder did not increase in LAR or leaf area in elevated [CO_2]. Growth stimulation was higher in the pioneer species compared with the conifer, however.

The fact that common alder responded more positively to elevated [CO_2], and experienced less photosynthetic acclimation than Scots pine, may be attributable to its capacity for fast, indeterminate growth and its ability to fix nitrogen. Conifers like Scots pine, on the other hand, are constrained in their growth response by

determinate growth patterns and slower growth rates than common alder. Barton (1997) found that Sitka spruce seedlings were able to initiate new shoots midway through the season when exposed to elevated [CO_2]. Growth in elevated [CO_2] has been found to enable second flushes of growth in several conifer species. This was only observed in a small percentage of the seedlings in this experiment, however. Thus, although some of the extra carbon was allocated to the stem (increased SBD) in Scots pine an appreciable amount of the carbon assimilated seems to have been exuded from the root system, thus inducing acclimation to elevated [CO_2].

The response of the fast-growing alder is made more complex by the capacity to fix nitrogen. Fertilised and unfertilised plants were stimulated in growth to an equal extent by exposure to elevated [CO_2]. The treatment without fertiliser enables one to look at how nitrogen fixation may have been reacting to the extra carbon availability without the added complication of a fertiliser addition. As many other authors have found (see Woodward 1992, Hood 1993) nitrogen addition seems to have suppressed nitrogen fixation in this experiment, and trees grown without fertiliser and therefore fixing nitrogen were not able to grow more than those with fertiliser. Ingestad (1980) found that inoculated grey alder (*Alnus incana*) grown without added mineral nitrogen, had optimum nitrogen status but only half the maximum SGR. It seems therefore that nitrogen fixation was not very efficient. As Ingestad found, however the maximum fixation rate has an upper ceiling compared with the total nitrogen uptake rate required for maximum growth. *Alnus glutinosa* in this study seems to have been able to fix more nitrogen in elevated [CO_2] but the extent of this fixation stimulation may have been limited by intrinsic limitations to nitrogen fixation such as phosphorus availability.

It is important to note that growth in pots may be an extra factor affecting results in this experiment. Experiments with plants grown in pots have found clear growth stimulation as well as those using plants grown in the ground (Pettersson and McDonald 1992, Kimball *et al.* 1993), although caution should be used when interpreting results from pot experiments since root restriction and nutrient limitation can affect the overall response to [CO_2] (Eamus and Jarvis 1989, McConnaughay *et*

al. 1993). This may have been the case in this short-term experiment. For a comparison of the same tree species in a long-term experiment without pots, see Chapters 4 and 5.

Conclusions

- Both species of tree had greater total biomass in elevated [CO_2] (20 % increase in alder and 10 % in pine) but the difference was not statistically significant. SBD was significantly higher in pine in elevated [CO_2], whereas stem height in elevated [CO_2] alder caught up with initially higher values in ambient [CO_2]. Thus there was a trend towards total biomass stimulation in elevated [CO_2] as well as a significant stimulation of SBD in pine and stem height in alder.

- Allocation of carbon changed in elevated [CO_2]. In common alder the extra carbon assimilated was allocated to the roots. In Scots pine more carbon was allocated to the roots in elevated [CO_2] and less was allocated to the stem and needles.

- Leaf morphology in alder changed in elevated [CO_2] since SLA was significantly lower.

- There was generally no interaction effect of elevated [CO_2] and nutrient addition on the state variables measured.

- The hypothesis that the alder would not suffer a source-sink imbalance to the same extent as the pine was supported. The faster-growing and nitrogen fixing species was better able to use the extra carbon to increase total biomass. Scots pine showed typical signs of having acclimated to elevated [CO_2], i.e. starch accumulation, reduced nutrient concentration and altered chlorophyll *a* and *b* ratios. The alder did not show these responses although SGR remained the same in elevated [CO_2] and biomass was not stimulated as much as expected. This could be because of the fact that nitrogen fixation is

limited in the extent to which it can produce growth stimulation. Another factor which could have played a role in each species' response to elevated [CO_2] was root restriction because of growth in pots, as well as genotypic determination a plant's overall development and growth.

- This experiment was short-term and seedlings were grown in pots which could have affected nutrient availability and root not directly in the ground. In order to be able to clearly differentiate between effects and their causes plant growth and development, longer-term experiments with plants grown directly in the soil are recommended.

CHAPTER 4

Growth and biomass, nutrient concentration and internal nitrogen cycling in *Alnus glutinosa* and *Pinus sylvestris* grown in elevated carbon dioxide concentration - a three-year experiment at the Glendevon field site

4.1 Introduction

Many impact studies on the effect of elevated [CO_2] on tree growth and physiology have been short-term experiments of less than one year in duration, with juvenile plants grown in pots (Eamus and Jarvis 1989, McConnaughay *et al.* 1993). Significant increases in plant biomass of *c.* 30-70 % have been reported (Idso and Kimball 1991, Mousseau and Saugier 1992, El Kohen *et al.* 1993, Kimball *et al.* 1993, Poorter 1993, Ceulemans and Mousseau 1994) as well non-statistically significant increases up to 20 % (Mousseau and Enoch 1989, Bazzaz *et al.* 1990, Kimball *et al.* 1993, Poorter 1993). In most short-term (up to one growing season) and longer-term experiments (from one growing season to five years) plants show increased photosynthesis and biomass accumulation in the first weeks or months of exposure to elevated [CO_2] (Gunderson and Wullschleger 1994, Reining 1994, Drake *et al.* 1997). There is generally a tendency to increased growth rates and shifting carbon allocation patterns towards roots (Chu *et al.* 1992, Idso and Kimball 1992, Rogers *et al.* 1992, Rogers and Runion 1994). The increase in photosynthetic capacity is engendered by a larger availability of CO_2, a major substrate of Rubisco in carboxylation and also an activator of Rubisco via carbamylation. The competition between carbon and oxygen (photorespiration) for the Rubisco active site also shifts in favour of carboxylation in elevated [CO_2]. All these factors stimulate the rate of photosynthesis.

Increased [CO_2] results in an increased demand by trees for other resources, such as nitrogen (Lee *et al.* 1998). Seedlings often undergo a negative feedback after a certain, species-specific time point (Grulke *et al.* 1993, Reining 1994). This negative feedback is manifested as a reduction in photosynthetic rate induced by either so-called down-regulation ('fine control') or acclimation ('coarse control'). The former

involves a reduction in photosynthetic rate without a change in photosynthetic leaf composition, whereas in the latter case photosynthetic capacity is reduced via diminished photosynthetic components (Delgado *et al.* 1994), such as Rubisco, chlorophyll or other enzymes. Photosynthetic acclimation has been associated with accumulation of carbohydrates because of low sink demand and / or reduction in Rubisco amount or activity (Stitt 1991). This longer-term acclimation effect of carbon dioxide enrichment varies in its time of onset, but short-term experiments, varying from days to one growing season, have shown a temporary increase in photosynthetic rate as predicted by leaf models. In longer term studies this enhancement may be reduced after a few weeks or months (Mousseau and Saugier 1992). Many hypotheses have been put forward to explain acclimation. These range from a) limitation of RuBP regeneration, b) nitrogen or phosphorus limitation, c) loss of extra carbon via root exudation, d) carbohydrate accumulation which induces a source-sink imbalance and sometimes chloroplast rupture, and e) canopy closure which limits the potential for increased assimilatory leaf material. Often the impact of elevated [CO_2] is closely inter-linked with and limited by other environmental variables, such as nitrogen, phosphorus, photon flux density (PPFD), temperature or water. The intricacies of these interactions need to be elucidated in order to have a better grasp of the mechanisms involved in response to elevated [CO_2]. Another complicating factor is that responses to elevated [CO_2] often appear to be species-specific, with some species exhibiting a large response and others barely any (Garbutt *et al.* 1990, Poorter 1993). This interspecific variation is difficult to assess accurately because of varied methodologies (e.g. some plants are grown for longer or shorter periods than others, some in inadequate rooting medium or constricting pots and with various CO_2 concentrations) as well as the use of various clones or trees of different age or seed provenance (Lee and Jarvis 1996). However, more and more mixed-species experiments, as well as better conducted ones, are showing that interspecific variation in response to elevated [CO2] is a reality and can be strikingly large (Oberbauer *et al.* 1985, Ziska *et al.* 1991, Duff *et al.* 1994, Norby *et al.* 1996). Different species seem to have different limits to their plasticity such as size constraints which are defined by their genetic makeup or by the environmental conditions.

The question arises as to whether species that grow relatively quickly under optimum conditions respond more strongly than those with lower maximum relative growth rates (Oberbauer *et al.* 1985, Poorter 1993)? This question will be addressed through a comparison between two native trees with quite different developmental and growth patterns. The common alder (*Alnus glutinosa* (L.) Gaertn) is a deciduous pioneer species which belongs to the group of actinorhizal nitrogen-fixing species which fix dinitrogen from the atmosphere through an endosymbiotic relationship with a bacterium of the *Frankia* genus. These filamentous, gram-positive bacteria known as actinomycetes, possess branched septate hyphae and sporangia which invade root hairs and develop root nodules in alder from within cortical cells (Hood 1993).

This species is contrasted with a slower-growing, mycorrhizal conifer, Scots pine (*Pinus sylvestris* L.). Previous short-term studies of the effects of elevated [CO_2] on alder seedlings suggested that nodulated plants had higher whole plant photosynthesis and nitrogenase activity, and larger nitrogen content and total biomass compared with nodulated plants grown in ambient [CO_2], or compared to non-nodulated plants grown in both ambient and elevated carbon dioxide concentrations (Norby 1987, Arnone and Gordon 1990). One possibility is that nitrogen-fixing trees grown in elevated [CO_2] are able to export extra photosynthate (sugars) to increase nodulation of roots and so be more independent of external nitrogen additions. While Scots pine does not have this advantage, being evergreen it can continue to fix carbon throughout the year when environmental conditions are suitable.

As discussed in Chapter 1, nitrogen cycling within plants enables the uncoupling of growth from nutrient uptake in the spring thus playing an important role in tree physiology and growth. Nitrogen budgets, previously used to estimate N cycling, have been replaced by isotopic studies, which are capable of more accurate quantification. The use of the stable ^{15}N isotope in experiments has shown that factors such as soil fertility affect the amount of N stored but have no direct effect on the amount of N remobilised (Millard 1995). To my knowledge, there is no experimental evidence to date on the reponse of internal N cycling in trees to elevated [CO_2]. Berntson and Bazzaz (1998) studied the effects of [CO_2] on below ground growth and N cycling in

two mesocosms containing a variety of tree species. Their research concentrated mainly on below ground processes such as remineralisation and uptake rates of N but did not quantify storage and remobilisation of N within the plants. The particular aim of this research on the other hand, was to see which tree tissues acted as storage organs over the winter, and which were sources of remobilised N for new growth in the spring. Considering that Scots pine is a mycorrhizal conifer and common alder a deciduous N-fixer, storage of N over the winter and remobilisation in the spring would be expected to be very different in the two species. In addition, the availability of elevated [CO_2] could influence the movement of N within the trees. The addition of a ^{15}N stable isotope spike to the fertiliser enabled these questions to be addressed.

The following long-term experiment using seedlings planted directly into the ground, had as an overall aim to investigate how elevated [CO_2] and nitrogen supply affected the growth, physiology and N cycling in *Alnus glutinosa* and *Pinus sylvestris*.

4.1.1 Hypotheses and Aims

The hypothesis addressed is that common alder, by fixing nitrogen will be able to take advantage of the extra carbon assimilated in the elevated [CO_2] conditions and increase its growth, biomass and photosynthetic efficiency. In addition, nitrogen limitation and nitrogen dilution as a result of the extra carbon is not expected to occur. Nitrogen fixation will increase and become more efficient in elevated [CO_2]. Nitrogen storage in alder over the dormant period of the year is expected to occur mainly in the stem but also in the roots. In addition, the allocation of nitrogen to new growth in the spring might show different patterns in elevated [CO_2].

The Scots pine, on the other hand, after an initial increase in growth in elevated [CO_2], may develop a source-sink imbalance in which excess starch and other carbohydrates induce a dilution of the nitrogen content. This may lead to negative feedback with the result that those trees without fertiliser addition in particular will show no significant differences in growth, physiology and biochemistry compared to those grown in ambient [CO_2]. An accumulation of starch and soluble sugars is likely

to be visible during part of or all of the initial growing season, but will not be sustained in further months or years.

The particular aims of this experiment were to elucidate :

1) whether Scots pine and common alder seedlings grown in elevated [CO_2] over a three-year time span would respond by increased growth and biomass, or changes in nutrient concentrations compared with those grown in ambient [CO_2];
2) whether the short-term stimulation of growth found in the short-term experiment (see Chapter 3) would be sustained over several growing seasons under field conditions;
3) whether the allocation of carbon would differ between the treatments, since trees grown in elevated carbon dioxide have been found to be ontogenetically more advanced than those grown in ambient conditions;
4) whether there would be any interactive effects of the environmental variables carbon dioxide and nutrients on growth, physiology and biochemistry of the two species;
5) whether the nitrogen-fixing alder and the mycorrhizal pine would respond differently to increased availability of carbon and possible concomitant dilution of nitrogen (creating a source-sink imbalance);
6) whether nitrogen fixation was occurring in individual trees and whether nitrogen fixation would increase and be more efficient in elevated [CO_2];
7) whether nitrogen would be stored mainly in the stem over the winter in alder, as in other deciduous species, whereas Scots pine should store nitrogen in the previous year's needles; and
8) whether elevated [CO_2] would alter the partitioning of nitrogen over the winter and remobilisation for spring growth?

4.2 Materials and Methods

4.2.1 Site Description

The Glendevon field site, near Yetts O'Muckart, Perthshire, Scotland (National Grid Reference NO311058, latitude 56 ° 12' N, longitude 36 ° W) was set amongst a large Forestry Commission Sitka spruce (*Picea sitchensis*) plantation. Prior to 1994 the open top chambers (OTC) on the site were extended to 3.5 m in height and modified for both larger trees (four to five-year old conifers) and one-year old seedlings. The trees were planted into the site in early May 1994 and grew in the chambers and control plots until autumn 1996 for the alder (until the trees outgrew the chambers) and autumn 1997 for the pine, with the advantage of at least three growing seasons worth of data. For more information on variables such as soil nutrients and soil water, pH, or meteorology, see section 2.3 in Chapter 2 Materials and Methods.

4.2.2 Experimental Design

Introduction

576 one-year old seedlings of common alder and Scots pine (from registered seed stand 89(2009) Elgin) were transplanted directly into the ground in eight open-top chambers (OTC), four at ambient [CO_2] (*c.*350 μmol mol^{-1}), four at ambient [CO_2] + 350 μmol mol^{-1} CO_2 and four control plots (see Figure 2.2 in Chapter 2). Since carbon dioxide concentrations vary diurnally, a constant amount of carbon dioxide was added to the elevated [CO_2] chambers allowing the concentration to track that in the ambient chambers and control plot. For more information on the design of the field site, [CO_2] and fertiliser performance, the prevalence of pests and their treatment, see Chapter 2, Materials and Methods.

4.2.3 Experimental Procedure

Growth

Seasonal growth of the trees was monitored by regular measurements of height (monthly) of a sub-sample of 192 trees (4 per quadrant, 16 per chamber). Stem basal diameters (SBD) were measured (the average of two measurements made at 90° to

one another using a digital micrometer) at the beginning and the end of each growing season on all trees. Data for stem heights and stem basal diameters were used to calculate the volume of trees at the beginning and the end of the growing season, by multiplying the diameter squared by the height. Two forms of growth rate were calculated (according to Beadle 1993): growth rate per day (cm) derived from stem heights, and specific growth rate (SGR or $\underline{G}$), the basic component of growth analysis. The latter variable represents the dry mass increase per unit dry mass present per unit of time, and is derived from biomass data using the following equation:

$$\underline{G} = \frac{1}{M} \int \frac{dM}{dt}, \qquad 4.1)$$

where M is total biomass, t is time. Instantaneous SGR is the slope of the curve of ln M against time, but mean SGR (G) is in practice measured between two discrete time intervals (t_1 and t_2) using the following equation:

$$G = \frac{\ln M_2 - \ln M_1}{t_2 - t_1} \qquad (4.2)$$

Since interpolation between total biomass at t_1 and t_2 (using stem basal diameter and / or stem height to calculate total biomass at a later time) was not very accurate (low r^2 values), trees harvested at t_1 and t_2 were paired according to the method of Hunt (1978) using total tree biomass, such that smaller trees at t_1 were paired with smaller trees at t_2 etc.. SGR was then calculated using the paired tree dry masses. In addition, to test for any ontogenetic effects, trees with similar mean biomass per [CO_2] treatment were picked to calculate SGR of trees of the same size. This was done by pairing two tree biomass means from different harvests for each [CO_2] treatment. Mean biomass of similar size (within 20 g of each other) was used for each [CO_2] treatment as well as between treatments (ambient [CO_2] mean biomass was *c.*65 g whereas mean biomass in elevated [CO_2] was *c.*110 g).

Sampling

Leaves for analysis were sampled from one tree per quadrant (i.e. from the minus and plus nutrients treatments, thus two per chamber per species) giving a chamber replication of four. Three discs (1 cm in diameter) were sampled from an alder leaf *c.* 20 cm from the top of the seedling, and three current-year needles, *c.* 5 cm from the tip of the leader shoot in pine, were sampled monthly during the growing season for chlorophyll, Rubisco and soluble protein analysis. During periods of low turgidity or fungal disease (see section 2.3.1 in Chapter 2) five leaf discs were taken. The Rubisco / soluble protein and chlorophyll samples were frozen directly in liquid nitrogen (using a cryoshipper) and kept in - 80 ° C and - 20 ° C freezers, respectively, until analysis. The carbohydrate / starch samples were kept on frozen-ice in the field, freeze-dried in the laboratory, and then kept in a vacuum until analysis. Universal 25 cm^3 vials were filled with needles or leaves because more material was required for carbohydrate and starch analysis.

Leaf Area Measurements

Dry leaf mass was measured at intervals in 1995 and converted to leaf area using mean specific leaf area (SLA) for each [CO_2] and nutrient treatment in July 1994. This method was deemed the most reliable considering that no direct leaf area measurements were made during the long-term experiment. It should be noted, however, that these calculations are based on the assumption that the mean SLA did not change substantially over time. This is a potentially fallible assumption and the results must be seen as relative amounts not absolutes. In addition, measuring needle area of the Scots pine using the leaf area meter was problematic and therefore the original pine leaf area data (see Chapter 3) and the leaf area converted from SLA data should also be seen primarily as comparative data. SLA was not ascertained for Scots pine since there were no direct leaf area measurements and SLA from the short-term experiment was used to calculate leaf area in the long-term experiment. A second point to note is that it was not found appropriate to express variables such as soluble sugars and starch on a leaf area basis since no leaf discs were taken for these analyses and no SLA data was available from which to calculate leaf area of samples. Although alder leaf discs were used for Rubisco and soluble protein analysis, no such leaf area

data were available for pine. For this reason it was decided to show these variables expressed on a dry mass basis only in the results. Leaf area ratio (LAR, total leaf area per total tree dry mass), leaf mass ratio (LMR, leaf area per unit total plant dry mass) and net assimilation rate (NAR) were also determined.

The mean NAR (N) between t_1 and t_2 is given by:

$$N = \frac{1}{t2-t1}\int_{t1}^{t2}\frac{1}{S}\times\frac{dM}{dt} \qquad (4.3)$$

where S is leaf area, M is total biomass and t is time.

The relationship between total biomass and leaf area was found to be linear (r^2 = 0.91 for Scots pine and r^2 = 0.81 for common alder, see Figures 4.1a and 4.1b) and total biomass and leaf area were not found to be discontinuous functions of time thus fulfilling the assumptions for integrating equation (4.3):

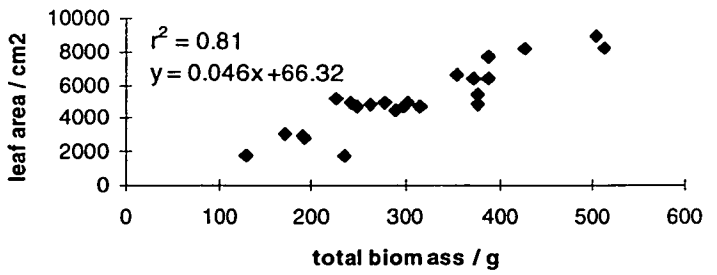


Figure 4.1a The relationship between total biomass and leaf area in common alder in August 1995. Each point is an individual tree measurement, and all treatments were pooled together.

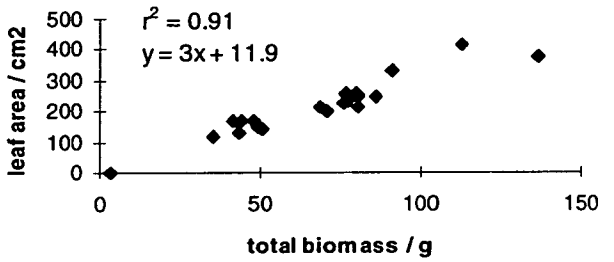


Figure 4.1b The relationship between total biomass and leaf area in Scots pine in August 1995. Each point is an individual tree measurement, and all treatments were pooled together.

Mean NAR between April and December 1995 was determined for both species by integrating equation (4.3) to give:

$$NAR = \frac{(M_2 - M_1)(\ln S_2 - \ln S_1)}{(S_2 - S_1)(t_2 - t_1)} \qquad (4.4)$$

where M is total biomass, t is days and S is leaf area.

Harvests

A total of nine harvests was made of common alder and ten harvests of Scots pine. As concerns the numbering of the harvests, the two harvest in the short-term experiment (Chapter 3) were harvest 1 and 2; the first harvest at Glendevon (September 1994) was therefore, called harvest 3. In September 1994 a sub-sample of 96 trees at

Glendevon was destructively harvested (harvest 3). Two trees within each quadrant in each chamber or control plot was selected for this purpose. The trees were divided into sub-sections (tissues) of root, stem 1993, stem 1994, needles 1993, needles 1994, or leaves 1994 (in alder), and lammas growth at the end of the season (in pine). The tissues were oven- dried for 48 hours at 80 °C and weighed. These masses were used to calculate total biomass per tree, biomass allocation and also for following the fate of labelled N within the trees. The trees were harvested and sampled by myself in 1994, 1995 and the first harvest in 1996. Dr. Gail Jackson and Diarmuid O'Neil continued the experiment during the rest of the 1996 growing season making three further harvests of above-ground biomass for the alder and two for the pine.

The leaves and 1994 needles were sub-sampled and ground for nutrient analysis (N, P, K, Mg, Ca) sampling from one tree per quadrant per chamber, using eight trees per [CO_2] treatment and four trees per combined nutrient and [CO_2] treatment. Tissues (root, leaves or needles 1993, 1994, stem 1993 or 1994) from the harvested trees grown in nitrogen-fertilised quadrants (+N, or 'H' in Figure 2.2 in Chapter 2) were also finely ground and sub-sampled for atomic absorption/mass spectrometric analysis of the ^{15}N and N concentrations. In subsequent growing seasons the number of tissue categories were increased accordingly (i.e. stem 1994, 1995 etc.).

In 1995 there were four more harvests of very similar type to harvest 3, except that 24 trees of each species were harvested instead of 48. All the + N trees were sub-sampled, ground in a centrifuge grinder and then a ball-mill and sent to MLURI, Aberdeen for mass spectrometry. In harvest 7 in October 1995, pine 1995 needles instead of 1994 needles were sampled for nutrient analysis since they have higher concentrations of nitrogen at this time of year. Earlier on in the season, the 1994 needles contained adequate, detectable levels of N, and consisted of enough material for sampling, without damaging the seedling too much (D.C.Malcolm *pers. comm.* 1995). In June 1995, because of competition between the alder seedlings, and hence increased fungal and aphid infestation, some trees were thinned out of the alder quadrants (approximately two from each quadrant, 51 trees total) and their fresh and dry mass measured. These data were incorporated into the calculation of cumulative

biomass of all trees of each species (see below for calculation details and Section 4.4 Total Biomass for results).

In early April 1996 48 trees were again harvested to compare storage and movement of labelled nitrogen in the trees after the winter and to identify potential patterns of nitrogen remobilisation. Cumulative biomass of all trees was calculated using mean total biomass data from the six harvests made by myself, including the thinned alder biomass data (see above).

Biochemical Analysis

Nutrient Analysis (N, P, K, Mg, Ca)

Nutrient concentrations were determined using an acid digest (Allen 1974). The samples were first oven-dried at 80 °C, ground and kept in desiccators until analysis. 100 (±10) mg of ground sample (per tree) was dissolved carefully in 100 vol. (2 × 0.75 cm^3) hydrogen peroxide and 2 cm^3 of boiling sulphuric acid (containing 2 % lanthanum chloride, LaCl2) and shaken. The sample tubes were then heated at 320 °C for six hours, cooled and then made up to 100 cm^3 in distilled water. Nitrogen concentration was determined by a gas diffusion method using a flow injection analyser (Fiastar, Tecator Ltd, Wilsonville, OR, USA). Phosphorus concentration was determined using a molybdenum blue method, using a flow injection analyser (Fiastar, Tecator Ltd, Wilsonville, OR, USA). Potassium, calcium and magnesium were determined by flame emission spectroscopy using an atomic absorbtion spectrometer (Unicam 919, Cambridge, UK). The values obtained for the samples were compared with standards of known concentration (of 20, 40, 60 mg dm^{-3} Ca ; 5, 10, 15 mg dm^{-3} Mg ; 10, 20, 40 mg dm^{-3} K). Each sample was measured three times, and a relative standard deviation based on the three measurements calculated to trace large inaccuracies. Based on 100 mg of dried plant material made up to a fixed volume of 100 cm^3, the concentration (mg g^{-1}) of a particular nutrient was calculated as:

$$\frac{c \times v}{1000 \times m} \qquad (4.5)$$

where c = measured concentration of sample (mg dm^{-3}),

v = volume used (50 cm^3), and

m = mass of sample (0.1 g).

Internal Nitrogen Cycling

In order to compare nitrogen (N) partitioning and potential remobilisation within the trees in the subsequent growing season(s), the nutrient solution was enriched with $^{15}NH_4NO_3$ in July 1994. Half the ground area of each OTC received nutrients and these were fed with 20 dm^3 of water containing 300 g of nitrogen enriched by 4.0901 atom % abundance ^{15}N (measured from a nutrient tank sample) (see Figure 2.2 in Chapter 2). This spike addition of enriched fertiliser enabled the partitioning of N over the winter of 1994 and 1995 to be studied in different treatments and any subsequent movement of labelled N within the trees from one tissue to another to be detected. Any subsequent uptake of nitrogen by the roots in 1995 would have been at natural abundance. This work was done in collaboration with Dr. Peter Millard of the Macaulay Land Use Research Institute (MLURI), Aberdeen.

A sub-sample from every oven-dried and finely ground tissue (see *Harvests* section for description of age classes) was sent to Aberdeen for mass spectrometric analysis. Total nitrogen and ^{15}N enrichment were determined using an ANA-SIRA mass spectrometer (VG Isogas, Middlewich, Cheshire, U. K.) coupled with an elemental analyser. One analysis was made per sample. The elemental analyser separated the sample into its gaseous components, by dropping it into a combustion furnace (He and O_2) and the products of combustion were taken through a reduction column where oxides of nitrogen were converted to nitrogen gas. During mass spectrometry gases were separated using a large, curved magnet and samples of varying masses were quantified : 28 ($^{14}N_2$) ; 29 ($^{14}N^{15}N$) ; 30 ($^{15}N_2$). The natural abundance of ^{15}N must been taken into account when calculating how much enriched nitrogen has fertiliser as its source. All natural sources of nitrogen, from the gas to complex organic material, contain a small amount of the stable isotope of nitrogen, ^{15}N as well as the major component ^{14}N. Thus, in enrichment studies one must subtract this natural abundance (delta, δ) from the enrichment measured by the mass spectrometer

to find out the actual enrichment of the sample. Natural abundance is *c.* 0.3663 atom % in N_2 gas in air.

Mass spectrometry provided the mass of nitrogen (mg), nitrogen % and the atom % enrichment of ^{15}N of each plant tissue. The contributions of labelled fertiliser N and of unlabelled (^{14}N) soil N to the N content of leaves, different age-classes of needle and stem, and roots were calculated for six harvests (September 1994 to April 1996) using the following equations:

$$A = CD / E \quad (4.6)$$

$$B = (1 - C / E) \times D \quad (4.7)$$

where A = labelled N and unlabelled N in tissue from fertiliser spike (mg), B = unlabelled soil N not derived from the fertiliser spike (mg), C = atom % ^{15}N excess in tissue, D = N content of tissue (mg) and E = atom % ^{15}N excess in fertiliser. Values of C and E were corrected for natural abundance (taken to be 0.366 atom % ^{15}N, International Atomic Energy Agency, 1983), hence the term 'excess'.

There were two main assumptions in this study. The first was that the ^{14}N isotope behaved in a similar manner to the ^{15}N-enriched isotope so that the contribution of the fertiliser to each tissue's growth could be determined. The second was that uptake of labelled N was finished by the autumn of 1994 so that by April 1995 any N taken up was at natural abundance. There are several points to take into consideration regarding this second assumption. Since the trees were not grown in sand but in natural field conditions, the uptake of native soil N at natural abundance at the same time as the uptake of fertiliser ^{15}N cannot be allowed for. This can cause an underestimation of nitrogen cycling. Thus it was not possible to quantify the *absolute* amount of N within the trees which was derived from the enriched fertiliser spike, nor to know if the trees had taken up all the labelled N in the soil. Some of the labelled N could have been immobilised by bacteria and fungi and have then been released later on in the experimental period. Plant size was therefore incorporated into the statistical

analysis as a co-variate, in order to see if any subsequent increase in total ^{15}N in whole trees was because of remineralistion of microbial ^{15}N, root exudates or the results of variation in plant size at different harvests. This experiment was a tracer experiment only, because of the difficulty in working with enriched fertiliser in field conditions. Two-way ANOVA was used to analyse labelled ^{15}N and unlabelled ^{14}N data over time in the two [CO_2] treatments (*p* values are given).

Nitrogen Fixation

When nodules were assumed fully developed in August 1995 (harvest 6), Dr. Sue Grayston from MLURI, Aberdeen, sampled half of the harvested alder roots for nodules, which were then analysed in Aberdeen for acetylene reduction, a test for the presence of nitrogen fixation. Nodules were sampled from the roots of all the non-fertilised alder trees harvested (12 trees) and the roots and nodules washed and weighed (fresh and dry mass). An acetylene reduction assay (ARA) was used to assess whether or not the alder seedlings were fixing nitrogen and to give an indication of the relative fixation rates of the different trees. Nitrogenase activity was expressed in moles of ethylene produced.

Statistical Analysis

All data were analysed for two-way ANOVA using a general linear model (glm) in SAS 6.11 (SAS Institute Inc., Cary, NC 27513, USA). Factors in this analysis were carbon dioxide concentration (*c.* 350 μmol mol^{-1} CO_2 versus ambient +350 μmol mol^{-1} CO_2), chamber presence (no chamber versus chamber; note that this factor is not independent of the CO_2 factor) and nutrient availability (no-fertiliser versus fertiliser). For further details of statistical considerations see Section 2.2.2.

4.3 Growth Results

Overall, any visible differences in growth and phenology varied depending on the time of the year. At the beginning of the season the control plot trees grew less vigorously than those in chambers, whereas later on there seemed to be a balancing-out effect. Although lammas growth (second flush) in the Scots pine did not occur in all trees in the late season of 1994 and 1995, the general trend was that trees grown in elevated

[CO_2] had a tendency to a second flush of growth, with a treatment average of between 1g and 2 g dry mass of lammas needles per tree in 1995 compared with ambient [CO_2] means of 0.6 g in one tree (this was the only occurrence of lammas growth in ambient trees, in August 1995). In contrast to the ambient [CO_2] seedlings, lammas growth was observed in the elevated [CO_2] seedlings in every late-season harvest. There was no conclusive evidence that elevated [CO_2] increased the length of the growing season, although the elevated [CO_2] seedlings initiated stem growth slightly earlier than the ambient [CO_2] seedlings (two of the four 'E-N' pine trees at the second harvest on 27 April 1995 had started producing new stem, compared with no stem elongation at this time in all other pine trees).

4.3.1 Stem Height

Scots pine and common alder stem heights responded very differently to elevated [CO_2]. In the common alder there was no significant difference between those plants grown in elevated [CO_2] and those grown in ambient [CO_2] ($p > 0.05$) although there was a trend towards taller trees in elevated [CO_2] in August 1994 on julian day 217 ($p = 0.085$) (see Figure 4.2a). There was no nutrient effect on stem height except on the 29th of July 1996 (julian day 940) (Figure 4.2b) when trees grown with fertiliser were taller than those without ($p = 0.018$). No interaction between [CO_2] and nutrients occurred. Thus the hypothesis that common alder seedlings grown in elevated [CO_2] conditions without fertiliser (i.e. stimulating nitrogen fixation) would grow taller than those in other treatments was rejected.

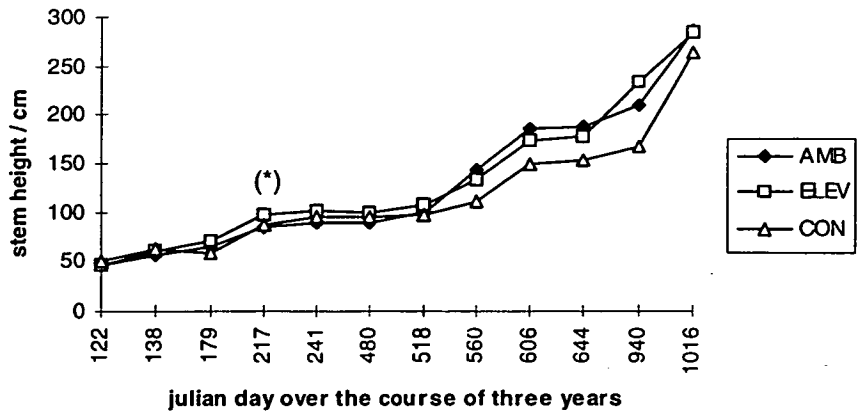


Figure 4.2a Seasonal course of mean stem height in *common alder* over three growing seasons (1994-1996). Time is shown as julian days (i.e. adding 365 days to each consecutive year). Elevated [CO_2] plus and minus nutrients = ELEV; ambient [CO_2] plus and minus nutrients = AMB; control plot plus and minus nutrients = CON; (sample size = 24). (*) = trend towards statistical significance at $p < 0.1$.

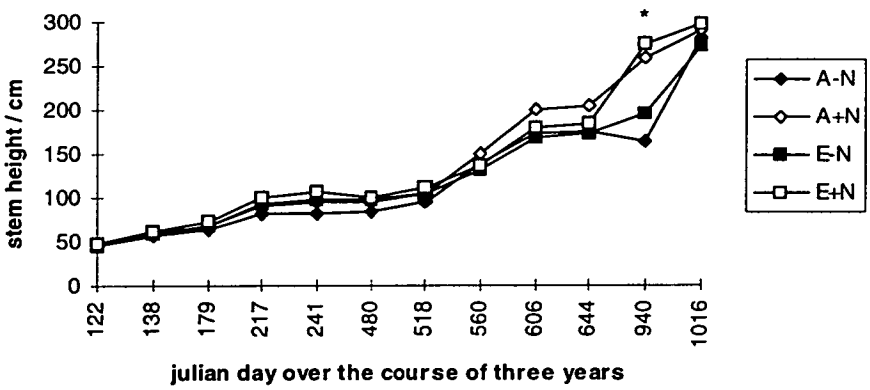


Figure 4.2b Seasonal course of mean stem height of nutrient and [CO_2] treatments in *common alder* over three growing seasons (1994-1996). Time is shown as julian days (i.e. adding 365 to each consecutive year). Ambient minus nutrients = A-N; ambient plus nutrients = A+N; elevated minus nutrients = E-N and elevated plus nutrients = E+N (sample size = 12). * = statsitically significant difference at $p < 0.05$.

In Scots pine, the effect of [CO_2] on stem height was significant at the start of the experiment and continued to be throughout the first two growing seasons (p = 0.0042 to 0.047 with the lowest probabilities in April 1994 and in autumn 1995). In 1996 however, there was no significant [CO_2] effect on height (p = 0.134 and 0.420 on julian days 955 and 1018 respectively) despite the gap between ambient [CO_2] and elevated [CO_2] stem heights widening with time after 1994 (see Figure 4.3a). A nutrient effect was only found on the 19 August (julian day 606) and the 6 October 1995 (julian day 644) (p = 0.045 and 0.02 respectively) (see Figure 4.3b). There was

no interaction between [CO_2] and nutrients. Thus, [CO_2] had a larger effect on stem height in Scots pine than in common alder. Overall, these data confirm the hypothesis that those trees grown in elevated [CO_2] and especially those with fertiliser grow better than seedlings in other treatments at first, followed by acclimation to elevated [CO_2]. Although stem heights in pine in 1996 were larger in elevated [CO_2], the difference was not significant, indicating possible acclimation to elevated [CO_2].

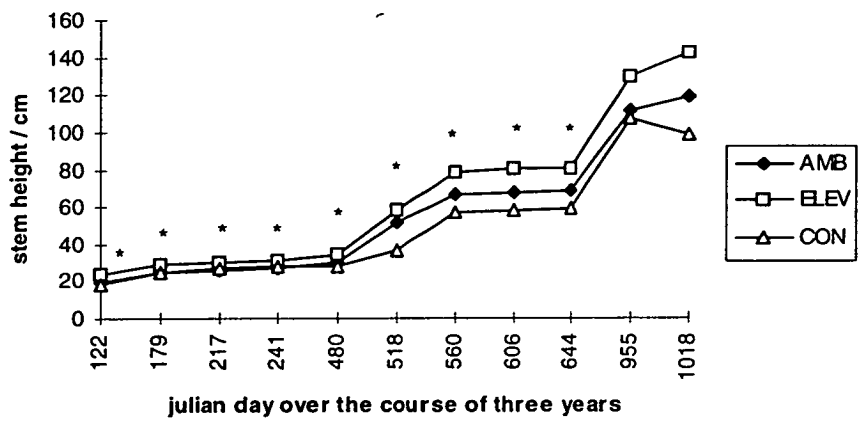


Figure 4.3a Seasonal course of mean stem height in *Scots pine* over three growing seasons (1994-1996). Time is shown as julian days (i.e. adding 365 days to each consecutive year). Elevated [CO_2] = ELEV; ambient [CO_2] = AMB; control plot = CON; (n = 24). * = statistically significant at $p < 0.05$.

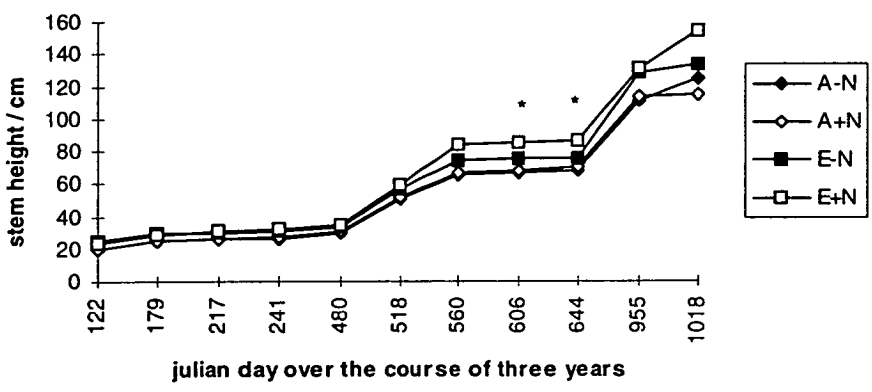


Figure 4.3b Seasonal course of mean stem height of nutrient and [CO_2] treatments in *Scots pine* over three growing seasons (1994-1996). Time is shown as julian days (i.e. adding 365 to each consecutive year). Ambient minus nutrients = A-N; ambient plus nutrients = A+N; elevated minus nutrients = E-N and elevated plus nutrients = E+N (n = 12). * = statistically

An increasing chamber effect on stem height in the common alder was visible in 1994 and 1995 ($p < 0.1$, mostly $p < 0.01$) although this was only a trend in 1996 (see Figure 4.2a). In the Scots pine the chamber effect was only significant after mid July 1995 ($p < 0.05$).

4.3.2 Height Growth Rate

Daily height growth rate in both common alder and Scots pine was increased by exposure to elevated [CO_2]. Elevated [CO_2] alder grew half a centimetre more than ambient [CO_2] alder per day (see Table 4.1). The high growth rate of the elevated minus nitrogen trees is in agreement with the hypothesis that the alder would be able to take advantage of the extra carbon to increase growth via nitrogen fixation. In Scots pine the growth rate per day in elevated [CO_2] was only a mean of 0.1 centimetres higher than in the ambient [CO_2]. This small difference between the ambient and the elevated [CO_2] treatments summed over a whole growing season supports the hypothesis that the Scots pine would not experience a sustained increase in growth (see Figure 4.3a).

Table 4.1 Height growth rate (cm d^{-1}) and mean tree volume in common alder and Scots pine in 1995. Height growth rate was calculated by dividing the difference in stem height between spring and autumn by the number of days in the time period.

height growth rate	alder	pine
Ambient	1.11	0.55
Elevated	1.64	0.65
Control	0.87	0.51
A-N	1.12	0.43
A+N	1.11	0.67
E-N	1.96	0.59
E+N	1.32	0.71

Mean tree volume (cm^3, with one standard error in brackets) was estimated by calculating the stem basal diameter (cm) squared, multiplied by the stem height (cm), or $d^2 \times h$. Sample size per [CO_2] treatment in common alder was n = 32 in April 1995 and 24 in October 1995; for Scots pine sample size per [CO_2] treatment was n = 32 in April 1995 and n = 26 in October 1995. Sample sizes per nutrient and [CO_2] treatment was half that of the [CO_2] treatment.

volume	April 1995	October 1995
common alder		
Ambient	51.45 (8.92)	121.06 (20.84)
Elevated	53.33 (4.08)	141.18 (11.57)
A-N	49.56 (10.22)	99.99 (22.95)
A+N	53.43 (11.41)	142.12 (22.30) *
E-N	50.41 (4.38)	120.54 (17.11)
E+N	56.24 (7.47)	161.82 (12.84) *

volume	April 1995	October 1995
Scots pine		
Ambient	26.14 (2.59)	193.48 (15.98)
Elevated	33.15 (3.12)	334.55 (27.84) *
Control	19.32 (2.40)	144.65 (17.69)
A-N	22.39 (2.62)	153.93 (18.70)
A+N	29.88 (3.96) *	233.00 (24.48) **
E-N	30.26 (2.89)	258.10 (19.73)
E+N	36.03 (3.78) *	411.01 (45.96) **

[CO_2] only had a statistically significant effect on mean tree volume in Scots pine in October 1995 (p = 0.0014), whereas there was no effect in common alder ($p > 0.05$). Nutrient addition on the other hand, had a significant effect in both species in October 1995 and in common alder in April 1995, with those trees given fertiliser growing

bigger than those without. There was no chamber effect on tree volume in either species ($p > 0.05$).

4.3.3 Stem Basal Diameter (SBD)

Stem basal diameter (SBD) in both species of tree responded positively to elevated [CO_2] over the course of the three growing seasons (see Figures 4.4a and b, and 4.5a and b). In common alder, although not initially, [CO_2] had a significant effect on SBD from autumn 1995 (October) onwards, continuing throughout 1996 ($p = 0.0349$, and $p = 0.0029$ in July '96, $p = 0.0136$ in October 1996). However, the mean ambient SBD mean was larger than the mean elevated SBD mean in October 1996. There was no nutrient effect on SBD, or interaction between nutrients and [CO_2] at any time during the experiment (see Figure 4.4b). These results support the hypothesis that the alder would grow bigger in response to the extra availability of carbon with respect to SBD, although there is an indication that acclimation was occurring in October 1996.

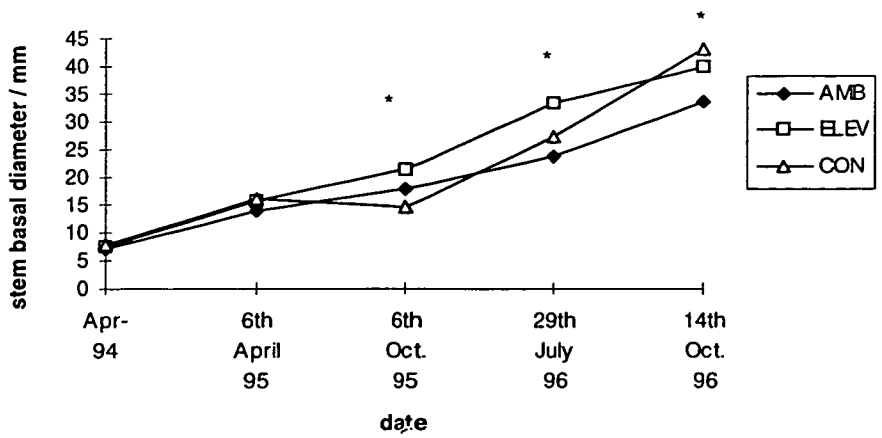


Figure 4.4a Seasonal course of mean stem basal diameter in *common alder* over three growing seasons. Elevated [CO_2] = ELEV; ambient [CO_2] = AMB; control plot = CON; n = 96 in 1994, 80 and 36 in 1995, and 8 in 1996. [CO_2] had a significant effect from October 1995 onwards (* = $p < 0.05$).

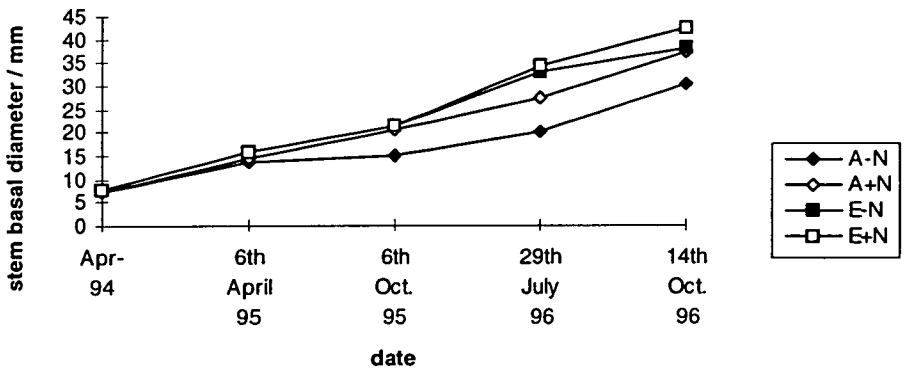


Figure 4.4b Seasonal course of mean stem basal diameter in *common alder* over three growing seasons for nutrient and [CO_2] treatments. Ambient minus or plus nutrients = A-N or A+N; elevated minus or plus nutrients = E-N or E+N. n = 48 in 1994, 40 and 18 in 1995 and 4 in 1996. There was no significant nutrient effect at any point ($p < 0.05$).

The Scots pine SBD data also showed a clear difference between the main [CO_2] treatments. Significant differences in SBD between elevated [CO_2] and ambient [CO_2] trees were found at the beginning of the experiment (p = 0.032), in October 1995 (p = 0.0068) and throughout 1996 (p = 0.040 and 0.037). There was a nutrient effect only in October 1995 (p = 0.0002) (see Figure 4.4b). On the same date there was an interaction between nutrients and [CO_2] (p = 0.06) as well as in April 1995 (p = 0.016). Overall, this does not support the hypothesis that the pine undergoes an initial spurt in growth which is subject to negative feedback after a certain time point.

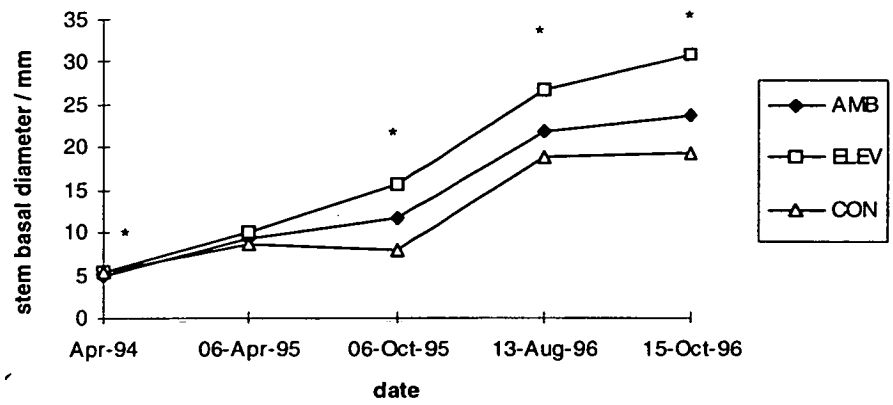


Figure 4.5a Seasonal course of mean stem basal diameter in *Scots pine* over three growing seasons. Ambient [CO_2] = AMB; elevated [CO_2] = ELEV; control plot = CON. n = 96 in 1994, 80 and 36 in 1995, and 8 in 1996. [CO_2] had a significant effect (* = $p < 0.05$) at all dates except April 1995.

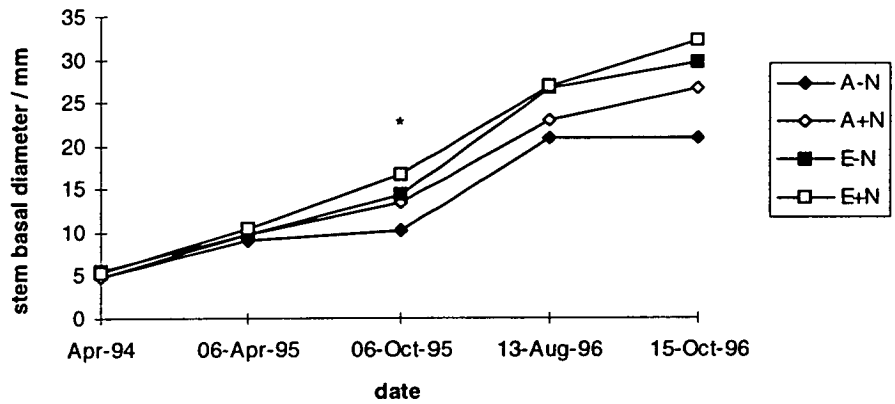


Figure 4.5b Seasonal course of mean stem basal diameter in *Scots pine* over three growing seasons for nutrient and [CO_2] treatments. Ambient minus nutrients, plus nutrients = A-N or A+N respectively; elevated plus or minus nutrients = E+N or E-N. n = 48 in 1994, 40 or 18 in 1995, and 4 in 1996. Nutrient effect was significant (* = $p < 0.05$) in October 1995 only.

The presence of chambers had a significant effect on alder at the beginning and at the end of the experiment (p = 0.078 and 0.0012, respectively), but on the pine only at the start of the experiment (p = 0.018 and 0.096 in the spring of 1994 and 1995, respectively).

4.3.4 Stem Basal Area (SBA)

Stem basal area (SBA) was consistently higher in elevated [CO_2] trees than in ambient [CO_2], although the difference was only statistically significant in October 1995 (p = 0.028 for alder and p = 0.0019 for pine). There was also a significant nutrient effect in October 1995 in both species (p = 0.041 in alder, and p = 0.0001 in pine), but again not in April 1995 ($p > 0.05$). There was an interaction effect in Scots pine in both April and October 1995 (p = 0.026 and 0.029, respectively).

Table 4.2 Mean stem basal area (mm^2) in common alder and Scots pine in April and October 1995. Values are the means (and one standard error in brackets) of 80 measurements in April in both species, and 28 measurements in alder, 36 measurements in pine in October for the [CO_2] treatments, sample size = half these values for the nutrient and [CO_2] treatments (i.e. A-N etc.).

Treatment	April	October
common alder		
Ambient	164.66 (9.160)	435.63 (36.94)
Elevated	205.82 (10.65)	554.65 (33.64)
Control	211.36 (9.10)	499.01 (24.49)
A-N	160.05 (14.93)	374.91 (49.59)
A+N	169.52 (10.50)	496.35 (51.64)
E-N	204.02 (13.80)	519.72 (43.33)
E+N	207.63 (16.40)	591.76 (51.70)
C-N	204.68 (12.29)	464.37 (30.45)
C+N	217.88 (13.22)	524.21 (35.24)

Table 4.2 continued

Treatment	April	October
Scots pine		
Ambient	73.04 (4.97)	221.84 (13.24)
Elevated	81.93 (3.44)	323.72 (18.81)
Control	63.37 (4.10)	197.62 (12.71)
A-N	66.71 (5.60)	179.13 (13.11)
A+N	79.37 (8.16)	262.41 (18.74)
E-N	76.85 (4.36)	259.07 (18.37)
E+N	87.14 (5.29)	394.54 (26.59)
C-N	70.84 (7.36)	205.41 (14.92)
C+N	55.90 (3.23)	190.43 (20.57)

4.4 Total Biomass

Cumulative biomass of all trees harvested (the sum of harvest means from September 1994 to April 1996) was higher in elevated [CO_2] in both species. Elevated [CO_2] cumulative biomass in alder was 1390.4 g compared with 1008.5 g in ambient [CO_2] and 1116.5 g in the control. In Scots pine the same treatment means were 375.5 g, 409.3 g and 289.3 g, respectively.

Overall, growth in elevated [CO_2] stimulated total biomass in common alder by an average of 59.1 % , and 33.5 % in Scots pine (see Table 4.3). Although these percentage increases in total biomass were considerable they were not always statistically significant. In common alder significant differences in total biomass due to [CO_2] were found in September 1994 and June 1995 (p = 0.059 and 0.002 respectively) but not in April, August or December 1995 ($p > 0.1$). No alder root data were available after April 1996 but the total above-ground biomass was statistically different in May and July (p = 0.084 and 0.042, respectively). There was a tendency towards a nutrient effect in April 1996 (p = 0.087) and an effect in July 1996 (p = 0.016). No interaction between [CO_2] and nutrients was found. These data over three

growing seasons indicate that the alder was, as hypothesised, able to grow larger in response to elevated [CO_2] although the difference in total biomass was not always statistically significant. In addition, mean total biomass of the elevated [CO_2] trees was lower than that in ambient in the spring of both seasons. The response was therefore very seasonal and was largest in September 1994 and June 1995. It should also be noted that the alder suffered fungal attack (*Taphrina tosquinetti*) in the summer of 1995 (peaking in August) such that the loss of vigour could have affected total biomass results.

Table 4.3 Percentage increase in total biomass in common alder and Scots pine over time. Values were calculated by subtracting the ambient [CO_2] mean from the elevated [CO_2] mean, dividing by the ambient mean and multiplying by 100.

date	alder	pine
September 1994	54.48	47.76
April 1995	66.91	48.26
June 1995	101.03	27.80
August 1995	23.32	5.27
Dec. / Oct 1995	49.85	16.11
April 1996	29.87 (negative)	13.71 (negative)
October 1996	-----	55.67
mean % increase	59.12	33.48

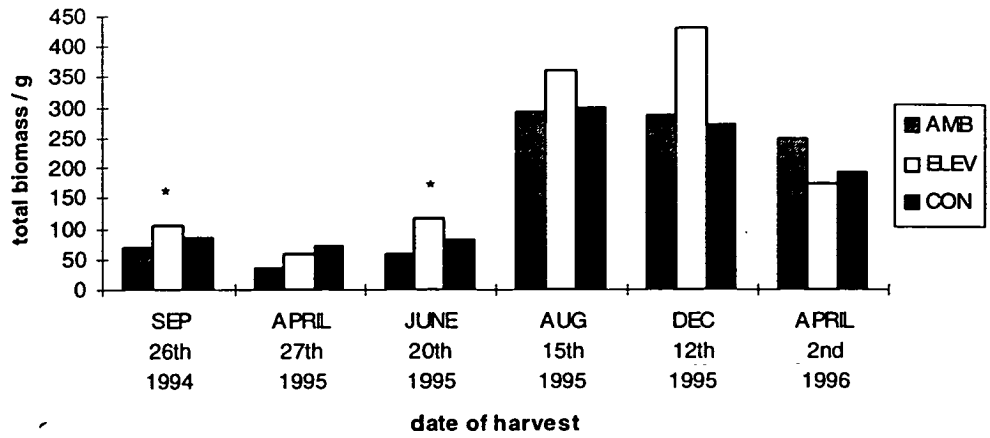


Figure 4.6a Seasonal course of mean total biomass (g) in *common alder*. Values are the means of eight replicates. Ambient [CO_2] = AMB; elevated [CO_2] = ELEV; contol plot = CON. [CO_2] had a significant effect in Sep. 94 and June 95 (* = $p < 0.05$).

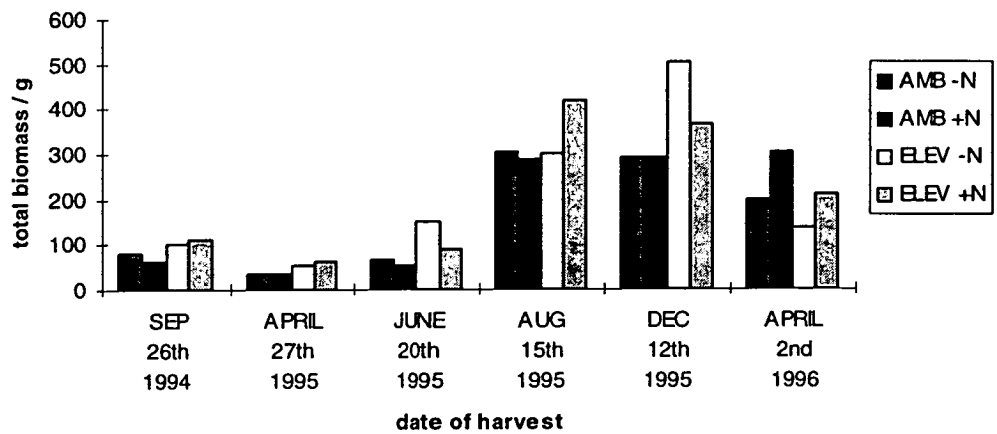


Figure 4.6b Seasonal course of mean total biomass (g) for nutrient and [CO_2] treatments in *common alder*. Values are the means of four replicates. Ambient plus and minus nutrients = A-N and A+N; elevated plus and minus nutrients = E-N and E+N. There was no nutrient effect at any time ($p < 0.05$).

In Scots pine there was a significant difference in total biomass between ambient and elevated [CO_2] trees in September 1994 ($p = 0.012$), in April 1995 ($p = 0.04$) and in October 1996 ($p = 0.061$), but not at any other time in-between (see Figures 4.7a and b). Although there were no data for total biomass in May and July 1996, aboveground pine biomass in July was significantly affected by [CO_2]. Nutrient treatments only had a significant effect on total biomass in October 1995 ($p = 0.030$) but there was a trend towards a nutrient effect in October 1996 ($p = 0.08$). No interaction between [CO_2] and nutrients was found. As in common alder, total biomass of Scots pine was

affected by [CO_2] differently depending on the time of year. The hypothesis that Scots pine shows an initial increase in growth followed by negative feedback is partly confirmed by these data since there was a significant difference in total biomass in September 1994 and in April 1995. On the other hand, there was a significant difference in October 1996 at the end of the experiment which does not support this hypothesis.

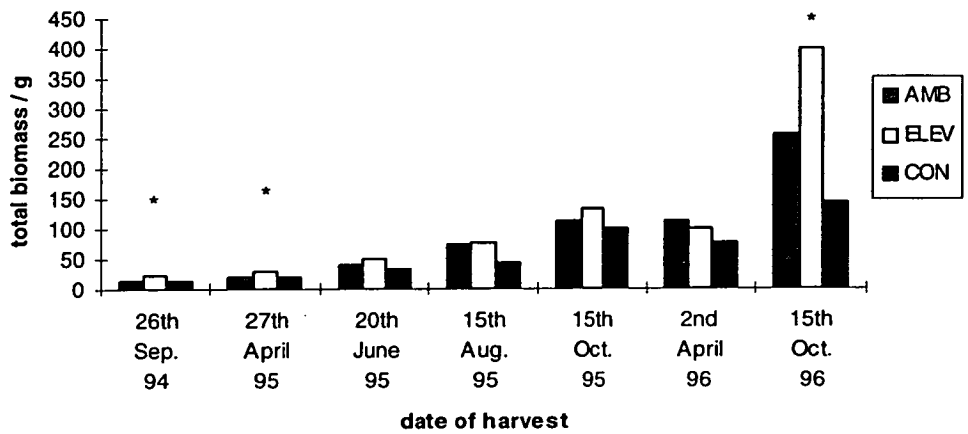


Figure 4.7a Seasonal course of mean total biomass (g) in *Scots pine*. Values are the means of eight replicates. Ambient [CO_2] = AMB; elevated [CO_2] = ELEV; contol plot = CON. [CO_2] had a significant effect in Sep. 94, April 95 and Oct. 96 (* = $p < 0.05$).

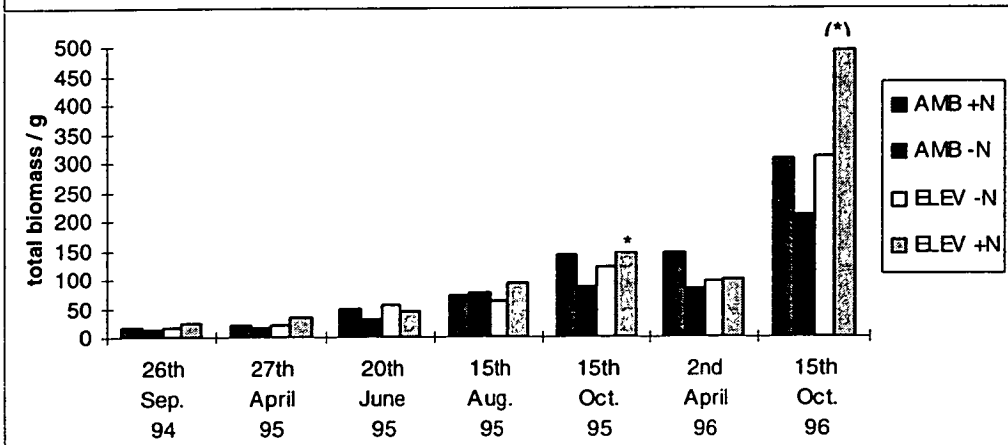


Figure 4.7b Seasonal course of mean total biomass (g) for nutrient and [CO_2] treatments in *Scots pine*. Values are the means of four replicates. Ambient plus and minus nutrients = A-N and A+N; elevated plus and minus nutrients = E-N and E+N. * = statistically significant at $p < 0.05$, (*) = a trend towards a significant effect at $p < 0.1$.

There was no chamber effect on total biomass in the common alder at any time in the three growing seasons ($p < 0.05$). In Scots pine there was tendency towards a chamber effect in June 1995 ($p = 0.065$) as well as an effect on the total above-ground biomass in July 1996 ($p = 0.025$). Overall, the presence of OTCs did not affect total biomass much.

4.5 Allocation

4.5.1 Biomass allocation

In common alder there was a significant [CO_2] effect on biomass allocation to leaves in April and June and August 1995 ($p = 0.054$, 0.0017 and 0.083, respectively) (see Table 4.4 and section on Leaf Mass Ratio below for more detail). In June 1995 there was a trend towards an effect on allocation to 1994 shoots ($p = 0.068$).

There was a nutrient effect on allocation to 1994 shoot in September 1994 ($p = 0.02$) and on allocation to leaves in June 1995 ($p = 0.0018$) but not on any other tissue allocation. There was no interaction effect of [CO_2] and nutrients.

Table 4.4 Biomass allocation to different tissues in common alder in the September 1994 harvest. Values are mean tissue biomass fractions (*R* root, *L* leaf, *S* shoot) with standard errors in brackets, of eight samples for [CO_2] and four samples for nutrient and [CO_2].

Treatment	*R*	*L*	1993 *S*	1994 *S*
Ambient	0.36 (0.057)	0.24 (0.044)	0.24 (0.032)	0.22 (0.081)
Elevated	0.40 (0.021)	0.16 (0.034)	0.25 (0.036)	0.23 (0.036)
Control	0.42 (0.039)	0.16 (0.026)	0.28 (0.023)	0.15 (0.043)
A-N	0.35 (0.044)	0.21 (0.021)	0.20 (0.039)	0.22 (0.081)
A+N	0.36 (0.069)	0.26 (0.067)	0.28 (0.025)	0.22 (0.081)
E-N	0.36 (0.038)	0.17 (0.031)	0.31 (0.082)	0.16 (0.032)
E+N	0.43 (0.040)	0.14 (0.036)	0.18 (0.032)	0.30 (0.039)
C-N	0.43 (0.040)	0.14 (0.039)	0.30 (0.017)	0.13 (0.049)
C+N	0.40 (0.037)	0.17 (0.012)	0.26 (0.029)	0.17 (0.037)

Photos 4.1- 4.3 Common alder at different stages of the 1995 growing season. 4.1 An alder seedling in an OTC in bud in April 1995. 4.2 Alder in full leaf in August 1995. 4.3 A harvested alder tree in December 1995.

In Scots pine there was no effect of elevated [CO_2] on biomass allocation at any time in 1995 except on allocation to 1993 needles in September 1994 (p = 0.024) (see Table 4.5). There was a trend towards a nutrient effect in September 1994 on allocation to 1994 needles (p = 0.068) and a significant effect in June 1995 (p = 0.03). There was also a trend towards a nutrient effect on 1994 stem and total needle mass in October 1995 (p = 0.075 and 0.071 respectively). There was an interaction effect of nutrients and [CO_2] on 1995 stem and 1995 needle allocation in June 1995 (p = 0.048 and 0.068 respectively). There was also trend towards an interaction effect on 1994 stem in October 1995 (p = 0.075).

Table 4.5 Biomass allocation to different tissues in Scots pine in the September 1994 harvest. Values are mean tissue biomass fractions (*R* root, *N* needle, *S* shoot) with standard errors in brackets, of eight sample for [CO_2] and four samples for nutrient and [CO_2]. S = shoot; N = needles.

Treatment	*R*	*1993 S*	*1994 S*	*1993 N*	*1994 N*
Ambient	0.19 (0.033)	0.14 (0.012)	0.17 (0.063)	0.09 (0.014)	0.48 (0.037)
Elevated	0.19 (0.020)	0.15 (0.019)	0.17 (0.039)	0.05 (0.017)	0.49(0.048)
Control	0.28 (0.073)	0.19 (0.032)	0.15 (0.0081)	0.081 (0.014)	0.40 (0.053)
A-N	0.15 (0.019)	0.14 (0.012)	0.16 (0.084)	0.071 (0.016)	0.51 (0.056)
A+N	0.22 (0.046)	0.14 (0.012)	0.18 (0.042)	0.10 (0.012)	0.45 (0.018)
E-N	0.17 (0.029)	0.13 (0.020)	0.14 (0.047)	0.066 (0.022)	0.50 (0.062)
E+N	0.20 (0.010)	0.16 (0.018)	0.20 (0.031)	0.034 (0.011)	0.48 (0.034)
C-N	0.20 (0.0051)	0.18 (0.0088)	0.15 (0.010)	0.077 (0.0035)	0.46 (0.055)
C+N	0.35 (0.14)	0.20 (0.055)	0.15 (0.0061)	0.084 (0.024)	0.33 (0.050)

Leaf Mass Ratio (LMR)

Exposure to elevated [CO_2] affected the mean allocation to leaves, LMR, in Scots pine and common alder differently. In alder elevated [CO_2] had a significant effect on LMR, lowering it throughout the whole of 1995 ($p < 0.05$) except in December (p = 0.13). Mean LMR in June 1995 in elevated [CO_2] was 0.24 g g^{-1} (SE 0.018) whereas in ambient [CO_2] it was 0.31 g g^{-1} (SE 0.019). In August 1995 mean LMR in elevated [CO_2] was 0.24 g g^{-1} (SE 0.009) versus 0.29 g g^{-1} (SE 0.051) in ambient [CO_2] and

0.30 g g^{-1} (SE 0.030) in the control trees. There was no nutrient effect or interaction effect of the former with [CO2] on LMR in alder ($p > 0.1$). Thus the alder was allocating less biomass to the leaves during the 1995 growing season.

In Scots pine there was no significant [CO_2] effect on LMR at any time in 1995 ($p > 0.1$). Mean LMR in August 1995 in elevated [CO_2] was 0.50 (SE 0.029) compared with 0.49 (SE 0.018) in ambient [CO_2] and 0.51 (SE 0.010) in the control. There was a trend towards a nutrient effect on LMR in October 1995 ($p = 0.072$). No interaction effect was found ($p > 0.1$).

The presence of chambers had a very significant effect on LMR in alder in April and June 1995 ($p = 0.0006$ and 0.0006, respectively). In Scots pine there was a significant effect in the same time periods ($p = 0.028$ and 0.042, respectively).

Number of Branches

Neither [CO_2], nutrient addition or the interaction between them had a significant effect on number of branches in either species of tree ($p > 0.1$). In July 1996 the mean number of alder branches in elevated [CO_2] was 41.13 (SE 3.56) compared with 40.53 (SE 3.55) in ambient [CO_2] and 43.83 (SE 5.31) in the control plot. In July 1996 the mean number of branches in Scots pine grown in elevated [CO_2] was 20.4 (SE 3.5) compared with 17.5 (SE 2.56) in ambient [CO_2] and 17.0 (SE 0.91) in the control plot. Elevated [CO_2] increased the number of branches in the pine, if not significantly, but not in the alder.

There was no chamber effect on branch number in either species ($p > 0.05$).

Leaf to Shoot Ratio (LF / SH)

Elevated [CO_2] had no significant effect on leaf to shoot ratio in June 1995 and August 1995 in Scots pine ($p > 0.1$). In common alder, however there was a significant [CO_2] effect on *LF / SH* in June 1995 ($p = 0.046$), if not in August 1995 ($p = 0.507$) (see Table 4.6). There was a nutrient effect and interaction effect between [CO_2] and nutrient addition in alder in June 1995 ($p = 0.019$ and 0.055, respectively).

There was no significant chamber effect on *LF / SH* except in alder in June 1995 ($p = 0.0016$).

Root to Leaf Ratio (RT / LF)

There was no significant effect of $[CO_2]$ on *RT / LF* in either species in June or August 1995 ($p > 0.1$). In August 1995 there was a trend towards a $[CO_2]$ effect on *RT / LF* in alder ($p = 0.094$) (see Table 4.6). There was no nutrient or interaction effect between the former and $[CO_2]$ on *RT / LF* in either species.

No significant chamber effect on *RT / LF* was found in either species in June or August 1995 ($p > 0.1$).

Table 4.6 Mean leaf to shoot ratio (*LF / SH*) and root to leaf ratio (*RT / LF*) in common alder and Scots pine in June and August 1995. Values are the means (with one standard error in brackets) of eight samples.

Treatment	June	August
LF / SH		
common alder		
Ambient	0.80 (0.058)	0.58 (0.02)
Elevated	0.59 (0.038)	0.54 (0.08)
Control	0.40 (0.091)	0.64 (0.04)
Scots pine		
Ambient	1.090 (0.060)	1.27 (0.07)
Elevated	1.020 (0.090)	1.32 (0.12)
Control	0.95 (0.070)	1.28 (0.03)

Table 4.6 continued

RT / LF		
common alder		
Ambient	1.12 (0.08)	0.81 (0.084)
Elevated	1.41 (0.12)	2.34 (1.08)
Control	3.92 (1.51)	0.91 (0.22)
Scots pine		
Ambient	0.30 (0.030)	0.24 (0.020)
Elevated	0.31 (0.043)	0.24 (0.028)
Control	0.36 (0.018)	0.25 (0.010)

4.5.2 Root to Shoot Mass Ratio (*R / S*)

The ratio of root to shoot was not significantly affected by elevated [CO_2] in either species of tree (see Table 4.6 and 4.7). In common alder, in August 1995 there was a tendency ($p = 0.063$) towards trees grown in elevated [CO_2] allocating more mass to the roots than those grown in ambient [CO_2], but not at any other harvest ($p < 0.1$). There was no nutrient effect or interaction effect of nutrients and [CO_2] on *R / S*. In Scots pine, there was no significant effect on *R / S* of either [CO_2] or nutrients or any interaction between them (see Table 4.7). Ontogenetic differences (which would be visible in trees of the same size but of different age) were not considered further, since there was no significant difference between ambient and elevated [CO_2] trees of the same age, possibly because differences in size were small.

Table 4.6 Mean root to shoot mass ratio including leaves (with one standard error in brackets) in common alder over time. Values are the means of eight samples for [CO_2] treatment, and four samples for the nutrient and [CO_2] treatments.

Treatment	1994	1995				1996
	September	April	June	August	December	April
Ambient	0.59 (0.095)	0.94 (0.12)	0.53 (0.034)	0.30 (0.030)	1.56 (0.56)	0.47 (0.047)
Elevated	0.67 (0.050)	0.99 (0.092)	0.49 (0.037)	0.45 (0.056)	0.61 (0.19)	0.78 (0.20)
Control	0.70 (0.070)	0.91 (0.083)	0.61 (0.041)	0.35 (0.083)	1.44 (0.81)	0.61 (0.12)
A-N	0.56 (0.12)	0.79 (0.083)	0.47 (0.070)	0.28 (0.032)	1.22 (0.82)	0.53 (0.054)
A+N	0.62 (0.17)	1.10 (0.14)	0.58 (0.15)	0.32 (0.05)	1.89 (0.84)	0.40 (0.070)
E-N	0.58 (0.95)	1.10 (0.14)	0.50 (0.070)	0.50 (0.10)	0.41 (0.03)	1.06 (0.47)
E+N	0.75 (0.012)	0.88 (0.11)	0.48 (0.039)	0.41 (0.05)	0.81 (0.37)	0.58 (0.05)
C-N	0.75 (0.12)	0.95 (0.13)	0.61 (0.041)	0.30 (0.041)	0.70(0.07)	0.47 (0.081)
C+N	0.67 (0.09)	0.86 (0.12)	0.61 (0.08)	0.40 (0.15)	2.17 (1.64)	0.84 (0.21)

Table 4.7 Mean root to shoot mass ratio including needles (with one standard error in brackets) in Scots pine over time. Values are the means of eight samples for [CO_2] treatment, and four samples for the nutrient and [CO_2] treatments.

Treatment	1994	1995				1996
	September	April	June	August	December	April
Ambient	0.24 (0.046)	0.26 (0.020)	0.15 (0.020)	0.13 (0.010)	0.36 (0.19)	0.20 (0.030)
Elevated	0.23 (0.025)	0.24 (0.009)	0.15 (0.015)	0.13 (0.010)	0.23 (0.070)	0.22 (0.030)
Control	0.63 (0.38)	0.24 (0.016)	0.16 (0.01)	0.14 (0.0057)	0.18 (0.015)	0.19 (0.010)
A-N	0.18 (0.026)	0.27 (0.039)	0.18 (0.028)	0.13 (0.021)	0.56 (0.38)	0.22 (0.055)
A+N	0.30 (0.083)	0.25 (0.015)	0.12 (0.0052)	0.13 (0.0081)	0.16 (0.019)	0.18 (0.012)
E-N	0.21 (0.042)	0.25 (0.015)	0.15 (0.092)	0.12 (0.022)	0.30 (0.130)	0.20 (0.017)
E+N	0.26 (0.024)	0.23 (0.011)	0.14 (0.031)	0.14 (0.019)	0.16 (0.015)	0.24 (0.054)
C-N	0.25 (0.0080)	0.22 (0.014)	0.17 (0.0060)	0.14 (0.012)	0.17 (0.015)	0.17 (0.016)
C+N	0.84 (0.060)	0.25 (0.029)	0.15 (0.026)	0.14 (0.0037)	0.19 (0.028)	0.20 (0.012)

4.6 Leaf Area, Leaf Area Ratio and Net Assimilation Rate

4.6.1 Leaf Area

There was no significant effect of $[CO_2]$ on alder leaf area in either April, June August or December 1995 ($p > 0.1$). There was also no nutrient or interaction effect of nutrients and $[CO_2]$ at any of these harvests ($p > 0.1$).

Elevated $[CO_2]$ had a temporary effect on needle area in Scots pine. There was a significant $[CO_2]$ effect on needle area in April 1995 ($p = 0.0073$) but not at later harvest dates ($p > 0.1$) (see Table 4.8). Nutrient addition had a significant effect in October 1995 ($p = 0.0019$), but not at any other time ($p > 0.1$). There was no interaction effect on $[CO_2]$ and nutrient addition at any time point ($p > 0.1$).

There was no chamber effect on leaf area in either species of tree at any harvest in 1995 ($p > 0.1$).

Table 4.8a Mean total leaf area (cm^2) in common alder in 1995 (with one standard error in brackets). Values are the means of eight samples per [CO_2] treatment and four samples per [CO_2] and nutrient treatment.

Treatment	April	June	August	December
common alder				
Ambient	135.85 (20.04)	1109.15 (151.72)	5257.58 (644.39)	--
Elevated	138.18 (28.50)	1511.79 (300.78)	4787.51 (744.26)	--
Control	117.25 (22.29)	805.75 (184.90)	5475.58 (763.63)	
A-N	129.78 (30.17)	1069.14 (291.76)	5680.97 (1119.54)	--
A+N	141.91 (30.64)	1149.16 (145.71)	4834.20 (751.60)	--
E-N	142.29 (44.78)	1965.56 (458.52)	3984.12 (737.39)	--
E+N	134.08 (27.67)	1058.02 (273.26)	5858.70 (1529.66)	--
C-N	127.51 (27.67)	503.25 (147.59)	6121.35 (964.35)	--
C+N	109.55 (36.19)	1108.25 (277.05)	4829.80 (1230.18)	--

Table 4.8b Mean total leaf area (cm^2) in Scots pine in 1995 (with one standard error in brackets). Values are the means of eight samples per [CO_2] treatment and four samples per [CO_2] and nutrient treatment.

Treatment	April	June	August	October
Scots pine				
Ambient	59.67 (4.77)	108.19 (22.12)	217.90 (13.18)	345.64 (58.80)
Elevated	106.89 (16.44)	153.75 (20.97)	256.23 (34.18)	403.71 (48.28)
Control	63.36 (7.60)	88.97 (16.56)	189.47 (32.98)	308.56 (33.60)
A-N	56.20 (5.19)	81.27 (23.24)	159.21 (37.41)	262.28 (40.65)
A+N	63.14 (8.44)	135.12 (35.49)	222.96 (11.48)	428.98 (99.27)
E-N	70.72 (16.24)	150.57 (24.02)	286.34 (29.94)	494.63 (42.09)
E+N	143.06 (11.15)	156.94 (38.32)	286.34 (29.94)	494.63 (42.09)
C-N	61.61 (8.70)	76.10 (12.68)	226.13 (62.85)	245.92 (22.68)
C+N	65.70 (15.70)	101.84 (31.76)	219.73 (55.88)	371.21 (46.23)

4.6.2 Leaf Area Ratio (LAR)

Elevated [CO_2] had a significant effect on LAR in common alder in the first half but not in the second half of 1995 (Table 4.6). Elevated [CO_2] had a significant effect on LAR in common alder in April and June 1995 (p = 0.0087 and 0.0057, respectively). In August 1995, however, there was only a trend towards an effect (p = 0.074 and 0.082, respectively). There was a nutrient effect and an interaction effect on LAR in June 1995 (p = 0.0018 and 0.052, respectively). Thus elevated [CO_2] reduced LAR in alder compared with ambient [CO_2] trees, i.e. the elevated trees had more biomass per unit leaf area than the ambient.

In Scots pine elevated [CO_2] had a significant effect on LAR in April and August 1995 (p = 0.022 and 0.016. respectively) but not in-between ($p > 0.1$). There was a nutrient effect on LAR in August and October 1995 (p = 0.013 and 0.029, respectively). There was only an interaction effect between [CO_2] and nutrient addition on LAR in October 1995 (p = 0.052).

There was a large chamber effect on LAR in alder in April 1995 (p = 0.0003) and in June 1995 (p = 0.0005) but not in August or December ($p > 0.1$). In Scots pine there was only a chamber effect on LAR in August 1995 (p = 0.047).

Table 4.10 Mean LAR (m^2 kg^{-1}) of [CO_2] treatments in common alder and Scots pine in 1995 (with one standard error in brackets). Values are the means of eight samples per [CO_2] treatment.

Treatment	April	June	August
common alder			
Ambient	4.09 (0.80)	18.71 (1.27)	17.60 (0.52)
Elevated	2.78 (0.52)	12.71 (0.67)	15.05 (1.27)
Control	1.74 (0.26)	10.26 (1.86)	17.79 (1.36)

Table 4.10 continued

Treatment	April	June	August	October
Scots pine				
Ambient	3.11 (0.083)	2.69 (0.072)	2.95 (0.080)	3.12 (0.25)
Elevated	3.70 (0.21)	3.02 (0.20)	3.40 (0.16)	3.05 (0.19)
Control	2.91 (0.30)	2.54 (0.22)	3.35 (0.13)	3.05 (0.19)

4.6.3 Net Assimilation Rate (NAR)

In common alder there was a significant effect of elevated [CO_2] on NAR between April and December 1995 ($p = 0.029$). There was no nutrient or interaction effect on NAR in alder ($p > 0.1$). In Scots pine on the other hand, elevated [CO_2] had no significant effect on NAR ($p = 0.45$), but there was a very significant interaction effect on NAR in Scots pine ($p = 0.0075$). There was however no nutrient effect on NAR in Scots pine ($p > 0.1$).

There was no chamber effect on NAR in pine ($p > 0.1$) and there were no control data for alder.

Table 4.11 Mean NAR (mg m^{-2} d^{-1}) in common alder and Scots pine between April 1995 and December (alder) or October 1995 (pine). Values are the means (one standard error in brackets) of eight samples for the [CO_2] treatments, and four samples for the nutrient and [CO_2] treatment.

Treatment	alder	pine
Ambient	14.80 (1.94)	33.05 (3.39)
Elevated	23.46 (1.53)	29.96 (2.99)
Control	--	32.17 (2.09)
A-N	14.33 (2.024)	28.53 (4.61)
A+N	15.28 (3.65)	37.58 (4.33)
E-N	23.06 (2.75)	37.00 (2.88)
E+N	23.86 (1.81)	22.92 (0.66)
C-N	--	33.17 (3.47)
C+N	--	30.84 (3.07)

4.7 Specific Growth Rate (SGR)

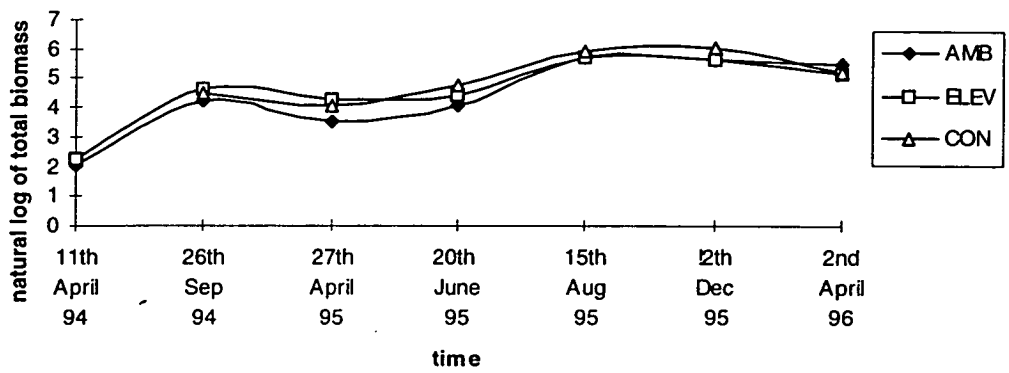


Figure 4.8a Natural log of total biomass against time in *common alder* over three growing seasons. Values are the means of eight replicates except for April 1994 where *n* = 6. AMB = ambient [CO_2] plus and minus nutrients; ELEV =elevated [CO2] plus and minus nutrients; CON = control plot plus and minus nutrients.

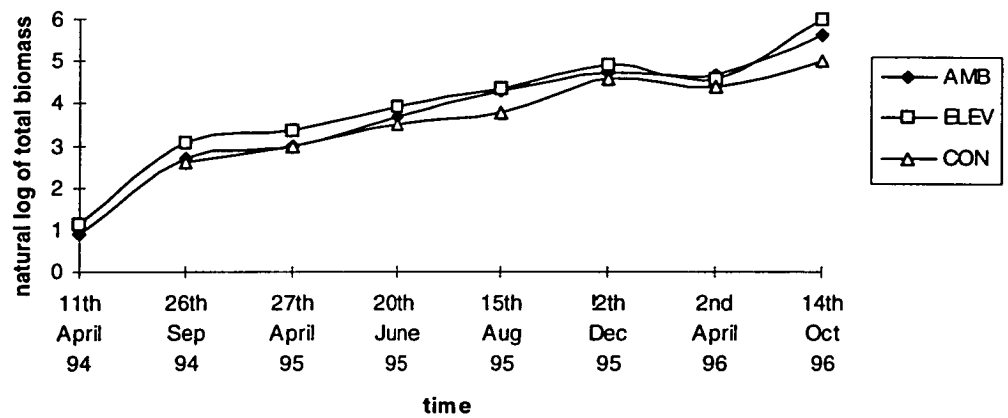


Figure 4.8b Natural log of total biomass against time in *Scots pine* over three growing seasons. Values are the means of eight replicates except in April 1994 when *n* = 6.

SGR in common alder was measured between April and December 1995. In Scots pine mean SGR of total biomass was measured between April 1994 and October 1996 ($p = 0.18$) as well as between the same 1995 months as for alder ($p = 0.54$). Elevated [CO_2] had no significant effect on SGR of total biomass, shoot biomass (including leaves) or root biomass in either species of tree ($p > 0.1$). In Scots pine, SGR of total biomass, shoot mass and root mass in elevated [CO_2] was lower than in ambient [CO_2] (see Table 4.12). In contrast, in alder mean SGR was higher in elevated than in ambient [CO_2] except in the roots, where mean SGR was lower (see Table 4.11). If one looks at

Figures 4.8a and 4.8b showing the natural logarithm of total biomass per harvest over time, however it is visible that SGR was slightly higher in the elevated [CO_2] treatment (with a steeper slope of the line) from April 1994 to about September 1994 in both species, but especially in pine. These data do not agree completely with SGR data calculated by pairing trees of similar stature, since the former values are mean total biomass values of all treatment trees over time (i.e. SGR of quite varied individual trees) and the latter involved a more accurate analysis of SGR in similar trees.

The only significant effect, interestingly, was on the interaction between [CO_2] and nutrients in both species. In alder there was an almost significant interaction effect on SGR of total biomass (p = 0.058). In pine there was a significant interaction effect on total biomass and shoot SGR (p = 0.0084 and 0.042, respectively). In both species it seems that those trees grown in elevated [CO_2] without fertiliser addition (E-N) were more stimulated in their specific growth rate, than would be expected by [CO2] or nutrient treatment alone. This was especially the case with the SGR of total biomass in both species, as well as with the SGR of shoots in pine.

Table 4.11 Mean SGR (mg g^{-1} d^{-1}) of total, shoot and root biomass in common alder between April and December 1995, with one standard error in brackets. Values are the means of data; sample size = eight per [CO_2] treatment and sample size = four per nutrient and [CO_2] treatment.

Treatment	total biomass	shoot mass	root mass
Ambient	9.51 (0.48)	8.93 (0.92)	9.26 (1.50)
Elevated	9.58 (0.91)	10.62 (1.15)	7.72 (1.15)
Control	5.88 (0.38)	5.67 (0.97)	5.46 (0.73)
A-N	9.43 (0.36)	8.76 (1.050)	9.03 (2.48)
A+N	9.59 (0.98)	8.76 (1.050)	9.49 (2.09)
E-N	10.77 (1.32)	12.48 (1.30)	8.27 (1.45)
E+N	8.38 (1.10)	8.76 (1.47)	7.18 (1.96)
C-N	5.02 (0.37)	5.60 (0.67)	4.34 (0.44)
C+N	6.75 (0.14)	5.75 (2.00)	6.58 (1.22)

Table 4.12 Mean SGR (mg g^{-1} d^{-1}) of total, shoot and root biomass in Scots pine between April and December 1995, with one standard error in brackets. Values are the means of data; sample size = eight per [CO_2] treatment and sample size = four per nutrient and [CO_2] treatment.

treatment	total biomass	shoot biomass	root biomass
Ambient	9.97 (0.84)	10.50 (0.80)	8.80 (0.71)
Elevated	9.37 (0.59)	9.47 (0.69)	8.25 (0.85)
Control	9.23 (0.40)	9.50 (0.40)	7.90 (0.40)
A-N	8.57 (0.93)	9.17 (0.73)	8.63 (1.25)
A+N	11.36 (1.050)	11.83 (1.14)	8.96 (0.86)
E-N	10.30 (1.01)	10.16 (1.37)	9.85 (1.25)
E+N	8.45 (0.19)	8.78 (0.20)	6.65 (0.34)
C-N	8.78 (0.62)	9.00 (0.60)	7.64 (0.66)
C+N	9.84 (0.27)	10.16 (0.17)	8.24 (1.36)

When trees of similar size were analysed for SGR, no difference was found in alder (0.53 mg g^{-1} d^{-1} in ambient and 0.47 mg g^{-1} d^{-1} in elevated [CO_2]). In Scots pine, it was more difficult to find trees of similar sizes at different harvests. Mean SGR for trees of similar size was 11.05 mg g^{-1} d^{-1} in ambient and 7.6 mg g^{-1} d^{-1} in elevated [CO_2]. The mean SGR is higher since the time period between the chosen harvests was smaller. Elevated [CO_2] pine of similar size had a lower mean SGR than in ambient [CO_2].

4.8 Foliar Nutrient Analysis

In both Scots pine and common alder growth in elevated [CO_2] did not significantly affect nitrogen concentrations in 1995 ($p > 0.1$) (see Table 4.13). Nutrient addition had no significant effect on nitrogen concentration in either species of tree ($p > 0.1$, see Appendix 6 for p values) except in September 1994 where there was a trend towards an

effect (p = 0.10) in Scots pine. There was no interaction effect of $[CO_2]$ and nutrient addition in either species.

There was a slight tendency for phosphorus concentrations in Scots pine to be increased by elevated $[CO_2]$ in September 1994 (p = 0.077), when mean [P] was 2.0 mg g^{-1} in elevated and 0.18 mg g^{-1} in ambient $[CO_2]$. No nutrient effect on phosphorus concentration was found in Scots pine (p > 0.1). There was, however an interaction effect between $[CO_2]$ and nutrients in September 1994 (p = 0.02).

In common alder there was no $[CO_2]$ or nutrient effect (p > 0.1) on phosphorus concentration. [P] ranged from 3.3 mg g^{-1} in both $[CO_2]$ treatments in April 1994 (first harvest), to 3.8 mg g^{-1} in ambient and 4.1 mg g^{-1} in elevated $[CO_2]$ in April 1995, and 1.2 mg g^{-1} in ambient and 1.4 mg g^{-1} in elevated $[CO_2]$ in December 1995. There was an interaction effect between $[CO_2]$ and nutrient addition in April 1995 however (p = 0.054).

The presence of chambers only had an effect on phosphorus concentration in alder in April 1995 (p = 0.02). In Scots pine a chamber effect on phosphorus concentration was found in September 1994 (p = 0.058) and there was also a between-chamber effect (p = 0.063). Chamber presence affected nitrogen concentration in September 1994 also (p = 0.0059) but not at any other harvest.

Table 4.13 Nitrogen concentration (mg g^{-1}) in leaves of alder and pine over time. Values are the means (one standard error in brackets) of eight samples for [CO_2] treatment and the means of four samples for nutrient and [CO_2]. (*) = trend towards a statistically significant effect ($p < 0.10$).

Treatment	Sep. 1994	April 1995	Aug. 1995	Dec. 1995
common alder				
Ambient	22.7 (2.1)	37.1 (2.6)	25.0 (2.0)	24.9 (2.9)
Elevated	22.5 (1.9)	37.1 (3.5)	22.0 (1.5)	20.2 (2.4)
Control	24.3 (0.6)	41.0 (1.8)	---	---
A-N	24.7 (2.2)	33.7 (2.8)	23.8 (1.5)	26.2 (2.4)
A+N	20.6 (2.0)	40.5 (2.3)	26.2 (2.7)	25.8 (3.4)
E-N	25.2 (2.5)	36.2 (3.0)	22.3 (1.9)	20.2 (1.5)
E+N	19.7 (1.2)	38.0 (3.9)	21.5 (1.0)	20.4 (3.3)
C-N	22.8 (0.9)	41.9 (2.1)	---	---
C+N	25.9 (2.3)	40.0 (1.4)	---	---

Treatment	Sep. 1994	April 1995	Aug. 1995	Oct. 1995
Scots pine				
Ambient	15.3 (1.0)	15.5 (2.0)	9.1 (0.5)	12.1 (1.0)
Elevated	16.1 (0.4)	15.7 (0.8)	10.3 (0.3)	11.2 (0.3)
Control	18.7 (0.3)	17.5 (0.4)	8.6 (0.4)	12.2 (1.0)
A-N	14.2 (0.4)	17.5 (3.1)	9.3 (0.3)	13.7 (1.1)
A+N	16.3 (1.6) (*)	13.5 (0.1)	9.2 (0.7)	11.8 (0.9)
E-N	15.4 (0.5)	15.6 (0.8)	10.5 (0.3)	11.8 (0.2)
E+N	16.7 (0.3) (*)	15.7 (1.4)	10.1 (0.3)	10.6 (0.3)
C-N	17.9 (0.3)	17.8 (0.7)	8.6 (0.8)	10.8 (1.9)
C+N	19.6 (0.2)	17.2 (0.06)	11.1 (0.3)	13.7 (1.1)

4.9 Allocation of Labelled Nitrogen Over Time

4.9.1 Labelled and Unlabelled Nitrogen Per Tree

This study was based on the assumption that uptake of labelled N was finished in the autumn of 1994 after the spike addition of ^{15}N labelled fertiliser in July 1994, and that in April 1995 any N uptake from the soil was at natural abundance. Some of the labelled N will have been lost through leaching and some immobilised in the soil by fungi and microbes, but the assumption is that further uptake of labelled N did not occur in the spring of 1995. Overall, the concentration of labelled N as a proportion of total plant N is expected to fall over time as unlabelled ^{14}N dilutes the labelled N. The amount of labelled N, however should stay approximately similar (approximately, since different individual trees were harvested each time). Figures 4.9a and 4.10a below show that the amounts of labelled N in trees of both species generally fell over time except in the spring-time when an increase occurred. The largest increase occurred between autumn 1995 and April 1996. ANOVA analysis of harvest differences in dry mass data for the trees at these times indicated that the spring 1996 increase in labelled N could not be attributed to larger trees in April 1996 compared with October or December 1995 (one-way ANOVA, alder $p = 0.12$, pine $p = 0.38$). Since the trees in this experiment were grown in soil, it is likely that some of the labelled N was immobilised in the soil by microbes and fungi, after the spike addition of fertiliser. As root turnover and remineralisation often takes place in the spring, labelled N previously immobilised in the soil could have been released and taken up by the plants, accounting for the increase in labelled N found in spring 1996. Allocation of labelled N in September 1994 and April 1995 was of most interest in this study, since the pulse of labelled N was only applied once and not over a longer period (see discussion for more details). The only increase of labelled N of note (but *ns* at $p = 0.24$) in April 1995 was in the ambient [CO_2] pine trees. However, since this increase in labelled N content was not statistically significant, the assumption that uptake of labelled N finished in autumn 1994 can be considered to have been met. In addition, there was no significant difference in labelled N per tree between harvests in either common alder or Scots pine. Pooled ambient and elevated data over time were not significantly different in either species (one-way ANOVA, $p = 0.28$ for alder, and $p = 0.14$ for pine). Equally, [CO_2] had no significant effect on alder ($p = 0.31$)

or pine (p = 0.41) labelled N contents. There was also no effect of time on labelled N in either species (p = 0.94 in alder, p = 0.17 in pine).

As far as unlabelled ^{14}N derived from the soil (and / or the atmosphere in alder) before, during and after the spike addition of labelled N was concerned, amounts increased over time, as expected (see Figures 4.9b and 4.10b). Most of the uptake occurred between June and August in common alder, when mean unlabelled N rose from *c.*1000 mg per tree to *c.* 4000 mg per tree. By comparison, in Scots pine most of the unlabelled N uptake took place in the late summer and autumn, when unlabelled N rose from *c.* 450 mg per tree to *c.*1250 mg per tree (see Figure 4.10b). There was increased N uptake by elevated [CO_2] alder between June and August 1995 compared with ambient [CO_2] trees (see Figure 4.9b). The pattern then changed in autumn however, with ambient [CO_2] alder continuing to take up N until December, whereas elevated [CO_2] alder stopped taking up N in August 1995. In Scots pine, the pattern of N uptake (see Figure 4.10b) was different with steady increase in N uptake from June to October. There was no clear pattern in which more N was taken up in elevated versus ambient [CO_2] trees.

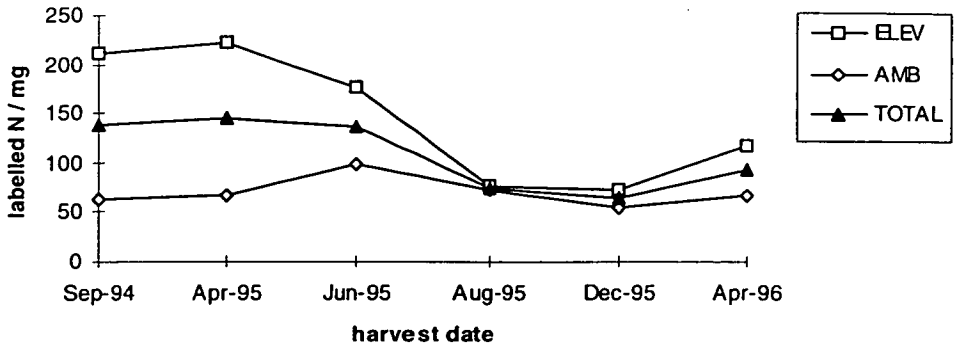


Figure 4.9a Mean amount of labelled N per tree in *common alder* over time. Each data point is the mean of four replicates for ELEV and AMB trees, and eight replicates for the total mean (see Table 4.14 for standard errors). Labelling occurred in July 1994.

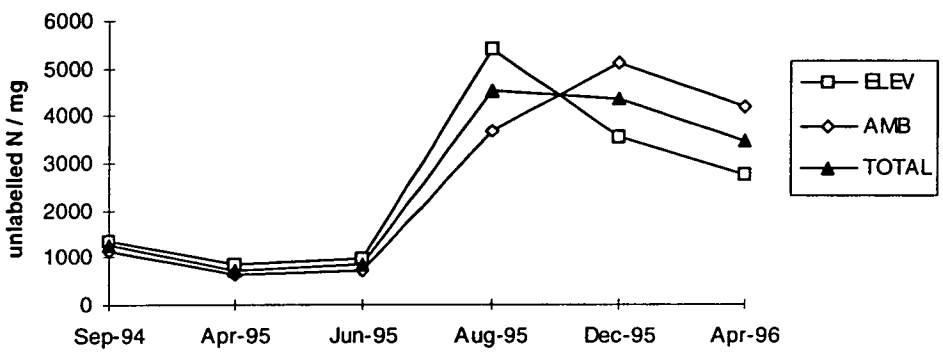


Figure 4.9b Mean amount of unlabelled N per tree in *common alder* over time. Each data point is the mean of four replicates for AMB and ELEV trees, and eight replicates for the TOTAL treatment (see Table 4.14 for standard errors).

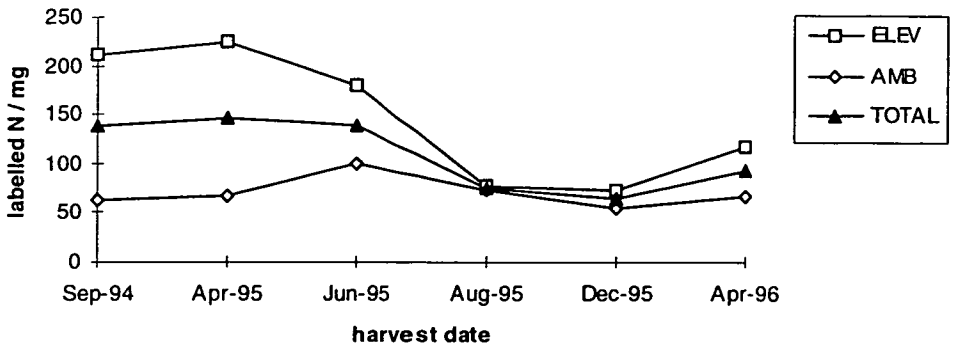


Figure 4.9a Mean amount of labelled N per tree in *common alder* over time. Each data point is the mean of four replicates for ELEV and AMB trees, and eight replicates for the total mean (see Table 4.14 for standard errors). Labelling started in July 1994.

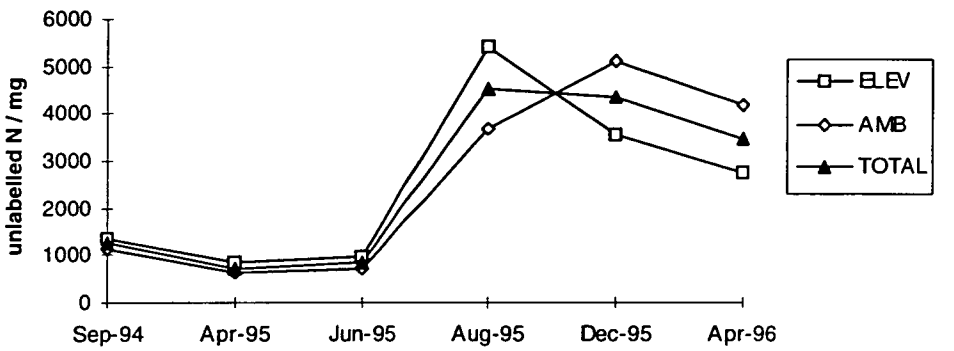


Figure 4.9b Mean amount of unlabelled N per tree in *common alder* over time. Each data point is the mean of four replicates for AMB and ELEV trees, and eight replicates for the TOTAL treatment (see Table 4.14 for standard errors).

4.9.2 Ratio of ^{15}N to ^{14}N

The second assumption upon which this research was based, was that the labelled N and the unlabelled N taken up during July to October 1994 were treated in the same way by the trees. This assumption also included the idea that labelled N and unlabelled N taken up in summer 1994 would be allocated to the tissues in a similar way the following spring. Biological processes usually discriminate against heavier isotopes, such that in this case one might find that ^{14}N was preferentially taken up over ^{15}N. Nevertheless, this error is very small compared with the magnitude of the enriched fertiliser 'signal' which was many times greater (at 4.09 atom % excess versus 0.366 atom %) than any natural ^{15}N abundance discrimination. Thus, one can make the assumption that any differences in

discrimination by the plants between ^{15}N and ^{14}N were negligible compared with the magnitude of the ^{15}N-enriched fertiliser spike.

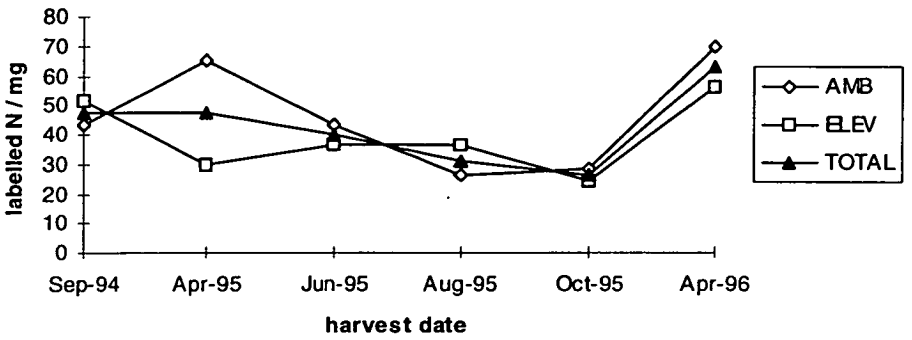


Figure 4.10a Mean amount of labelled N per tree in *Scots pine* over time. Each data point is the mean of four replicates for AMB and ELEV trees, and eight replicates for the TOTAL treatment (AMB + ELEV) (see Table 4.14 for standard errors). Labelling occurred in July 1994.

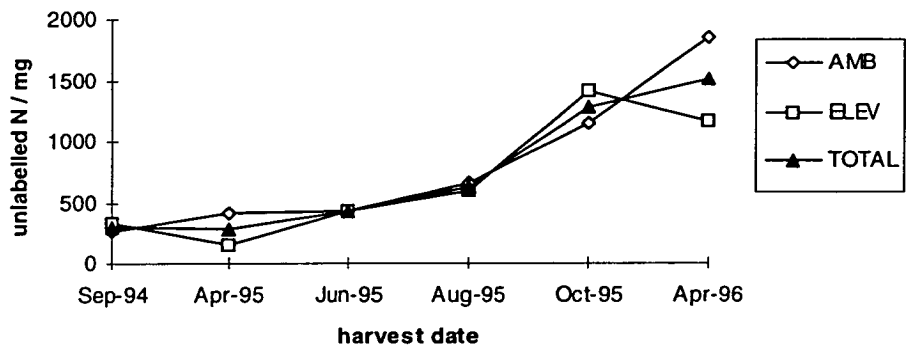


Figure 4.10b Mean unlabelled N per tree in *Scots pine* over time. Each data point is the mean of four replicates for AMB and ELEV trees, and eight replicates for the TOTAL treatment (AMB+ELEV) (see Table 4.14 for standard errors).

Table 4.14 Single standard errors (±) for mean labelled N and unlabelled N per tree over time (see Figures 4.9a and b and 4.10a and b for means) in common alder and Scots pine (mg per tree). Values are the standard errors of four (occasionally three) samples per [CO_2] treatment. Pooled = ambient and elevated data pooled together.

Treatment	1994 Sep.	1995 April	June	Aug.	Oct.	1996 April
alder						
labelled N						
Ambient	27.83	38.72	55.29	12.70	6.45	11.76
Elevated	7.81	26.47	65.45	26.66	18.02	28.70
Pooled	35.46	36.57	42.29	13.69	11.14	17.23
unlabelled N						
Ambient	144.78	174.93	132.52	423.73	598.20	779.46
Elevated	538.43	277.37	255.63	954.28	1825.34	369.27
Pooled	253.16	155.62	141.40	583.92	1087.10	484.02
pine						
labelled N						
Ambient	14.83	24.83	19.10	8.58	5.72	19.71
Elevated	12.23	7.34	6.48	17.27	12.69	10.78
Pooled	9.020	13.73	9.43	9.14	6.48	10.71
unlabelled N						
Ambient	46.20	25.08	109.61	17.95	221.78	426.72
Elevated	34.38	16.59	75.34	177.06	154.93	137.13
Pooled	28.64	49.04	61. 58	83.29	134.49	244.81

4.9.3 Labelled N Allocation

Up to 20 % of total N was labelled in common alder trees in September 1994, compared with up to 14 % in Scots pine (see Tables 4.15 to 4.18) . In both species these percentages fell to c. 2 % by the end of 1995. This dilution effect was most likely caused by the uptake of further unlabelled N into the trees after the July 1994 spike addition of labelled N fertiliser but also by the decline in labelled N content shown in Figures 4.9a and 4.10a. Elevated [CO_2] did not seem to change the percentage of plant N derived from uptake of labelled fertiliser in Scots pine, but in alder this percentage was consistently higher in elevated [CO_2] trees from September 1994 to June 1995 (13.4 % in elevated [CO_2] versus 5.2 % in ambient [CO_2] alder in September 1994; in April 1995 elevated [CO_2] alder had 21 % labelled N versus 9.5 % in ambient [CO_2]). By August 1995 however, at which point most of the N uptake had taken place (see section on Labelled N and Unlabelled N Per Tree), the effect of elevated [CO_2] on percentage labelled N was neutralised. This was probably caused by continuing uptake in ambient [CO_2] alder into the autumn, compared with the end of uptake in elevated [CO_2] alder (see previous section on Labelled N and Unlabelled N Per Tree).

Labelled N taken up in July 1994 contributed 8.2 % to ambient [CO_2] alder leaf growth in April 1995 compared with 26.6 % in elevated [CO_2] alder. Labelled N also contributed 9.6 % to new shoot growth in June 1995 in ambient [CO_2] alder compared with 14.3 % in elevated [CO_2]. In Scots pine, the labelled N contribution to 1995 needles was 13.3 % in ambient [CO_2] compared with 25.1 % in elevated [CO_2]. Labelled N contribution to 1995 shoot growth was 10.1 % in ambient compared with 8.0 % in elevated [CO_2]. Thus up to 25 % of the N used for new growth in 1995 was derived from N which was taken up after the spike addition of labelled fertiliser in July 1994 and allocated to certain tissues over the winter. In this experiment any reference to remobilisation is remobilisation of N derived from the enriched fertiliser, and it is defined as any labelled N recovered in new growth (leaves or stem) in 1995. The proportion of labelled N that was remobilised for spring leaf growth was greater in elevated [CO_2] trees in both species. This was also the case for the proportion of N remobilised for 1995 shoot growth in alder but not in pine.

Effect of Elevated [CO_2] on Labelled N Allocation in Different Tissues

As far as ^{15}N allocation to tissues was concerned, elevated [CO_2] had no significant effect on the different tissues of Scots pine at any harvest (from September 1994 to April 1996) ($p > 0.05$) (see Appendix 8 for ANOVA results, Table 4.17 for data). In common alder on the other hand, a significant [CO_2] effect was found in September 1994, in 1993 and 1994 shoot tissue ($p = 0.01$ and 0.022, respectively) with more labelled N present in the elevated [CO_2] tissues (see Table 4.16). In April 1995 [CO_2] also had a significant effect on the labelled N contents of 1993 and 1994 shoots ($p = 0.017$ and 0.024, respectively) and in the leaves ($p = 0.03$). Again, the elevated tissues contained more labelled N than the ambient ones. Differences in dry mass of the ambient and elevated [CO_2] tissues in question were not significant in September 1994 and April 1995 ($p > 0.05$) indicating that the significant differences in labelled N were not due to elevated [CO_2] tissue being larger. In April 1994, however there was a trend towards the elevated [CO_2] 1994 shoot dry mass being larger than the ambient [CO_2] dry mass ($p = 0.15$), which could account for the larger amount of labelled N in elevated [CO_2] 1994 shoots.

During the summer of 1995 elevated [CO_2] had no significant effect on the labelled N content of alder trees ($p > 0.05$). In April 1996, however, there was a trend towards a [CO_2] effect on the labelled N content of roots ($p = 0.07$) with more labelled N present in the elevated [CO_2] roots. This was not because the elevated [CO_2] trees has larger roots than ambient [CO_2] trees ($p = 0.90$), therefore one can conclude that the elevated [CO_2] roots contained more labelled N per unit root mass than ambient [CO_2] roots.

Overall, therefore, more labelled N was present in elevated [CO_2] alder shoots in autumn 1994 and spring 1995 than in ambient [CO_2] shoots. However, since the April 1995 harvest was made after bud-burst it was not possible to determine which tissues had been used to store N over the winter since remobilisation had probably already occurred. What we see here, does nevertheless indicate increased allocation of nitrogen over the autumn and winter to shoots in elevated [CO_2] alder trees. In contrast, elevated [CO_2]-grown Scots pine showed no significant difference in labelled N allocation at any time during the three growing seasons.

The Origin of Labelled N Found in New Growth

Common alder

The harvests of particular interest in this respect were September 1994 and April 1995, since one could be sure that any labelled N found in new leaf or shoot growth had been remobilised from other tissues. Table 4.15 shows that remobilisation of winter-stored N was complete by June 1995, since the largest amounts of labelled N in leaves were found at this time. The same table shows that in elevated [CO_2] trees, labelled N content of both roots and 1993 shoots increased slightly from September 1994 to April 1995 but then was reduced between April and June. In alder, 1994 shoot was the only category in which the amount of labelled N decreased. Statistically, there were no significant differences in labelled N content in any tissues in this time period ($p > 0.05$). There was a drop in labelled N in 1994 shoot and a rise in labelled N in root and 1993 shoot, however. Looking at the unlabelled N alder data in Table 4.17, shows that the amount of N found in the new leaves was equivalent to the amount lost from 1994 shoot over this time period (*c.* 84 mg). However, the amount of unlabelled N in the elevated [CO_2] roots (Table 4.17) also fell from September 1994 to April 1995. The unlabelled N for new growth could, therefore, have been derived from the roots in elevated [CO_2] trees, although the fact that labelled N levels in the rootss remained similar from the autumn to the spring does not confirm this possibility.

The mean dry mass of elevated [CO_2] alder 1994 shoot decreased from 31.3 g in September 1994 to 12.7 g in April 1995 (t-test $n = 4$, $p = 0.03$). In addition, the ratio of mg of labelled N to g dry mass in 1994 shoot increased from September to April in the elevated [CO_2] treatment (from 1.25 to 6.16). This is not what one would expect if there had been a net transfer of labelled N from 1994 shoot to new growth. On the other hand, the lower dry mass of 1994 shoot in the spring compared with the autumn could have swamped any possibility of detecting a net labelled N transfer. The April 1994 shoot dry mass data were very variable. This could have been because of natural variation in the trees harvested or an artefact of the experiment, since it had started to become difficult to distinguish between 1993 shoot and 1994 shoot during April 1995 harvesting. Deciduous trees store N in woody tissues over the winter (Millard 1996). Bark storage proteins (BSP) which are synthesised in the autumn and broken down in spring shoot

growth, have recently been isolated in deciduous trees (reviewed in Millard 1996). Roots of young trees have also been shown to store N (Millard 1996). Taking all the above factors into account, therefore, the N for new shoot and leaf growth in elevated [CO_2] alder seems to have come from the 1994 shoot or possibly the roots. It is not possible to clearly state from which tissue the nitrogen was derived.

In alder trees grown in ambient [CO_2] the allocation of labelled N between September 1994 and April 1995 was similar to that of the unlabelled N (see Tables 4.15 and 4.17). The only drop in labelled N over this time period was found in 1994 shoot (p = 0.63). Changes in labelled N in all tissues were again not statistically significant ($p > 0.05$), although there were biologically significant increases and decreases in different tissues. 1994 shoot lost *c.* 4 mg of labelled N and 9.2 mg were recovered in the leaves. 1994 shoot also lost a similar amount of unlabelled N from September to April to that found in the new leaves (*c.*100 mg were lost from 1994 shoot and 100 mg recovered in the leaves). As with the elevated [CO_2] alder, 1994 shoot dry mass was significantly lower in April 1995 compared with September 1994 (p = 0.047) and again, this could have been natural variation in the trees or an artefact of the experiment because of the difficulty in discriminating between 1993 and 1994 shoot. The ratio of mg labelled N to g dry mass of 1994 shoot also increased in ambient [CO_2], but not as much as in elevated [CO_2] (from 0.23 in autumn to 0.38 in spring). These changes are biologically significant because the variability of the data was high and the replication small, such that rigorous statistical tests such as ANOVA will not necessarily pick up processes of biological significance. In this case the data agree with the findings of other authors on N translocation patterns in evergreen and deciduous trees (reviewed by Millard 1996) lending credibility to the results.

In contrast to 1994 shoot, root and 1993 shoot labelled N and unlabelled N amounts in ambient [CO_2] remained similar between September 1994 and April 1995. On balance, it is more likely that the labelled N for new leaf and shoot growth came from 1994 shoot, although it is not possible to state this with certainty. This is in agreement with the findings of other authors that deciduous trees store N over the winter primarily in their stems (Millard 1996). It is possible that the N was stored as bark storage protein (BSP).

Because the harvest in April 1995 was made after bud-burst one can only talk of labelled N allocation over the winter not N storage, since remobilisation had probably already occurred.

Scots pine

In Scots pine, remobilisation of N for growth to 1995 needles was complete by April 1995, when the largest amount of labelled N was found in the new needles (*c.* 15 mg in ambient and *c.* 25 mg in elevated [CO_2]). The movement of unlabelled N compared with labelled N between tissues from September 1994 to April 1995 followed similar patterns (in both [CO_2] treatments) (see Tables 4.16 and 4.18). The change in labelled N in all pine tissues over this time period was not significantly different ($p > 0.05$), but biologically there were some significant changes in labelled N in different tissues. In elevated [CO_2] pine, the only big drop in labelled N from September 1994 to April 1995 was in 1994 needles ($p = 0.52$). Labelled N in 1994 shoot also decreased over this period but only very slightly (*ns* at $p = 0.78$). Labelled N in roots remained the same in both harvests ($p = 1.0$), whereas 1993 shoot and 1993 needles both had increased labelled N ($p = 0.22$ and 0.61, respectively). If one looks at the dry mass data for these tissue in the respective harvests, one finds that no changes in dry mass from autumn to spring were significant ($p > 0.05$), except in elevated 1993 needles ($p = 0.03$). The mass of 1993 needles however was very low and variable, such that no clear conclusion can be drawn from the increase in labelled N in spring of 1993 needles despite unlabelled N and dry mass decreasing.

Most the N used in new needle growth in elevated [CO_2] pine came from 1994 needles. The dry mass of 1994 needles increased in elevated [CO_2] (from a mean of 4.0 to 6.4 g). One can conclude that a net movement of N from 1994 needles to 1995 needles occurred in the elevated pine needles over the winter, since the amount of labelled N in 1994 needles decreased from 30.6 mg to 17.8 mg from September to April, despite the dry mass going up during the same period.

In ambient [CO_2] pine there were no statistically significant differences in labelled N from autumn 1994 to spring 1995 ($p > 0.05$). In contrast to in elevated [CO_2] pine, labelled N

and unlabelled N decreased in all tissues in ambient [CO_2] pine. The biggest drop in labelled N in ambient [CO_2] pine from autumn 1994 to spring 1995 was again in the 1994 needles (from 24.9 mg to 8.8 mg, *ns* at $p = 0.14$ but showing a trend towards a significant difference). The 17 mg of labelled N lost from 1994 needles is closely equivalent to the 15 mg present in the new needles. The dry mass of all tissues over the same time period showed no significant differences ($p > 0.05$), except in 1994 needles ($p = 0.02$). 1994 needle dry mass decreased over the time period in ambient [CO_2] (from a mean of 7.3 to 4.0 g). The ratio of labelled N to dry mass also decreased from autumn to spring (from 3.32 mg g^{-1} to 2.5 mg g^{-1}), however. Had the decrease in labelled N only been due to a decrease in dry mass of the tissue, the ratio of labelled N to dry mass would have remained the same or have increased. Since the opposite was the case, one can be relatively sure (given that the unlabelled N behaved in a very similar manner) that the N for new needle growth was derived from 1994 needles. These results are in agreement with other research, where it was found that evergreen trees store N in the foliage over the winter, and that conifers store N in the previous year's needles (Proe and Millard 1994, Millard 1996).

Table 4.15 Mean labelled ^{15}N, mg, per tissue (with one standard error in brackets) in common alder over time. Values are the means of four replicates (occasionally three). R = root; S = shoot; L = leaf. After August 1995 it was no longer possible to distinguish between 1993 shoot and 1994 shoot, so they were pooled.

Tissue	1994 Sep.	1995 April	June		Aug.	Dec.	1996 April
Ambient							
R	26.43 (13.62)	40.75 (26.11)	35.18 (17.09)	**R**	19.95 (3.76)	23.15 (8.88)	20.61 (4.88)
1993S	9.66 (5.22)	12.66 (6.91)	12.05 (8.02)	**93+94S**	30.62 (4.10)	10.96 (10.80)	33.94 (5.09)
1994S	8.75 (7.21)	4.81 (3.14)	7.14 (3.89)	**95S**	5.59 (0.60)	5.39 (one #)	12.91 (2.98)
1995S	--	0.37 (one #)	5.54 (2.78)	**L**	22.49 (3.89)	7.97 (2.33)	--
L	12.09 (6.68)	9.19 (3.21)	40.29 (24.33)				
Elevated							
R	78.68 (33.97)	84.50 (20.48)	75.42 (28.37)	**R**	24.53 (9.18)	35.85 (20.79)	42.23 (8.68)
1993S	35.84 (3.44)	43.42 (6.29)	22.51 (9.88)	**93+94S**	31.43 (12.09)	43.11 (11.00)	62.24 (17.80)
1994S	52.89 (12.52)	40.00 (11.27)	22.51 (9.88)	**95S**	5.88 (3.69)	10.11 (0.16)	13.42 (6.95)
1995S	--	1.22 (0.26)	6.94 (2.43)	**L**	15.83 (6.46)	5.79 (1.69)	--
L	65.90 (44.17)	30.39 (6.47)	55.79 (19.75)				

Table 4.16 Mean labelled ^{15}N per tissue, mg, (with one standard error in brackets) in Scots pine over time. Values are the means of four replicates (occasionally three). R = root; S = shoot; N = needles.

Tissue	1994 Sep.	1995 April	June	Aug.	Oct.	1996 April
Ambient						
R	6.42 (3.02)	4.89 (1.19)	3.52 (1.31)	1.58 (0.53)	1.84 (0.80)	4.71 (2.07)
1993S	7.23 (4.31)	3.36 (0.31)	2.87 (1.17)	1.00 (0.34)	1.35 (0.31)	3.27 (1.17)
1994S	3.54 (1.16)	3.04 (0.31)	3.31 (1.43)	1.32 (0.46)	2.87 (1.81)	3.53 (0.93)
1995S	--	--	7.23 (2.90)	3.79 (1.44)	3.51 (0.33)	8.55 (2.66)
1993N	2.37 (1.40)	1.25 (0.12)	3.57 (1.93)	0.17 (0.10)	0.26 (0.18)	0.95 (0.69)
1994N	24.85 (7.74)	8.75 (0.94)	12.52 (4.92)	3.41 (1.15)	9.57 (6.89)	19.44(15.14)
1995N	--	15.32 (1.80)	11.63 (5.24)	14.75 (4.83)	9.01 (2.92)	30.26(11.32)
Elevated						
R	8.65 (4.39)	8.64 (2.97)	4.01 (1.61)	2.22 (0.72)	2.65 (1.27)	5.86 (1.45)
1993S	2.31 (0.92)	6.04 (2.60)	2.40 (0.56)	1.79 (0.80)	1.22 (0.62)	5.60 (2.10)
1994S	6.84 (1.87)	5.90 (0.27)	3.30 (0.74)	2.54 (1.16)	1.66 (0.91)	2.42 (0.92)
1995S	--	--	5.20 (0.55)	5.47 (2.25)	4.80 (1.93)	11.17 (2.69)
1993N	1.63 (0.42)	1.95 (0.41)	1.65 (0.78)	0.29 (0.10)	0.13 (0.10)	0.31 (0.16)
1994N	30.58 (9.35)	17.78 (5.99)	10.25 (3.73)	6.38 (2.93)	2.46 (1.39)	4.03 (2.25)
1995N	--	24.72 (11.14)	10.69 (2.77)	17.95 (9.59)	11.60 (7.60)	27.21 (4.80)

Table 4.17 Mean unlabelled ^{14}N, mg, (with one standard error in brackets) in common alder tissues over time. Values are the means of four replicates (occasionally three). R = root; S = shoot; L = leaves. Note that after August 1995, 1993S and 1994S are pooled and known as 94S.

Tissue	1994 Sep.	1995 April	June		Aug.	Dec.	1996 April
Ambient							
R	321.4 (112.4)	345.8 (112.8)	239.7 (41.6)	**R**	482.8 (52.1)	1669.8 (332.4)	1023.1 (317.3)
1993S	134.7 (31.9)	139.7 (25.5)	95.1 (11.3)	**1994S**	884.8 (151.6)	1993.2 (81.7)	1933.1 (357.2)
1994S	155.1 (54.5)	55.1 (18.9)	63.3 (8.3)	**1995S**	417.6 (94.1)	--	1228.5 (203.9)
1995S	--	75.7 (71.3)	51.8 (18.8)	**L**	1992.1 (295.9)	937.9 (104.8)	--
L	396.5 (115.2)	102.9 (24.0)	337.6 (74.2)				
Elevated							
R	654.8 (143.8)	377.5 (130.7)	378.2 (111.2)	**R**	1145.2 (374.5)	1361.8 (235.8)	923.5 (136.9)
1993S	155.5 (91.1)	266.0 (76.8)	128.6 (38.2)	**1994S**	1232.0 (348.3)	2045.8 (445.3)	1163.7 (228.8)
1994S	219.1 (91.2)	135.8 (50.7)	107.5 (37.1)	**1995S**	703.8 (260.9)	1001.1 (319.0)	649.7 (96.8)
1995S	--	4.9 (2.0)	41.2 (10.7)	**L**	2309.2 (709.2)	783.8 (211.5)	--
L	406.5 (338.8)	84.1 (30.3)	338.7 (65.5)				

Table 4.18 Mean unlabelled ^{14}N, mg, (with one standard error in brackets) in Scots pine tissues over time. Values are the means of four replicates (occasionally three). R = root; S = shoot; N = needles.

Tissue	1994 Sep.	1995 April	June	Aug.	Oct.	1996 April
Ambient						
R	40.1 (11.2)	35.6 (7.5)	42.5 (7.7)	38.5 (3.9)	93.5 (22.0)	173.5 (38.4)
1993S	31.9 (15.1)	22.1 (6.6)	31.4 (4.9)	19.8 (1.2)	41.5 (8.0)	80.1 (15.7)
1994S	25.6 (7.3)	13.9 (1.3)	34.6 (6.0)	26.5 (1.7)	75.6 (35.7)	102.9 (22.8)
1995S	--	--	64.1 (22.2)	113.9 (23.5)	164.9 (48.7)	286.8 (51.8)
1993N	24.2 (6.7)	10.3 (3.9)	44.0 (28.0)	2.8 (0.9)	6.9 (5.6)	306.1 (268.6)
1994N	147.9 (23.9)	37.3 (3.9)	108.3 (20.6)	44.7 (7.4)	194.8 (134.4)	336.4 (300.2)
1995N	--	99.4 (15.2)	138.2 (36.4)	401.1 (15.7)	609.3 (71.4)	560.3 (221.7)
Elevated						
R	62.0 (16.7)	60.8 (4.9)	48.3 (5.5)	32.0 (5.2)	117.0 (25.1)	121.9 (17.1)
1993S	23.1 (3.3)	49.0 (6.0)	29.2 (7.0)	21.5 (4.5)	52.9 (10.2)	83.8 (25.2)
1994S	40.0 (9.2)	33.6 (4.2)	36.4 (8.7)	38.6 (7.3)	93.7 (10.5)	39.0 (11.0)
1995S	--	--	59.5 (10.5)	88.0 (19.3)	218.7 (24.20	237.8 (43.6)
1993N	14.3 (2.0)	10.3 (3.9)	17.7 (5.6)	3.2 (0.7)	6.0 (3.4)	4.0 (1.6)
1994N	176.6 (28.9)	108.5 (8.4)	96.2 (25.8)	50.6 (9.6)	52.1 (7.5)	40.7 (17.6)
1995N	--	73.8 (42.1)	130.0 (38.9)	352.2 (127.8)	857.4 (109.3)	639.9 (88.7)

4.10 Nitrogen Fixation in *Alnus glutinosa* Grown in Elevated [CO_2]

Upon harvesting the trees, the nodules on elevated [CO_2]-grown roots were visibly smaller, more numerous and less clumped than those on ambient [CO_2] roots (see Photos 4.5 and 4.6). This was confirmed by Dr Sue Grayston, who determined the acetylene reduction of the nodules in August 1995. Total nodule mass was larger in elevated [CO_2] - N nodules, although not significantly so (6.89 g in ambient versus 7.28 g in elevated [CO_2], $p = 0.95$, $n = 4$), whereas total nodule mass was less in elevated [CO_2] (again not significantly) when fertilised nodules were also included in the analysis (with a mean of 6.15 g in ambient [CO_2] versus 5.88 g in elevated [CO_2], $p = 0.89$, $n = 8$). Root mass was larger in elevated [CO_2] than in ambient [CO_2] but not significantly so ($p > 0.1$).

Acetylene reduction, effected by the nitrogenase enzyme and thus an indication of the extent of nitrogen fixation, was also different in the different [CO_2] treatments. Nodules from the elevated [CO_2] treatment reduced more acetylene per mass of nodule per hour than did those in the ambient [CO_2] treatment (see Figure 4.11, data from Sue Grayston) although not significantly. When calculated on a whole plant basis, more acetylene was also reduced per plant per hour in elevated [CO_2] conditions, although not significantly so (Figure 4.11).

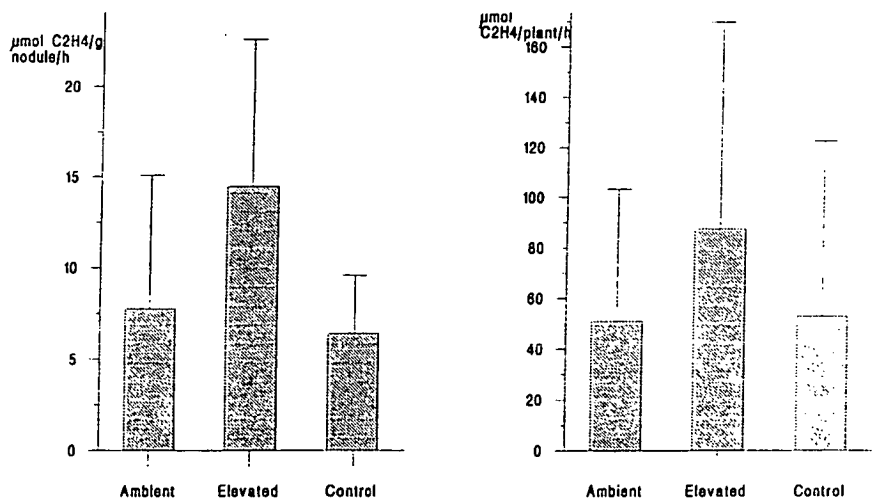
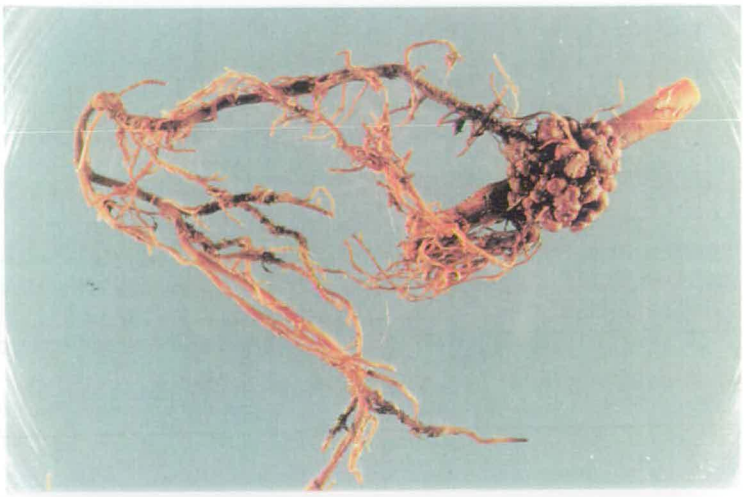
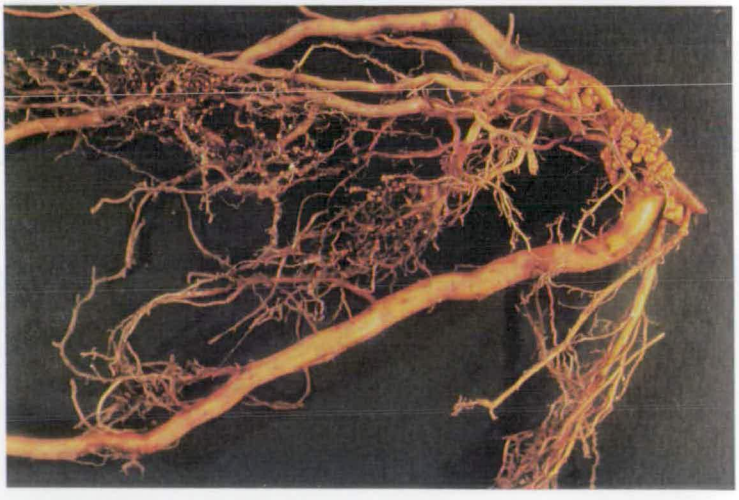


Figure 4.11 Mean ethylene reduction per gramme of nodule and per plant from alder trees growing at ambient and elevated [CO_2]. Values are the means of four samples.

Photo 4.4 Nitrogen-fixing root nodules on root of common alder in summer 1994.
Photo 4.5 Common alder root nodules from a tree grown in elevated [CO_2] and harvested on the 2nd April 1996.
Photo 4.6 Common alder root nodules from a tree grown in ambient [CO_2] in the control plot and harvested on the 2nd April 1996.





4.11 Discussion and Conclusions

Tables 4.19 and 4.20 are summaries of the main effects of elevated [CO_2] and nutrition on common alder and Scots pine seedlings. They enable a quick overview of the main effects but do not include interactions between [CO_2] and nutrient addition. These results are discussed in more detail in the subsequent sections.

4.11.1 Growth and Biomass

Longer-term experiments with trees grown in the ground are becoming more widespread and generally show increased growth over a certain time period which then becomes reduced in response to various limiting factors (Stitt 1991, Rey 1997, Tissue *et al.* 1997). In addition, such experiments indicate that every species has an individual response to elevated carbon dioxide. The response of the deciduous alder and the coniferous pine in this study agree closely with Poorter's 1993 review, which found that deciduous species and pioneer species were more stimulated than coniferous or slower-growing species (*c.* 54 % increase in biomass for the former versus *c.* 23 % for the latter, compared with an average biomass increase of 59 % in alder and 33 % in pine in this study). Norby (1987) also found that total biomass of *Alnus glutinosa* was stimulated 49 % in elevated [CO_2].

Alder

Elevated [CO_2] did not significantly affect specific growth rate of biomass during the third year of growth, although the interaction of [CO_2] and nutrient addition increased SGR in E-N plants. This suggests a slight stimulation of growth rate in the E-N treatment but no significant [CO_2] or nutrient effect alone. Norby *et al.* (1996) also found no change in SGR in white oak and yellow poplar and attributed this fact to decreased LAR, as in this experiment. Rey (1997) found very similar results in her long-term experiment with four-year old *Betula pendula* grown in the field, where SGR was not stimulated and yet total biomass (up 58 %), stem height and basal diameter were increased in elevated [CO_2].

Table 4.19 Summary of main effects of growth in elevated [CO_2] on *common alder* in the two nutrient treatments, fertiliser supply, F and no fertiliser supply, NF , combined and separately. The table shows significant statistical differences and how a variable in elevated [CO_2] compared with the ambient [CO_2] control. ++ large increase, + increase, (+) slight increase, ~ no change, (--) slight decrease, -- decrease, --- large decrease, ? not measured. Stars * indicate that the result was statistically significant ($p < 0.05$) at least at some time during the experiment. (*) = trend towards a significant difference between treatments ($p < 0.1$). *R* = root; *L* = leaf; *S* = shoot (without leaves); *R / S* = root to shoot ratio; *R / L* = root to leaf ratio; *L / S* leaf to shoot ratio.

Variable	F + NF together	F	NF
stem height	~	+ *	~
growth rate per day	+	+	+
SBD	+ *	+	+
stem basal area	+ *	+ *	+ *
total biomass	++ *	+	+
cumulative biomass	+	?	?
leaf allocation	-- *	-- *	-- *
root allocation	~	~	~
branch allocation	~	~	~
stem allocation	(+) (*)	+ *	+ *
R / S	(+) (*)	~	~
R / L	(+) (*)	~	~
L / S	-- *	-- *	-- *
leaf area	~	~	~
LAR	-- *	-- *	-- *
NAR	+ *	~	~
SGR	~	~	~
[N]	~	~	~
[P]	(+) (*)	~	~
ethylene reduction	+	?	?
nodule no.	+	?	?
labelled N in shoots	+	?	?
labelled N in roots	~	?	?
labelled N in leaves	+	?	?
N for new growth	from previous year's stem and branches		?

Table 4.20 Summary of main effects of growth in elevated [CO_2] on *Scots pine* in the two nutrient treatments, fertiliser supply, F and no supply, NF, combined and separately. The table shows significant statistical differences and how a variable in elevated [CO_2] compared with the ambient [CO_2] control. ++ large increase, + increase, (+) slight increase, ~ no change, (--) slight decrease, -- decrease, --- large decrease, ? not measured. Stars * indicate that the result was statistically significant ($p < 0.05$) at least at some time during the experiment. (*) = trend towards significant difference between treatments ($p < 0.1$).

Variable	F + NF together	F	NF
stem height	+ *	+ *	+ *
growth rate per day	+	+	+
SBD	+ *	+	+ *
stem basal area	+ *	+ *	+ *
total biomass	+ *	+	+ *
cumulative biomass	+	?	?
leaf allocation	-- *	-- *	-- *
root allocation	~	~	~
branch allocation	~	~	~
stem allocation	~	~	~
R / S	~	~	~
R / L	~	~	~
L / S	~	~	~
leaf area	+ *	~	~
LAR	-- *	-- *	-- *
NAR	~	~	~
SGR	~	~	~
[N]	~	~	~
[P]	(+) (*)	~	~
labelled N in shoots	~	?	?
labelled N in roots	~	?	?
labelled N in leaves	~	?	?
N for new growth	from previous year's needles		

The fact that fertiliser addition did not have a significant effect on alder growth indicates that those trees given no fertiliser and hence fixing nitrogen were able to grow as well as those supplied with full nutrients to the soil. The nodules exposed to elevated [CO_2] were smaller and therefore their nitrogenase activity higher (Wilson and Coutts 1985). Wilson and Coutts (1985) indicated that evidence for a strong correlation between nodule dry mass and growth was accumulating. [N] was not decreased in elevated [CO_2] in either nutrient treatment despite the increase in total biomass, corroborating this idea.

Pine

The reponse of Scots pine to elevated [CO_2] was not as large as that in alder. As in alder, SGR was not stimulated despite increase in total biomass, stem basal diameter and area. In contrast to the short-term experiment in this long-term experiment stem height was enhanced in elevated [CO_2] in pine but not in alder. Stem elongation per day was also higher in elevated [CO_2]. This reponse, particularly that of increased stem basal diameter, was very similar to that found by Ewa Jach (University of Antwerpen, *pers. comm.*) in her experiment with Scots pine. Very varied reponses of different pine species to elevated [CO_2] have been reported in the literature with *Pinus ponderosa* and *Pinus taeda* in North America, *Pinus sylvestris* in Europe and *Pinus radiata* in Australia. All-round positive responses during the first or second growing season have been shown in *P. taeda* (Lewis *et al.* 1996, Tissue *et al.* 1997) but in contrast Griffin et al. (1993) only found seedling growth of *P. taeda* to be stimulated when soil nitrogen was high. Higginbotham *et al* (1985) found an all-round positive response in lodgepole pine, *P. contorta*, grown in pots in chambers, whereas Grulke *et al.* (1993) found no stimulation of stem height or diameter in *P. ponderosa.* In *Pinus radiata*, Conroy *et al.* (1990) found that elevated [CO_2] increased whole plant dry mass only when phosphorus was in adequate supply.

These results highlight how different species in the same genus can respond very differently to elevated [CO_2], and these differences derive mainly from different developmental strategies (Mousseau and Saugier 1992) as well as possible genetic restraints to growth (Poorter 1993). Another example of this is the work of Duff *et al.* (1994) in which *Eucalyptus tetrodonta* increased its total biomass significantly under

elevated carbon dioxide conditions, whereas *Eucalyptus miniata* did not. These results confirm the suggestion that increased SGR may not persist for long periods (DeLucia *et al.* 1994). As Pettersson and McDonald's work on *Betula* seedlings (1992) pointed out, slightly higher SGR values during the first few weeks of development in birch seedlings were sufficient to produce important differences in biomass over a period of several growing seasons. This is probably what happened in this long-term experiment. Unfortunately it was not possible to measure SGR during the first and second growing seasons because of lack of harvests. The most common plant response to elevated [CO_2] is that growth and total biomass are increased but the growth stimulation is offset after a certain time period (varying from days to a few years) by various limiting factors. Such acclimation is often species-specific and the mechanism not yet clearly known, although down-regulation of photosynthesis occurs when carbon sources outstrip carbon sinks (Stitt 1991). Other authors have postulated that increased growth during the first developmental stages causes increased demand for nutrients and concomitant dilution of nitrogen which in turn causes NAR and allocation to the leaves to be reduced (Pettersson and McDonald 1992). Although NAR was stimulated in 1995 in alder, it is probable that the effect was reduced in 1996 (since there were signs of acclimation), especially since there was no increase in leaf area over the entire experimental period and [N] was reduced in 1996. The relatively long period of growth in alder (from 1993 to approximately August 1995 when canopy closure occurred) before acclimation set in, seems to have been a result of adequate nutrient availability from the soil for the trees grown with fertiliser, and adequate supply of nitrogen fixed from the atmosphere in trees grown without fertiliser. This enabled the trees to maintain the same [N] until 1996 when there was a significant reduction in [N] in alder leaves. Johnson et al. (1996) found that increases in growth of *Pinus taeda* were accompanied by a more efficient use of N only in the first six months of growth. It seems that this was the case in both alder and pine in this experiment, although the period of increased nitrogen use was at least two growing seasons long.

4.11.2 Allocation

Alder

Biomass allocation was much less affected by elevated [CO_2] in pine than in alder. There is evidence that the extra carbon in alder tended to be allocated to the stem and branches (collectively called shoots). Carbon was allocated away from the leaves in elevated [CO_2] throughout 1995, whereas increased allocation to the shoot only occurred significantly in June 1995. Field *et al.* (1992) pointed out that plants grown in elevated [CO_2] often have decreased allocation to the leaves so that respiratory costs for growth and maintenance are reduced, thus offsetting any extra respiratory costs caused by increased growth and maintenance. This could be the case with the alder in this study.

Root to shoot ratio has often been found to increase in elevated [CO_2], although this has often been in experiments with nutrient limitation, in which extra carbon is allocated to the roots (Bazzaz 1990, Rogers *et al.* 1992). This seems to be more a response to poor nutritional conditions rather than to elevated [CO_2] however, which is backed up by many tree experiments with adequate fertilisation in which *R / S* was unchanged in elevated [CO_2] (Bazzaz 1990, Chu *et al.* 1992, Tissue *et al.* 1996, Tissue *et al.* 1997). There was only a temporary tendency for alder *R / S* to increase in August 1995, whereas during the rest of the experiment there was no significant difference in *R / S*. This supports the idea that those trees growing without fertiliser addition were able to keep up a high enough supply of N to support increased growth in elevated [CO_2], via fixation of N from the atmosphere. A point to also keep in mind is that the alder were quite densely planted within each quadrant and as they grew larger, the root systems may have been restricted (see Norby *et al.* 1996). Leaf to shoot ratio (*L / S*) was reduced in June but not August 1995, whereas *R / L* ratio was, curiously, not affected by elevated [CO_2]. The reduction in *L / S* in June 1995 fits with evidence of increased allocation to the shoot in the same month, whereas allocation away from the leaves occurred throughout 1995. Leaf mass ratio (LMR) was reduced in elevated [CO_2] throughout most of 1995. This is to be expected since total biomass was stimulated but leaf area remained the same.

Pine

In Scots pine the only change in biomass allocation in elevated [CO_2] was in the 1993 needles and the effect only occurred in September 1994. Less biomass was partitioned to the needles at this time. Otherwise there were no differences in allocation in trees grown in elevated [CO_2], neither in *R/S*, *L/S*, *R/L*, LMR, or in allocation to other plant tissues at any time during the experiment. Jarvis (1995) stated that carbon allocation is barely affected if the nutrient supply is maintained. This seems to be the case, especially in Scots pine where hardly any changes in biomass allocation were found. It is rather puzzling, however, that the trees grown without fertiliser were also not affected in their allocation of biomass to different tissues.

4.11.3 Assimilatory Material and Net Assimilation Rate

Alder

Norby (1987) found increased leaf area but decreased LAR in *Alnus* grown in elevated [CO_2], which indicated increased NAR, as was found here, despite leaf area not increasing significantly. Gail Jackson found that alder SLA in the present study was also not significantly affected in elevated [CO_2] in 1996 (unpublished data). SLA during 1995 was not affected by elevated [CO_2], unlike in the short-term experiment. Overdieck and Forstreuter (1995) found no change in beech (*Fagus sylvatica*) leaf area grown in elevated [CO_2] for three years. El Kohen and Mousseau (1994) on the other hand, found that leaf area of sweet chestnut (*Castanea sativa*) was only increased in elevated [CO_2] supplied with fertiliser. In a study with yellow poplar (*Liriodendron tulipifera* L.), leaf area was reduced in elevated [CO_2] (Norby and O'Neill 1991) and increased total biomass was wholly ascribed to an increase in NAR. This is a similar result to that found in this experiment on alder. Norby *et al.* in their long-term experiment on *Liriodendron tulipifera* and *Quercus alba* (1996) found species-specific leaf responses to elevated [CO_2]. Leaf area in the former species was reduced by 10 % compared with a 95 % increase in the oak. LAR was reduced in both species however. As a result growth stimulation in oak was very large compared with no reponse in poplar (despite increased photosynthetic rates). In this case the slower growing species benefited much more from elevated [CO_2] than the faster growing one. In his study of ten fast-growing species, Poorter (1993), on the other hand, found increased LAR compared with slower-growing

species, but NAR and the rate of photosynthesis were stimulated to the same extent. This was not the case with Scots pine and common alder in this long-term experiment, where LAR was decreased in both species but NAR was only enhanced in alder. These varied results once again indicate how species-specific and different tree reponses to elevated [CO_2] can be.

Another very important aspect of tree response to elevated [CO_2] in the field, is canopy closure. There were 12 alder seedlings per quadrant at the beginning of the experiment, which meant that despite harvesting and the June 1995 thinning, canopy closure occurred in summer 1995. Unfortunately it was not possible to ascertain whether canopy closure occurred earlier in elevated [CO_2] plants or not. Norby et al. (1996) postulate that the only lasting effect of early growth enhancement in elevated [CO_2] may be a shortening of the time requires to reach canopy closure. However, they also state that although biomass production may not continue to the same degree after canopy closure, there is no particular reason why the relative effect of elevated [CO_2] on growth efficiency would decline after canopy closure. Interestingly, signs of a reduction in growth stimulation, i.e. acclimation, appeared around August 1995 when [N] and [P] changed from being optimum to being intermediate, root mass was significantly larger in elevated [CO_2] and *R / S* tended to increase. LAR was significantly lower in April and June 1995 but not after this date, whereas leaf to shoot ratio was lower in June but not in August.

Pine

In Scots pine leaf area increased in elevated [CO_2] in April 1995 but not at any other date, whereas LAR was reduced in April and August 1995 (but not at any harvest in-between). In contrast to alder, NAR was not stimulated by elevated [CO_2]. DeLucia *et al.* (1994) found a similar effect on *Pinus ponderosa* grown in a phytotron, where increases in biomass were offset by decreases in LAR.

These results differ from many [CO_2] experiments, in which leaf area was found to increase, along with leaf biomass and thickness (Mousseau and Saugier 1992, Jarvis 1995). Saralabai et al. (1997) postulate that the common increase in leaf area in elevated [CO_2] is caused by more extensive branching in dicotyledons. It should be kept in mind

however, that total leaf area is often not measured directly, but calculated from a sub-sample of leaves or from previous relationships between leaf area and SLA as in this experiment. As such it is difficult to be sure if the lack of effect of elevated [CO_2] on leaves in this experiment was an artefact of the methodology or a real [CO_2] effect.

4.11.4 Leaf Nutrient Concentration

Alder

[N] in alder was not affected by elevated [CO_2] although there was a tendency for the concentration to be lower in elevated [CO_2] trees (see Table 4.13). According to Van den Burg's (1985) compilation of tree nutrient data, the alder leaves had neither deficient nor optimum concentrations of N, although some from the elevated [CO_2] treatment were in the deficient range (2.2 to 2.9 %). Concentrations dropped to deficient in elevated [CO_2] in August 1995 and continued to be so in December. In contrast, [P] was optimum at the beginning of the experiment until August 1995, when [P] became intermediate. [P] also increased slightly in elevated [CO_2] (if *ns*). These results are very different from other experiments with *Alnus* spp, in which [N] was decreased (Norby 1987) or increased in elevated [CO_2] (Arnone and Gordon 1990). Since the [N] remained similar in elevated [CO_2] and total biomass increased, the amount of N must have increased along with the amount of carbon in order for N dilution not to have taken place. Tracking of carbon amounts by nitrogen could have been brought about by the ability to fix nitrogen in the alder (see section 4.9.6 on Nitrogen Fixation for more discussion of this). A key question that springs to mind is, how did the alder (and pine) manage to increase [P] in the leaves in elevated [CO_2], since there was no nutrient addition effect on [P] (in either species)? I suggest that the extra availability of carbon increased mycorrhizal growth and activity which in turn lead to positive feedback for the plants.

Pine

[N] in pine was not affected by elevated [CO_2] but [N] was deficient (1 to 2 %) to intermediate (1 to 3 %). Interestingly, [N] was deficient in August 1995 which seems to be a possible date for the start of acclimation in both species. [P] in pine needles was optimum to intermediate and, as in alder, the drop from optimum to intermediate

occurred in August 1995 (or possibly in June since there were no data for nutrient concentrations in the June harvest). [P] was also slightly higher in elevated [CO_2]. Many experiments with trees grown in elevated [CO_2] have found that foliar nutrient concentrations decrease (Chu *et al.* 1992, Ceulemans and Mousseau 1994, Rogers *et al.* 1994), although it needs to be pointed out that many of these experiments involved plants grown in pots or otherwise nutrient limited. Rogers *et al.* (1994) found in their review that when plants were supplied with large amounts of nutrients, nutrient concentration and nutrient uptake efficiency were generally not significantly affected by elevated [CO_2]. This seems to be the case in this study, although it is difficult to explain why those trees grown without fertiliser had similar [N] concentration in elevated [CO_2] to ambient [CO_2]. See section 2.4.2 in Chapter 2 for details of soil nitrogen concentrations within the chambers.

4.11.5 Labelled N Partitioning

Trees store N during winter and summer and remobilise the stored N in the spring to support new growth (Millard 1996). The pattern of N storage depends on whether trees are evergreen or deciduous. Deciduous trees tend to remobilise nitrogen from perennial woody tissues (stem, bark and sometimes roots) in the spring (Kang and Titus 1980a). Bark storage proteins (BSP) which are synthesised during the autumn and broken down in the spring have been isolated (reviewed in Millard 1996) and gene expression for BSP has been associated with both short-day photoperiod and high N availability. This suggests a role for such proteins in seasonal storage of N and during periods of excess N availability (Millard 1996). Conifers, on the other hand, often store nitrogen in the previous year's needles during the winter (Nambiar and Fife 1991, Millard 1994, Millard 1996), mostly in the form of Rubisco (the most abundant C_3 plant protein). Camm (1993) found a decline in photosynthetic capacity in mature Douglas fir needles during leaf flushing suggesting that the photosynthetic apparatus could be serving as an N source for leaf growth. Both storage strategies enable plants to uncouple their ability to grow in the spring from the availability of nutrients in the soil (Millard 1996). In the summer, both evergreen and deciduous trees store N in their leaves. During autumn senescence of deciduous leaves, removal of this stored N occurs, mostly in the form of Rubisco (up to 90 %) (Kang and Titus 1980a).

Alder

In this ^{15}N spike experiment most uptake of unlabelled N occurred between June and August 1995 in alder, compared with late summer and autumn in pine. Elevated [CO_2] trees showed increased N uptake compared with ambient [CO_2] trees, during the period of maximum uptake. This pattern was reversed in autumn. Labelled N found in new leaves in April 1995 contributed 8.2 % to leaf growth in ambient [CO_2] compared with 26.6 % in elevated [CO_2]. Elevated [CO_2] seems to have increased the proportion of N in new leaves derived from winter stored N. Millard (1994) found that remobilised N contributed about one third of the N for new leaf growth in *Acer pseudoplatanus* in the spring. Feigenbaum *et al.* (1987) found evidence for a substantial contribution of remobilised N (*c.* 75 %) to new growth in mature citrus trees. This confirms that mature trees are less dependent on nutrient uptake and more dependent on internal nutrient cycling than seedlings.

Labelled N allocated to other tissues was generally not affected by elevated [CO_2], however, except in September 1994 and April 1995, in 1993 and 1994 shoot. In addition there was more labelled N in elevated [CO_2] leaves in April 1994 (as stated above). The increase in labelled N present in the 1993 and 1994 shoots in April 1995 may have been the result of the increase in dry mass in these tissues, whereas in September 1994 dry mass variation could not account for the extra labelled N. This corroborates the suggestion that more labelled N in elevated [CO_2] was being allocated over the winter into the shoot than in ambient [CO_2]. Increased allocation to the shoot in alder (see section 4.9.2 on Allocation) confirms this finding.

Remobilisation of N for new growth in the spring of 1995 was complete by June 1995 (when the largest amounts of labelled N were found in the leaves). In contrast, in Scots pine remobilisation was complete by April 1995. These findings are in agreement with those of Millard (1994) from his work on *Acer pseudoplatanus* and *Picea sitchensis* in which remobilisation in the conifer occurred before bud burst, whereas in sycamore remobilisation occurred between bud burst and root uptake of N.

As regards the origin of the labelled N found in new growth, it is not entirely clear where it came from. It is most likely (see 4.5.3) that the N for leaf growth in spring 1995 was derived from the previous year's shoot (stem and branches). Feigenbaum *et al.* (1987) also found evidence of N storage in wooded branches of mature citrus trees over the winter, and little evidence for storage in the roots. In contrast, Marmann *et al.* (1997) showed that in ash (*Fraxinus excelsior*) most of the N remobilised in the spring for new growth came from the roots and not from the stem. Bark storage proteins (BSP) or Rubisco may have been the primary form of N stored in this way over the winter and remobilised to new leaves and stem in the spring. Millard *et al.* (1998) found a ten-fold increase in citrulline and glutamine in the xylem sap of *Betula pendula* during spring remobilisation. Kang and Titus (1980b) also identified glutamine as a key amino acid in N storage and remobilisation.

Pine

Labelled N contributed more to new needle growth in elevated [CO_2] than in ambient (13.3 % versus 25.1 %), whereas there was little difference in the contribution of labelled N to new shoot growth (10.1 % versus 8.0 %, respectively). Allocation of labelled N to different pine tissues was not otherwise significantly affected by elevated [CO_2] at any time point from September 1994 to April 1996. Remobilisation of N to the new needles was complete by April 1995. There were no statistically significant changes in labelled N in the different tissues between September 1994 and April 1995, but the drop in labelled N found in 1994 needles is indicative of the origin of remobilised N for new needle growth. This drop occurred in both ambient and elevated [CO_2] trees.

Different patterns of N storage and remobilisation in evergreen and deciduous trees were confirmed in this experiment. Common alder has the added characteristic that it can fix nitrogen from the atmosphere. One would possibly expect the alder therefore, to be less dependent on remobilised N (of fertiliser origin in the previous summer) for new growth than the pine. A major constraint to this could be that nodule development is not compete by early spring. In evergreen nitrogen fixing plants the ability to fix nitrogen could have an effect on remobilisation of stored N. Interestingly, Thornton *et al.* (1995) found that the ability to fix nitrogen did not affect the proportion of N remobilised in the

spring in *Ulex europaeus*. Alder in this experiment did not seem to be less dependent on remobilised N, however. N partitioning and remobilisation in common alder responded more than Scots pine to elevated [CO_2]. The proportion of labelled N remobilised for spring *leaf* growth was larger in elevated [CO_2] trees of both species, whereas the proportion of labelled N remobilised for spring *shoot* growth was only larger in elevated [CO_2] alder trees. In addition, in alder more labelled N was allocated to 1994 shoots over the winter elevated [CO_2] than in ambient [CO_2]. In Scots pine there were no differences in [CO_2] treatments in allocation of labelled N at any time during the experiment. These results are in agreement with the general reponse of alder and Scots pine to elevated [CO_2], in that the alder had a much larger response than the latter. The mechanisms by which more N was remobilised for spring growth in alder in elevated [CO_2], and why more labelled N was allocated to the 1993 and 1994 elevated [CO_2] shoots in alder than in pine are not clear.

4.11.6 Nitrogen fixation

The classification of *Frankia* species, the bacteria which form a symbiosis with *Alnus* species, has changed with the advent of advanced bacterial classification methods such as nucleic acid content, biochemical and morphological characteristics which has enabled researchers to again classify the bacteria at the species level. Originally classification at the species level was abandoned when it was found that the symbiont was capable of infecting a number of different host species (Sprent and Sprent 1990). Nowadays *Frankia alni* and *F. elaeagni* are commonly used.

Frankia are long-lived organisms, surviving up to eight years and there is evidence that the actinomycete microbe *Frankia* can travel in soil from one plant to another (Wilson and Coutts 1985). In the actinorhizal symbiosis with common alder a recognition system (as yet little understood) enables root hairs to be infected by *Frankia* bacteria producing branched root hairs (as opposed to curled root hairs in legumes). Considerable amounts of secondary wall material are then produced in and around the infection site on the roots (Sprent and Sprent 1990).

During the chemical process of nitrogen fixation uncombined nitrogen from the atmosphere is reduced to ammonium by combining with hydrogen. This occurs via MoFe- and Fe-proteins which form a nitrogenase complex, coupled with a suitable source of ATP and reducing power, with oxygen usually acting as the acceptor molecule. The first product of this process is ammonia which is protonated to ammonium. The latter represses nitrogenase activity making rapid assimilation of ammonium crucial. Ammonium is initially transformed into glutamate via reaction with glutamic acid and ATP which enables rapid assimilation of ammonium pools at low concentrations (Sprent and Sprent 1990). Glutamate is in turn transformed into a number of export products in vascular plants, the main ones in *Alnus* being asparagine, citrulline and allontoin. These products are exported via the vascular bundle to the plant's sinks (Sprent and Sprent 1990).

As well as the direct transfer of nitrogenous compounds to the host plant, nitrogen fixation in *Alnus* plays an indirect role in forests. Leaf fall, excretion from roots of organic compounds such as phenols, fatty acids and amino acids and nodule decay all may contribute to soil nitrogen, improve growth of free-living nitrogen-fixing organisms and reduce soil pathogens (Sprent and Sprent 1990).

The importance of a supply of photosynthetic products from the plant host for symbiotic nitrogen fixation has long been recognised (Gordon and Wheeler 1978). Gordon and Wheeler (1978) demonstrated with *Alnus glutinosa* of different genetic stock and grown in varying environmental conditions, that carbon and nitrogen fixation are closely correlated in young alder. As mentioned earlier, nitrogen-fixing plants with limited access to soil nutrients are expected to respond the most to elevated [CO_2] (Körner 1995). This is because the addition of nitrate to soil inhibits nitrogen fixation (Woodward 1992), and N-fixing trees should be able to use the extra carbon for increased N-fixation and growth. In addition the indirect release of nitrogenous compounds via nodule decay or from the roots would be expected in the longer-term to improve soil status with a positive feedback on plant growth. Thus it would be expected that in conditions of elevated [CO_2] when the ability to fix nitrogen is hypothesised to stimulate growth (see section 4.1.1), this stimulation would be limited to the non-fertilised plots. Unfortunately,

acetylene reduction was only determined in nodules from roots in unfertilised plots, giving no indication of whether added nitrate did inhibit nitrogen fixation. These trees grown without fertiliser seemed all to be fixing nitrogen.

On the other hand one would expect those trees in unfertilised plots to grow larger and have higher nitrogen concentrations than those in the fertilised plots. This was not the case for stem height, SBD or total biomass in alder in both the plus or minus nutrient treatments, except in June and December 1995 when the total biomass of E-N trees was larger than in all other treatments (see Figure 4.5b). Thus the hypothesis that the ability to fix nitrogen in conditions of excess CO_2 would stimulate growth was not supported, although total biomass, SBD and stem height were all greater in elevated [CO_2]. As mentioned earlier (section 4.9.1), it seems that the ability to fix nitrogen gave the alder grown without fertiliser the ability to respond positively to elevated [CO_2], and the addition of adequate fertiliser enabled the alder to also respond positively. Ingestad (1980) found that inoculated grey alder (*Alnus incana*) grown without added mineral nitrogen, had optimum nitrogen status but only half the maximum SGR. This response could not be explained by the energy costs of nitrogen fixation. It seems therefore that nitrogen fixation was not very efficient. This experiment showed that the alder was more stimulated in its growth than the pine, and that this extra growth could well be attributable to the ability to fix nitrogen. As Ingestad found the maximum N fixation rate probably had an upper ceiling compared with the total nitrogen uptake rate required for maximum growth. The *Alnus glutinosa* trees in this study were able to fix more nitrogen in elevated [CO_2] but the extent of this stimulation was probably limited by limitations to nitrogen fixation such as phosphorus availability.

Wilson and Coutts (1985) point out that nodules of *Frankia* are long-lived, unlike mycorrhiza, and that their nitrogenase activity per unit dry mass decreases with increase in nodule size. The alder nodules in this experiment accord with this view: the smaller but more numerous nodules in elevated [CO_2] had higher nitrogenase activity per unit dry mass. This was also the case in 1996 (Gail Jackson, *pers. comm.*) even if the difference was again not significant.

There is a trend in the data for nitrogen fixation to have become more efficient in elevated [CO_2]. The rate of reduction of acetylene per unit mass of nodule as well as per plant was higher in elevated [CO_2] than in ambient [CO_2]. This is in accordance with work by Norby (1987) on *Alnus incana* in which plants and nodules grown under nutrient-poor conditions were larger in elevated [CO_2], and total nitrogenase activity assayed by the acetylene reduction method was significantly higher. It is noteworthy, however, that in Norby's work, specific nitrogenase activity was not significantly affected. Higher nitrogenase activity, nodule and plant dry mass were also found by Arnone and Gordon (1990) in *Alnus rubra,* although in their experiment the significant differences were concomitant with changes in allocation to above-ground biomass. In this study, allocation of biomass was not much affected by [CO_2], and total biomass was not always significantly stimulated. Nitrogen fixation seems therefore to have been positively affected by elevated [CO_2] but to a lesser extent and with less of an impact than found by other authors. Nevertheless, these data do support the premise that nitrogen limitations does not exclude growth stimulation by elevated [CO_2] in nitrogen fixing woody plants.

Conclusions

- Both species of tree showed stimulated growth and biomass accumulation (up to 100 % in alder) in elevated [CO_2]. Stem basal diameter and area were increased in both species, whereas stem height was only increased in pine (despite stem elongation per day increasing in both species). Specific growth rate in 1995 was not affected by elevated [CO_2] in either species, whereas NAR was increased in alder. Leaf area remained the same in alder and was temporarily increased in pine. [N] was unaffected in either species, although [P] temporarily increased in elevated [CO_2] in pine.

- The stimulation of biomass accumulation and carbon assimilation did not persist throughout the three year experimental period, with signs of acclimation of photosynthesis and growth in August 1995 in both species.

- Changes in allocation mostly occurred in alder, with reduced carbon allocation to the leaves, and increased allocation to the shoots. Although root biomass was significantly larger until August 1995, R / S was not generally affected by elevated [CO_2]. Leaf to shoot ratio was reduced in elevated [CO_2]. In Scots pine the only change in allocation of carbon was a reduction in biomass allocation to the needles.

- In general there was little interaction effect of [CO_2] and fertiliser addition on the variables measured. Overall there was no clear nutrient effect on the response to elevated [CO_2].

- Common alder experienced more growth and biomass stimulation than Scots pine, although acclimation of photosynthesis seems to have occurred at around the same time. The faster-growing and nitrogen fixing species was better able to use the extra carbon to increase total biomass, net assimilation rate and nitrogen use efficiency (see Chapter 5 for more details on NUE).

- Nitrogen fixation was found to be occurring in the alder and seemed to be made more efficient in elevated [CO_2].

- Nitrogen allocation to different tissues over the winter and remobilisation for spring growth was different in pine and alder, with N being allocated to and remobilised from the previous year's stem in alder, but from the previous year's needles in pine. There was no [CO_2] effect on this pattern.

- In alder in elevated [CO_2], more N was partitioned to the shoot (stem and branches) over the winter than in ambient [CO_2] whereas in Scots pine there was no [CO_2] effect on partitioning of N over the winter.

- In order to clarify the mechanism for acclimation of photosynthesis in elevated [CO_2] and the effect of nutrient addition on the response, further experiments are needed.

- In particular, a detailed analysis of the effect of different fertiliser addition and nitrogen fixation is necessary in order to have a better grasp on the whole plant response.

CHAPTER 5

Biochemistry of *Alnus glutinosa* and *Pinus sylvestris* grown in elevated carbon dioxide concentration - a three-year experiment at the Glendevon, Perthshire field site

5.1 Introduction

As discussed in Chapters 3 and 4, plants grown in elevated [CO_2] usually show an enhanced rate of carbon fixation and increased biomass production (Eamus and Jarvis 1989, Mousseau and Saugier 1992, Kimball et al. 1993, Poorter 1993), although this change in photosynthetic rate does not necessarily translate into a sustained increase in growth (Stitt 1991, Stitt and Schulze 1994). Such acclimation responses have not been found in all experiments on plant growth and CO_2 and have in the past sometimes been attributed to restricted growth in pots (Eamus and Jarvis 1989, McConnaughay *et al.* 1993) or unnatural conditions in growth chambers (Körner 1995, Norby *et al.* 1996), although recent evidence from longer-term, field experiments is mounting that acclimation in plants grown in elevated CO_2 is not / not only an artefact of pot growth or root restriction but a reality (Tissue *et al.* 1993, Johnson *et al.* 1996, Rey 1997). Acclimation is a common response to plant growth in elevated [CO_2] (Sage *et al.* 1989, Besford 1990, Van Oosten *et al.* 1992, Sicher *et al.* 1994, Jacob *et al.* 1995), and on the whole acclimation seems likely to be the rule (Bowes 1991, Reining 1994) since trees in particular generally live in nutrient-limited environments (Linder and McDonald 1993, Drake *et al.* 1997, Rey 1997) and the photosynthetic process behaves homeostatically bringing any non-limiting components back into balance with limiting components (Stitt and Schulze 1994).

Growth and photosynthetic responses of plants have been the main focus of research on the impacts of elevated [CO_2] on plants over the past twenty years, with particular attention to classical growth analysis and gas exchange measurements (Cure and Acock 1986, Eamus and Jarvis 1989, Mousseau and Saugier 1992, El Kohen *et al.* 1993, Kimball *et al.* 1993, Long *et al.* 1993, Poorter 1993, Ceulemans and Mousseau 1994, Delgado *et al.* 1994). Fewer studies have concentrated on metabolic responses in leaves (Sage 1990, Bowes 1991, Stitt 1991, Sicher *et al.* 1994, Stitt and Schulze

1994) although the metabolic activity in leaves largely regulates plant growth and development. Acclimation to elevated [CO_2] in trees and other plants comes in a wide variety of forms ranging from morphological and anatomical to physiological and biochemical. Plants respond from the leaf to the whole plant or stand scale to elevated [CO_2]. The intricacies of response at each level need to be elucidated in order to have a better grasp of the overall mechanisms involved in response to elevated [CO_2].

On a biochemical level much attention has been focused on Rubisco, the world's most abundant enzyme accounting for up to 50 % of total soluble protein in leaves (Lawlor 1993), which catalyses the carboxylation of ribulose-1,5-bisphosphate (RuBP), the first molecule in the photosynthetic carbon reduction (PCR) cycle. This enzyme constitutes the largest nitrogen sink in the photosynthetic apparatus, often existing at higher concentrations than is necessary for maximum photosynthesis at high irradiance, suggesting a possible additional role as a nitrogen store (Stitt 1991). Rubisco is a rather slow enzyme with a low turnover and low affinity for CO_2 (Miziorko and Lorimer 1983, Bowes 1991). Oxygen and carbon dioxide both compete for the active site of this enzyme. Given these characteristics, Rubisco limits the rate of photosynthesis in C_3 plants at current atmospheric [CO_2] and [O_2] (Lawlor 1993). The short-term increase in photosynthetic capacity in C_3 plants grown in elevated [CO_2] is engendered by a higher availability of CO_2, a major substrate of Rubisco in carboxylation and also an activator of Rubisco via carbamylation. The competition between carbon (photosynthetic carbon reduction, PCR cycle) and oxygen (photorespiration or carbon oxidation PCO cycle) for the Rubisco active site also shifts in favour of carboxylation in elevated [CO_2]. All these factors stimulate the rate of photosynthesis.

A common response of trees and other plants to longer-term growth in elevated [CO_2] is acclimation in the form of decreased leaf [N], in both chlorophyll and Rubisco, and accumulation of non-structural carbohydrate in the leaves (Eamus and Jarvis 1989, Mousseau and Saugier 1992, Poorter 1993, Ceulemans and Mousseau 1994, Wilkins *et al.* 1994, Norby et al. 1996). In these cases photosynthetic capacity is reduced via diminished photosynthetic components (Delgado *et al.* 1994, Wilkins *et*

al. 1994), such as Rubisco, chlorophyll or other enzymes. There are several proposed mechanisms for this acclimation, for example whether acclimation is mediated via Rubisco and the PCR cycle, or a source-sink imbalance caused by accumulation of starch and soluble carbohydrates in the chloroplast which in turn causes down-regulation of photosynthetic components. In models of photosynthesis describing the regulation of Rubisco, electron transport and triose phosphate use in response to PPFD and / or [CO_2], the main assumption is that non-limiting processes are brought back into balance with limiting processes (Farquhar 1979, Farquhar *et al.* 1980, Sage 1990, Bowes 1991, von Caemmerer *et al.* 1994). The location of the main limiting step in such models depends on the prevailing conditions (Stitt 1991). It is hypothesised that RuBP regeneration limits photosynthesis at high [CO_2] or in low PPFD (Farquhar *et al.* 1980), whereas at low [CO_2] or in high light the [RuBP] is in excess of the number of binding sites on Rubisco, and photosynthesis is limited by Rubisco activity or concentration (Bowes 1991). A third limitation to photosynthesis often proposed in models is triose phosphate limitation of sucrose synthesis (Bowes 1991, Stitt 1991).

Stitt and Schulze (1994) cite mounting evidence that photosynthetic metabolism is regulated in highly complex and interactive ways, which cannot be assigned to just one rate-limiting step dependent on prevailing conditions. They postulate that control over a pathway can be shared between two enzymes and the relative distribution of this control varies depending on the prevailing conditions. In addition, in conditions of high [CO_2] the PPFD-saturated rate of photosynthesis may be restricted by the rates of sucrose and starch synthesis (Stitt and Quick 1989). This form of limitation of photosynthesis is one of the direct ways in which starch and carbohydrate accumulation could be regulating photosynthesis, along with direct chloroplast disruption (Stitt 1991). An indirect and more adaptive way in which photosynthesis could be limited by carbohydrate accumulation is via reduction in protein levels and other photosynthetic components. This can be viewed as more adaptive because it engenders a readjustment of the source-sink imbalance and allow for the reallocation of nitrogen and other components from the leaves to new or already established sinks. Recent experiments have also highlighted the potential importance of canopy closure

and restriction of rooting volume (Drake *et al.* 1997). All of these sometimes conflicting hypotheses need further investigation in order to elucidate the role a particular protein plays in the acclimation of plants to long-term growth in elevated [CO_2].

The long-term response of trees to elevated [CO_2] in nutrient limited conditions is an important question, since these are the conditions in which most forest trees grow (e.g. Linder and McDonald 1993, Norby *et al.* 1996, Drake *et al.* 1997). This chapter concentrates on the biochemical responses of common alder and Scots pine to long-term exposure to elevated [CO_2] and nutrient addition, to evaluate whether and how leaf composition changes in order to gain a better grasp of the potential mechanisms underlying long-term acclimation to elevated [CO_2]. Specific aims of the experiment were to elucidate whether:

1) Scots pine and common alder would respond to elevated [CO_2] by reductions in concentrations of Rubisco, soluble protein and chlorophyll in leaves as found in other investigations of trees grown in elevated [CO_2];
2) there would be a species-specific biochemical response to long-term growth in elevated [CO_2], particularly in common alder where inorganic nitrogen could be fixed from the atmosphere and the trees thus be less dependent on soil nitrogen sources;
3) whether alder would exhibit less of a source-sink imbalance than Scots pine since nitrogen-fixing nodules could act as a large sink for any extra carbon fixed;
4) whether nutrient addition would affect the biochemical response to elevated [CO_2]; and
5) whether starch and soluble carbohydrate accumulation would occur in the leaves of alder and pine grown in elevated [CO_2].

5.2 Materials and Methods

5.2.1 Sampling Procedure

Details of the Glendevon experimental design and procedure are given in Chapter 4. Samples of leaves for biochemical determinations were taken monthly starting in August 1994 and continuing throughout 1995. Gail Jackson and Diarmuid O'Neill continued with monthly sampling in the 1996 growing season. The same kind of leaves were taken for biochemical analysis throughout the experimental period. Leaves were sampled from one tree from each of the fertilised and non-fertilised quadrants, thus two per chamber per species. Three discs (1 cm in diameter) were sampled from an alder leaf, *c.* 20 cm from the top of the seedling and three current-year needles *c.* 5 cm from the tip of the leader shoot in pine were sampled monthly close to midday during the growing season for chlorophyll, Rubisco and soluble protein. During periods of low turgidity or fungal disease (see Chapter 2) five leaf discs or needles were taken. The Rubisco / soluble protein and chlorophyll samples were frozen directly in 0.2 cm^3 screw top microcentrifuge tubes (Camlab Ltd., Cambridge, UK) in liquid nitrogen (using a cryoshipper) and kept in - 80 °C and - 20 °C freezers, respectively, until analysis. A second set of leaf samples was collected at the same time from the same leaves. The fresh and dry mass of the second set of leaves was determined (M_f and M_d) and these data were used in the calculation of Rubisco, soluble protein and chlorophyll concentrations. This second leaf sample was necessary since a period of lengthy weighing leaves away from a freezer would leave the protein denatured. Rubisco activity in Scots pine fluctuates periodically over the year and depending both on needle-age and on the prevailing climate conditions (Gezelius 1986). This may have introduced extra variability into the results, although by consistently sampling new needles from near the top of the trees it was hoped to reduce this extra variation.

For carbohydrate and starch determination Universal 25 cm^3 vials were filled with needles or leaves (since more material was required than for the other biochemical analyses). Carbohydrate and starch samples were kept on frozen-ice in the field, freeze-dried in the laboratory, ground in a centrifuge grinder (Retsch, Glen Creston, Stanmore, UK) and then kept in a vacuum until analysis.

5.2.2 Chlorophyll Determination

Chlorophyll *a*, chlorophyll *b* and total chlorophyll were determined spectrophotometrically (Series 2, Cecil Grating, Cambridge, UK) in N, N-dimethyl formamide (DMF). Intact leaf tissue was immersed in DMF in darkness for four to seven days until the chlorophyll was fully extracted. Absorbances were measured at 647, 664 and 750 nm with DMF as a blank reference. The following equations derived from Porra *et al.* (1989) were used to calculate chlorophyll concentrations ($\mu g\ g^{-1}$):

$$\text{Chl } a = \frac{12 \times (A_{664} - A_{750}) - 3.11 \times (A_{647} - A_{750}) \times 3}{M_d} \quad 5.1)$$

$$\text{Chl } b = \frac{20.78 \times (A_{647} - A_{750}) - 4.88 \times (A_{664} - A_{750}) \times 3}{M_d} \quad 5.2)$$

$$\text{Chl } a + b = \frac{17.67 \times (A_{647} - A_{750}) + 7.12 \times (A_{664} - A_{750}) \times 3}{M_d} \quad 5.3)$$

where M_d = dry mass of leaf discs (alder) or needles (pine)
A = absorbance (nm) at a particular wavelength

For expression on a leaf area basis the above concentrations were multiplied by:

$$M_d / r \quad 5.4)$$

where r = area of leaf discs (cm^2)

5.2.3 Soluble Carbohydrate and Starch

Soluble Sugar Concentration

Soluble carbohydrates were analysed using anion-exchange chromatography with pulsed amperometric detection (Dionex Corporation, Sunnyvale, CA, USA). A sample of 50 mg (± 1 mg) of freeze-dried and ground leaves in 10 cm^3 of double distilled water were extracted once by shaking and placing in a 30 °C water bath for 15 minutes. After centrifuging at 4500 rpm for 15 minutes, the supernatant was removed, and filtered using Whatman 0.2 µm nitro-cellulose membrane filters

(Maidstone, England). The retrieved solution was diluted 1 : 10 and then analysed by ion chromatography using a Carbopak PA carbohydrate column (Ion Chromatography System hplc, DX500).

Each sample was run for one hour through the Dionex machine according to the following protocol: prior to sample injection the machine was washed with double distilled water, then 200 mmol dm^{-3} NaOH (100 % of the eluent) was injected into the system for the first 15 minutes of the 1 hour run (per sample), followed by 16 mmol dm^{-3} NaOH (i.e. 8 % of the eluent) and double distilled water (92 % of eluent) for the last 45 minutes. This method was used on all the samples analysed. A set of ten pure sugars and polyols (inositol, manitol, fucose, rhamnose, arabinose, galactose, glucose, xylose, fructose and sucrose) was run as a standard with each set of 10 samples. Unknown peaks were not included in the results since the peaks were consistently very small. On exception to this was pinitol found in pine needles. Pinitol's peak was found very close to that of inositol but it was possible to distinguish between the two, so the sugars were discussed separately in the results section 5.3.2. Peaks obtained during the analysis were integrated using Peaknet Dionex Software (see Appendix 5 for details of integration parameters used to calculate peak area for each sample) followed by a linear calibration. The concentration of sugars in the samples (mg g^{-1}) was calculated as follows:

$$\frac{c \times d \times v}{M_d} \qquad 5.5)$$

where d = dilution factor

M_d = sample dry mass

c = concentration provided by integration calculation (mg dm^{-3})

v = volume used in extraction (cm^3)

Starch Concentration

Chemical methods for the analysis of starch are faster and cheaper than their enzymatic counterparts but are highly unspecific, such that one can not be sure that

the results relate only to starch. Certain acids used in the process extract not only starch but cellular components such as other insoluble carbohydrates (cellulose, hemicellulose etc.) leaving all but lignin left in the pellet (Dr. M. Forstreuter, *pers. comm.*). In addition, the use of dangerous chemical reagents renders the process less attractive as a laboratory method. For this reason I intended to determine starch using the enzyme method. Unfortunately, many of my 1995 freeze-dried samples were found to be mouldy and could no longer be analysed. I have thus included Gail Jackson and Diarmuid O'Neill's 1996 starch results using the chemical iodine and acid method in my results, to give an indication of how starch changed over time.

Table 5.1 shows a comparison of the two starch methods, in order to assess their compatibility. The higher values for starch using the iodine method are probably indicative that not just starch has been measured but also cellulose and hemicellulose etc. This should be taken into account when discussing the 1996 results.

Table 5.1 A comparison of [starch] (mg g^{-1}) in common alder leaves in July 1994 determined by the iodine method (Allen 1989) and the enzyme method (Böhringer Mannheim 1994).

Treatment	Sample	Iodine method	Enzyme method
E-N	1	76.02	19.31
E-N	2	87.81	16.16
E+N	3	240.27	55.50
E+N	4	48.19	17.85
A-N	5	122.51	25.86
A-N	6	64.56	33.74
A+N	7	2.02	1.96
A+N	8	71.77	26.95

In 1995 starch concentration was determined in ethanol extracts by the Böhringer Mannheim (1994) enzyme method using amyloglucosidase (AGS) which split the

starch into D-glucose, followed by glucose phosphorylation and the production of NADPH upon reaction with NADP (determined colorimetrically using a spectrophotometer). Starch was extracted from 100 (± 10) mg freeze-dried and ground samples, in 80 % ethanol by heating in a water bath (60 °C) for 30 minutes. The pellet was then centrifuged (at 4000 rpm) and again washed with 80 % ethanol, shaken and centrifuged. 10 cm^3 of distilled water was added to the pellet and the samples were then autoclaved for one hour, cooled and again centrifuged. The supernatant was analysed colorimetrically for starch as follows: 200 mm^3 of amyloglucosidase (AGS) and 100 mm^3 of extracted sample (or distilled water blank) were added to each cuvette and the cuvettes incubated for 20 minutes in a water bath at 50 °C. Then 2.7 cm^3 of pH 6.9 buffer (made up of 250 mmol m^{-3} triethanolaminehydrochloride, 10 mmol m^{-3} $MgSO_4$, 1 mmol m^{-3} NADP and 1 mmol m^{-3} ATP) were added to each cuvette, the solution was mixed and the absorbance read at 340 nm (E_0) after three minutes. Finally, 20 mm^3 of hexokinase and G6P-dehydrogenase was added to the corner of each cuvette, the solution was mixed and read at 340 nm (E_1) after 15 minutes. Absorbance E_1 was subtracted from absorbance E_0 to give E_A. Each sample was measured twice in this way and the average E_A used to calculate starch concentration (mg g^{-1} dry mass) as follows:

$$\frac{0.77705 \times E_A}{M_d \times v}$$

where v = volume of water in which sample was analysed

E_A = the difference between the two absorbances measured

M_d = dry mass of sample

In 1996 Diarmuid O'Neil and Gail Jackson determined starch in perchloric acid extracts by the iodine method described by Allen, modified (1989). The blue colour produced when starch reacts with iodine is measured colorimetrically. Starch was extracted in $HClO_4$ (32 % v / v) for 30 minutes at room temperature. 1 cm^3 aliquots were diluted in distilled water in 25 cm^3 flasks. Absorbance of the solution was

measured at 610 nm after having added 0.1 N HCl (four drops) and 2 % iodine solution (250 mm^3). Standards of known starch concentration were analysed with each set of samples. These standards were used to make a calibration curve from which the concentration (mg g_{-1}) of starch in the samples was calculated as follows:

$$\frac{c}{v_3} \times \frac{v_1}{M_d} \times v_2 \qquad 5.7)$$

where c = concentration of starch from calibration curve (mg cm^{-3})
M_d = dry mass of freeze-dried sample (mg)
v_1 = volume of flask (cm^{-3})
v_2 = volume of perchloric acid added (cm^{-3})
v_3 = volume added to flask (cm^{-3})

5.2.4 Rubisco Concentration

Determination of Concentration of Rubisco Standard

The concentration of Rubisco standard was determined according to the method of Wishnick and Lane (1971). An aliquot of purified Rubisco was diluted 1:100 in Tris buffer (pH 7.4) and centrifuged at 14 000 rpm in a microfuge. The solution was measured in the ultra-violet range on a UV and visible double beam spectrophotometer (Cecil CE 594) at 280 nm (A_{280}) and then 260 nm (A_{260}). The following calculation (5.8) enabled the extinction coefficient (E) of the protein to determined as:

$$\frac{1.55 \times A_{280} - 0.77 \times A_{260}}{10} \qquad 5.8)$$

The concentration (mg / cm^3) of protein was obtained by multiplying the extinction coefficient by the absorbance at 280 nm :

$$E \times A_{280} \qquad 5.9)$$

The ratio of absorbance at 280 nm / 260 nm is 1.9 for pure protein. Lower ratios indicate contamination with interfering substances such as nucleic acids. The aliquots of Rubisco standard used for this research had ratios of between 0.73 and 1.68.

Optimisation of Enzyme -Linked Immunosorption Assay (ELISA)

In order to make the ELISA work with Scots pine and common alder samples the assay was optimised for common alder and Scots pine by running a series of differing sample and antibody dilutions over a period of months. The results of these different assay conditions are outlined below in Table 5.2.

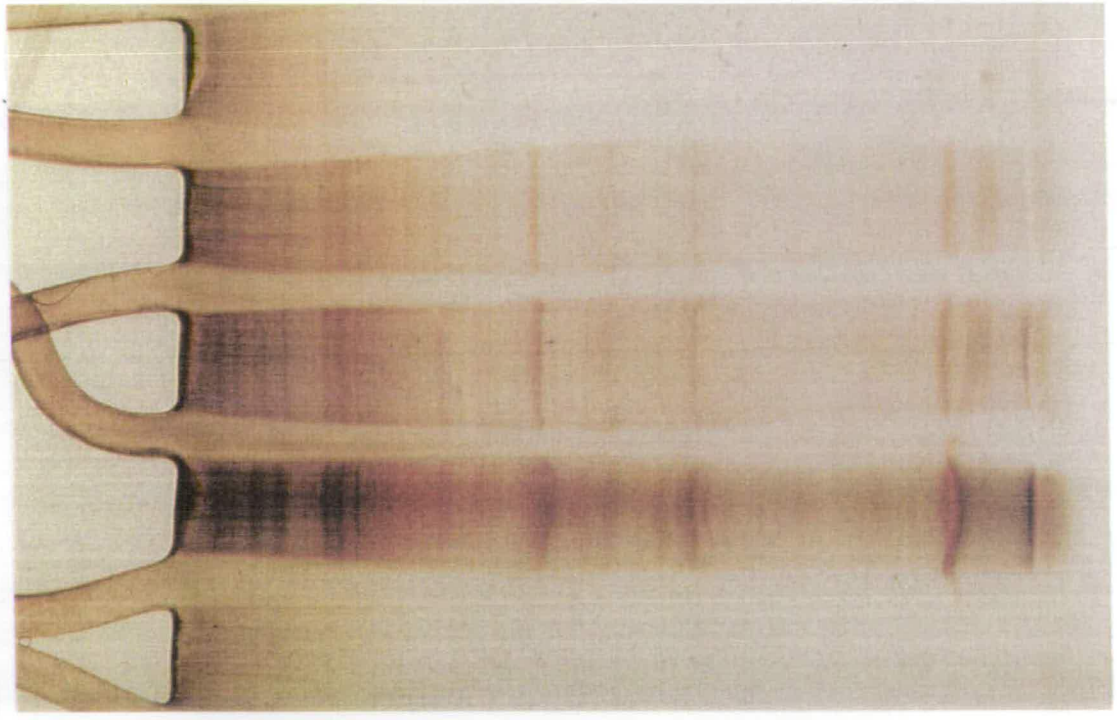
Enzyme Concentration Determination using ELISA

A modified immunological assay specific to the plant protein Rubisco developed from conventional ELISA assays by Catt and Millard (1988) was used to determine Rubisco. Catt's and Millard's assay differs from the conventional sandwich assays (primary antibody, protein, antigen) in that the protein is the first to bind to the plate, followed by the primary and then secondary antibodies. The assay is a positive assay and takes two days. The primary antibody, specific for Rubisco, was obtained by injecting pure Rubisco into a sheep and extracting polyclonal antiserum after a certain period.

Rubisco Extraction

Four leaves or needles were ground to a powder in liquid nitrogen, 4 cm^3 of PVPP, 4 cm^3 of cold extraction buffer (pH 7.4) containing 50 mmol m^{-3} HEPES,
5 mmol m^{-3} $MgCl_2$, 1 mmol m^{-3} EDTA, 1 mmol m^{-3} EGTA, 10 % (v/v) glycerol,
20 mmol m^{-3} DTT and (10 mm^3 per cm^3 of buffer) of benzamidine, E-Aminocaproic acid and PMSF. The extract was left to thaw, centrifuged in a microcentrifuge (3 minutes at 1400 rpm), the supernatant retrieved poured into two micro-centrifuge tubes, one for total soluble protein analysis (see section 5.2.4), the other for Rubisco determination. The latter was diluted appropriately (1:1000 for pine, 1:5000 for alder; see Table 5.2 above on Optimisation of ELISA) in 50 mmol m^{-3} carbonate / sodium bicarbonate buffer (pH 9.6) and 100 mm^3 aliquots used to coat individual wells on an ELISA plate.

Photo 5.1 SDS Gel of purified *Prunus avium* Rubisco. The bands of the large and small sub-units (at the end of the gel) are visible. Photo 5.2 An Elisa Plate with rows of Rubisco standard (no samples), at the end of the assay, showing the yellow colour formation in various grades according to Rubisco concentration.



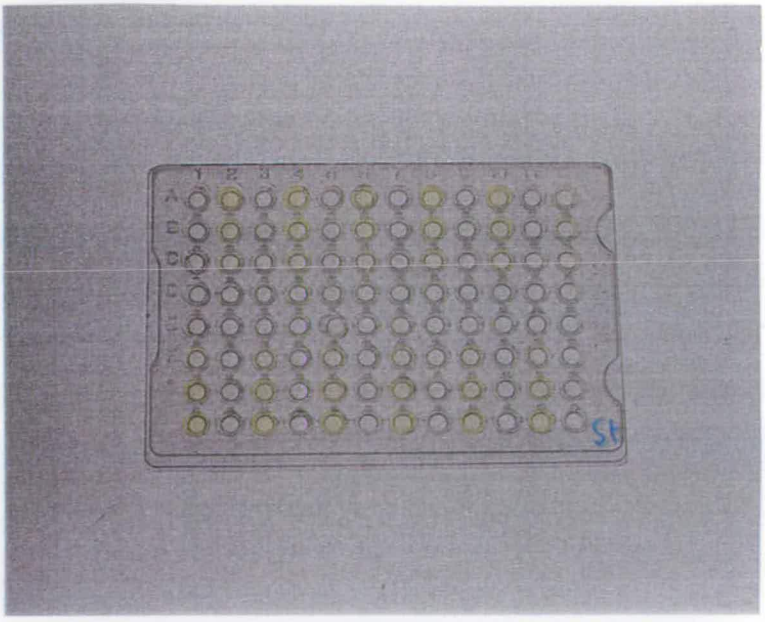


TABLE 5.2 ELISA ASSAY OPTIMISATION 1995 / 1996										
test number	*test date*	*no. of plates*	*extract. buffer (ml)*	*pine sample dilution*	*alder sample dilution*	*starting standard conc.*	*1st antibody dilution*	*2nd antibody dilution*	*mg of test leaf*	*success?*
1	22-23 Nov. 1995	2								
2	28-29 Nov. 1995	6 (3 pine, 3 alder)	4	1 : 5000 1: 10000 1: 20000	1 : 5000 1 : 10000 1 : 20000	A = 6.66 µg /ml	1 : 200	1: 300	16.46 mg (pine) 14.6 mg (alder)	NO (neg. values)
3	1-2 Dec. 1995	1	4	1: 5000 1: 10000 1: 20000	same as for pine	A = 6.66 ug / ml	1 : 100	1: 300	approx. 10, 15, 20 mg per species	very few positive results
4	5- 6 Dec. 1995	2	4	1: 1000 1: 5000 1 : 10000 1 : 20000	same as pine	B = 3.33 ug / ml	1 : 100	1 : 200	55. 5 mg of one pine needle	NO (plate tipped slightly)
5	7-8 Dec. 1995	2	4	1 : 1000 1 : 5000 1 : 10000 1 : 20000	same as pine	B = 3.33 ug / ml	1 : 100	1 : 300	58 mg of one pine needle	YES (but low absorb.)
6	30-31 Jan. 1996	2	4	1 : 1000 1 : 5000 1 : 10000	same as for pine	C = 1.66 ug ml	1 : 100	1 : 300	55.2 = p1 55.6 = p2 40.6 = a1 44..3 =a2	YES (pine best 1 : 1000, alder 1 : 5000)
7	7-8 Feb. 1996	1	4	1 : 1000	1 : 5000	B = 3.33 ug / ml	1 : 200	1 : 300	44.5 mg = alder 52 mg = pine	YES

The ELISA Assay

ELISA plates, which are conventionally used for immunological protein assays, have 8 rows (labelled A-H) and 12 columns (labelled 1-12) with a total of 96 wells, each holding 150 mm^3, see Photo 5.2 (in *Determination of Concentration of Rubisco Standard*). Since there was variability across plates and between wells, one sample was pipetted into one column per plate and two or four columns were filled with Rubisco standard (also diluted in carbonate buffer, with progressive dilution down the column in order to generate a calibration curve). For a schematic description of the ELISA assay see Appendix 9. All wells on a plate were coated with 125 mm^3 of appropriately diluted samples and Rubisco standard, and the plate was incubated for 18 hours at 4 °C in moist conditions (the protein sticks to the wells). Day 2: after 18 hours the plates were inverted and shaken to remove samples from the wells, and the plates were washed three times with Tris / NaCl Buffer (pH 7.4) containing 10 mmol m^{-3} Tris and 150 mmol m^{-3} NaCl. Any remaining active sites on the plates were blocked by incubating each well with the Tris buffer (above) containing 1 % (w / v) bovine serum albumin (BSA) and 0.01 % (v / v) Tween-20 for one hour at room temperature. The plates were again washed three times with Tris buffer, but this time with added BSA and Tween-20 to remove any unattached protein. 150 mm^3 of the primary antibody (sheep) diluted 1:200 in the Tris / BSA / Tween-20 buffer, was pipetted into each well and left to incubate for two hours at room temperature. The same washing procedure as above was repeated and the plates then coated with 150 mm^3 of the 2nd antibody (anti-sheep alkaline phosphatase conjugate, Sigma A5187, St Louis, MO 63178, USA) diluted 1:300 in Tris / BSA / Tween-20 buffer, and left for one hour at room temperature. The plates were again washed three times with Tris / BSA / Tween-20 buffer. 150 mm^3 of 50 mmol m^{-3} carbonate / sodium bicarbonate buffer (pH 9.6) containing 0.1 % p-Nitrophenolphosphate was added to each well and left to develop colour. The p-Nitrophenolphosphate removed the phosphate tail on the second antibody which produces a yellow colour. The reaction was stopped by the addition of 10 mm^3 of 3 mol m^{-3} NaOH to each well. The optical density of each well was read at 405 nm using an ELISA plate reader (Softmax automatic plate reader, Softmax, UK).

Rubisco Calibration Analysis

Absorbance means (at 405 nm) for each sample (each sample was present in one column per plate, therefore in eight wells) and for the standards (each standard concentration was present in three wells per plate) were calculated from the output of the automatic plate reader. A calibration curve, derived from the standard averages, was plotted on a half-log scale (x axis) against absorbance (y axis) (see Figure 5.1). Sample absorbances were mostly in the linear part of the curve, such that equations of the lines of best fit were obtained using linear regression analysis, from which the sample absorbance was then calculated.

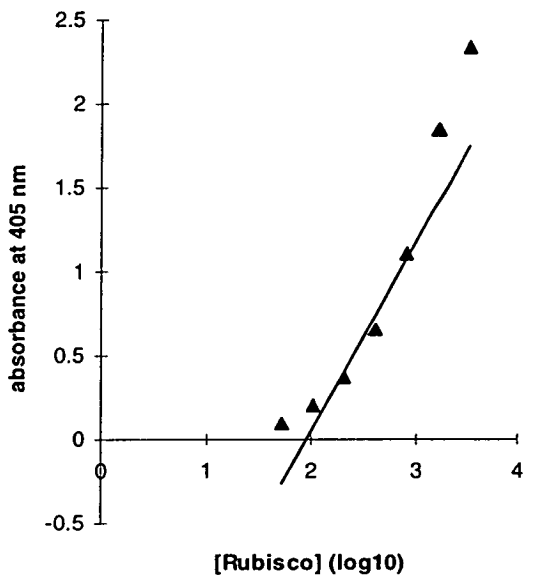


Figure 5.1 Rubisco standard calibration curve (ng / cm^3, log 10) against absorbance, derived from an ELISA assay on 15 February 1996 and used for Scots pine April 1995 samples. Each data point is the mean of three standard samples at one concentration.

The sample absorbance in question was read from the calibration curve and the dilution and fresh or dry mass or mg cm^{-2} of leaf area (in alder) of the sample taken into account in calculating the concentration of Rubisco (mg g^{-1}). It was decided to report Rubisco and soluble protein data on a fresh mass basis since fresh leaf material was used, and water content did not affect the pattern of monthly change in Rubisco.

Protein concentrations expressed on a dry mass and area basis were nevertheless also analysed statistically and the results of these tests are given in the text.

The following regression equations derived from standards on different ELISA plates (date of assay in brackets) were used to calculate the Rubisco concentrations in common alder:

April 1995

$y =$	$-2.38 + 1.21x$	($r^2 = 0.76$)	(13 February 1996)
$y =$	$-2.35 + 1.26x$	($r^2 = 0.93$)	(15 February 1996)
$y =$	$-1.35 + 1.05x$	($r^2 = 0.88$)	(8 May 1996)

June 1995

$y =$	$-1.66 + 1.19x$	($r^2 = 0.86$)	(8 May 1996)
$y =$	$-2.02 + 1.05x$	($r^2 = 0.85$)	(8 May 1996)
$y =$	$-1.64 + 1.17x$	($r^2 = 0.86$)	(8 May 1996)
$y =$	$-1.44 + 1.09x$	($r^2 = 0.86$)	(8 May 1996)

August 1995

$y =$	$-2.38 + 1.20x$	($r^2 = 0.86$)	(13 February 1996)
$y =$	$-1.70 + 1.10x$	($r^2 = 0.89$)	(6 May 1996)
$y =$	$-2.02 + 105x$	($r^2 = 0.85$)	(8 May 1996)
$y =$	$-1.64 + 1.17x$	($r^2 = 0.86$)	(8 May 1996)
$y =$	$-1.44 + 1.09x$	($r^2 = 0.86$)	(8 May 1996)

September 1995

$y =$	$-2.07 + 1.28x$	($r^2 = 0.99$)	(1 May 1996)
$y =$	$-2.02 + 1.24x$	($r^2 = 0.98$)	(1 May 1996)

October 1995

$y =$	$-2.38 + 1.21x$	($r^2 = 0.86$)	(13 February 1996)
$y =$	$-2.37 + 1.28x$	($r^2 = 0.92$)	(15 February 1996)
$y =$	$-2.02 + 1.05x$	($r^2 = 0.85$)	(8 May 1996)

The following regression equations (date of assay in brackets) were used to calculate the Rubisco concentration in Scots pine:

April 1995

$y =$	$-2.17 + 1.11x$	($r^2 = 0.85$)	(13 February 1996)

$y =$	$-2.39 + 1.27x$	($r^2 = 0.92$)	(15 February 1996)
$y =$	$-1.34 + 1.05x$	($r^2 = 0.88$)	(8 May 1996)

August 1995

$y =$	$-2.17 + 1.11x$	($r^2 = 0.85$)	(13 February 1996)
$y =$	$-2.02 + 1.34x$	($r^2 = 0.94$)	(6 May 1996)
$y =$	$-1.95 + 1.28x$	($r^2 = 0.95$)	(6 May 1996)
$y =$	$-1.70 + 1.17x$	($r^2 = 0.95$)	(6 May 1996)

September 1995

$y =$	$-2.07 + 1.28x$	($r^2 = 0.99$)	(1 May 1996)
$y =$	$-2.02 + 1.24x$	($r^2 = 0.98$)	(1 May 1996)

October 1995

$y =$	$-2.41 + 1.28x$	($r^2 = 0.84$)	(13 February 1996)
$y =$	$-2.17 + 1.11x$	($r^2 = 0.91$)	(15 February 1996)
$y =$	$-2.02 + 1.05x$	($r^2 = 0.85$)	(8 May 1996)

5.2.5 Total Soluble Protein

This assay is an optimised version of Bradford's total protein assay (Read and Northcoate 1981), using Bovine Serum Albumin (BSA) and BIO-RAD dye (phosphoric acid and methanol). Since BSA is an animal protein and Rubisco a plant protein, a comparison between Rubisco and soluble protein (i.e. the proportion of total protein that is Rubisco) will not necessarily be very accurate, since the use of BSA as a standard underestimated the concentration of soluble protein in the leaves. Using pure Rubisco as a standard could possibly have produced better absolute results, although the sample absorbance was often higher than the range of the Rubisco calibration curve. Also, lack of enough purified Rubisco enzyme did not allow for the use of Rubisco as a standard.

Three BSA standards (250, 500 and 750 mm^3 per cm^3 of water) were made up from a standard stock solution of 1 mg BSA / 1 cm^3 water (purified by Millipore). Two replicates per sample were analysed for soluble protein, one supernatant diluted 1:10, the other undiluted supernatant. The diluted samples gave inconsistent results and so were not used for the calculation of total soluble protein. 5 cm^3 of diluted BIO-RAD

(1:5 from stock) was added to 100 mm^3 of the standards, the sample and the blank, the contents mixed well, and left to develop colour over 5 minutes after which the samples were read in plastic cuvettes in a double beam spectrophotometer at 598 nm. Linear regression of the calibration curve of the standards (absorbance against concentration, $\mu g\ cm^{-3}$) was used to calculate the concentration of protein (mg g^{-1}) in the samples, taking into account the fresh mass, dry mass, water content and area of the sample and how much extraction buffer was used in the extraction. The following linear regression equations were used to calculate the concentration of protein in alder leaf samples:

April 1995

$y =$	$0.0057 + 0.00097x$	($r^2 = 0.997$)	(13 February 1996 assay)
$y =$	$-0.0010 + 0.0012x$	($r^2 = 0.996$)	(15 February 1996 assay)
$y =$	$0.13 + 0.0085x$	($r^2 = 0.996$)	(6 May 1996 assay)

June 1995

$y =$	$0.13 + 0.0010x$	($r^2 = 0.994$)	(6 May 1996 assay)

August 1995

$y =$	$0.023 + 0.00094x$	($r^2 = 0.996$)	(13 February 1996 assay)
$y =$	$0.13 + 0.00085x$	($r^2 = 0.996$)	(6 May 1996 assay)
$y =$	$0.13 + 0.0010x$	($r^2 = 0.99$)	(8 May 1996 assay)

September 1995

$y =$	$0.13 + 0.00078x$	($r^2 = 0.98$)	(3 May 1996 assay)

October

$y =$	$-0.0010 + 0.0012x$	($r^2 = 0.997$)	(15 February 1996 assay)
$y =$	$0.0057 + 0.00097x$	($r^2 = 0.997$)	(13 February 1996 assay)
$y =$	$0.13 + 0.0010x$	($r^2 = 0.99$)	(8 May 1996 assay)

The following linear regression equations were used to calculate the concentration of protein in pine needle samples:

April 1995

$y =$	$-0.0010 + 0.0012x$	($r^2 = 0.996$)	(15 February 1996 assay)
$y =$	$0.10 + 0.00090x$	($r^2 = 0.997$)	(13 February 1995 assay)
$y =$	$0.13 + 0.00085x$	($r^2 = 0.996$)	(6 May 1996 assay)

August 1995

$y =$ $0.12 + 0.0078x$ ($r^2 = 0.98$) (13 February 1996 assay)

$y =$ $0.13 + 0.00085x$ ($r^2 = 0.996$) (6 May 1996 assay)

September 1995

$y =$ $0.13 + 0.00078x$ ($r^2 = 0.98$) (3 May 1996 assay)

October 1995

$y =$ $0.10 + 0.00090x$ ($r^2 = 0.997$) (13 February assay)

$y =$ $-0.0010 + 0.0012x$ ($r^2 = 0.996$) (15 February 1996 assay)

$y =$ $0.13 + 0.0010x$ ($r^2 = 0.994$) (8 May 1996 assay)

5.2.6 Statistical Analysis

All data were checked for homogeneity of variance (F-test or Levene's test) and normality (test of fitted values versus residuals) and met these assumptions, except for the ratio of chlorophyll *a* / *b* which were transformed logarithmically. Since the variance of the Rubisco and soluble protein data was sometimes higher than the mean, the data was analysed twice, once with non-transformed data and once with log transformed data, but the results were the same. This indicates the power of SAS glm models in dealing with very variable data.

5.3 Results

5.31 Chlorophyll

In Figures 5.2a and 5.2b total chlorophyll concentration in alder on a leaf dry mass and leaf area basis of fully expanded leaves is reported for each [CO_2] treatment over time. Figure 5.3 shows total chlorophyll concentration on a needle dry mass basis over time per [CO_2] treatment. Tables 5.3 and 5.4 show alder chlorophyll *a*, chlorophyll *b* and the ratio of *a* / *b* on a dry mass basis and an area basis, respectively. Table 5.5 shows the same data for Scots pine on a mass basis.

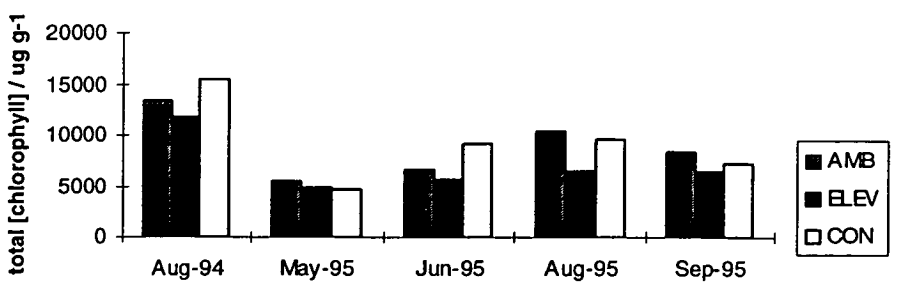


Figure 5.2a Seasonal course of mean total [chlorophyll] (ug g^{-1}) on a mass basis in *common alder*. Values are the means of eight replicates. AMB = ambient [CO_2] plus and minus nutrients; ELEV = elevated [CO_2] plus and minus nutrients; CON = control plot. [CO_2] had a significant effect in August 1995 (p = 0.018).

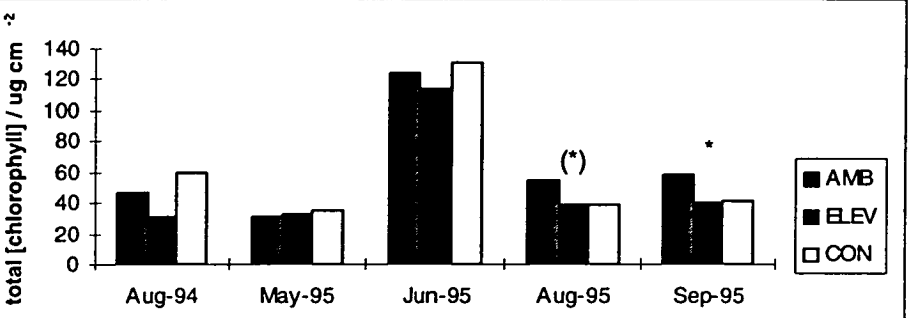


Figure 5.2b Seasonal course of mean total [chlorophyll] (ug cm^{-2}) on an area basis in *common alder*. Values are the means of eight replicates. AMB = ambient [CO_2] plus and minus nutrients; ELEV = elevated [CO_2] plus and minus nutrients; CON = control plot. [CO_2] had a significant effect in September 1995 (p = 0.016) and a trend towards an effect in August 1995 (p = 0.06).

Total Chlorophyll Concentration

Concentration of chlorophyll was highest in common alder in August 1994, followed by August and September 1995 (dry mass basis), and in June 1995 on an area basis (see Figure 5.2a and 5.2b). In Scots pine the highest concentrations were also found in August and September on a dry mass basis (see Figure 5.3).Total chlorophyll in both species was consistently lower in elevated [CO_2] although the difference was rarely significant. The elevated [CO_2] treatment showed a decrease in total chlorophyll of between 9.9 and 14 %, except in May 1995 (on an area basis) when there was a slight increase in total chlorophyll. There was no other clear seasonal pattern.

Nutrient addition had no significant effect on total chlorophyll on a dry mass basis ($p > 0.05$), but there was a significant nutrient effect in August 1995 ($p = 0.004$) and in September 1995 ($p = 0.01$) on total chlorophyll on an area basis. Interestingly, there was an interaction effect of [CO_2] and nutrients on total chlorophyll in alder in May 1995 ($p = 0.02$ on a mass basis, $p = 0.003$ on an area basis), but not at any other time ($p > 0.05$).

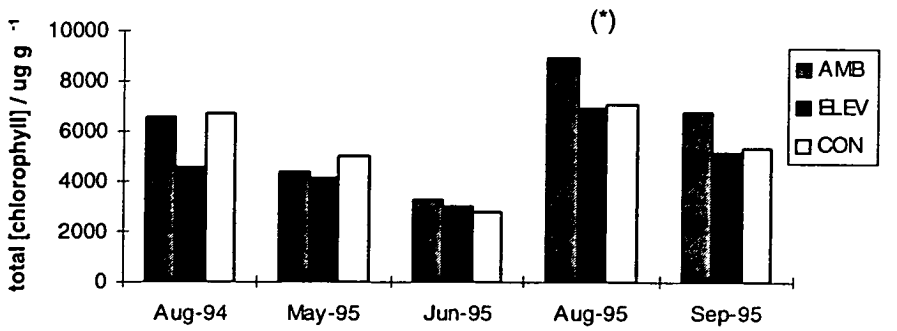


Figure 5.3 Seasonal course of mean total [chlorophyll] (ug g^{-1}) on a mass basis in *Scots pine*. Values are the means of eight replicates. AMB = ambient [CO_2] plus and minus nutrients; ELEV = elevated [CO_2] plus and minus nutrients; CON = control plot. [CO_2] had a trend towards a significant effect in August 1995 (*). ($p = 0.11$) only.

In Scots pine, [CO_2] had no significant effect on total chlorophyll on a dry mass basis at any time, although in August 1995 there was a trend towards an effect ($p = 0.11$). Total chlorophyll (on a dry mass basis) decreased by 35.3 % in August 1994, 8.24 5 in June 1995, and 22.0 % and 23.57 % in August and September 1995, respectively. Total chlorophyll on an area basis was only measured in August 1994 and no significant [CO_2] effect occurred ($p > 0.05$). There was only a nutrient effect on total chlorophyll (dry mass basis) in August 1995 ($p = 0.02$). There was no interaction effect of [CO_2] and nutrient addition on total chlorophyll (dry mass basis) at any time during 1994 or 1995 ($p > 0.05$).

There was a significant chamber effect in alder in June 1995 on total chlorophyll on a dry mass basis ($p = 0.05$). On an area basis in alder there was a significant chamber effect on total chlorophyll in September 1995 ($p = 0.04$) and a trend towards an effect

in August 1995 ($p = 0.07$). There was no chamber effect on total chlorophyll in Scots pine at any time ($p > 0.05$).

Chlorophyll a and Chlorophyll b

In common alder both chlorophyll *a* and chlorophyll *b* on a mass basis were consistently lower in elevated [CO_2] than in ambient [CO_2], although mostly not significantly (see Appendix 10, Table 5.3). Chlorophyll *a* was more affected by [CO_2] than chlorophyll *b*. Percentage decrease in chlorophyll *a* on a dry mass basis in elevated [CO_2] ranged from 13.8 % in August 1994, 6.56 % in May 1995 to 44.26 % in August 1995 (significant at $p < 0.05$). Percentage decrease in chlorophyll *b* on a dry mass basis in elevated [CO_2] was highest in September 1995 (27.97 %). In alder, chlorophyll *a* on both a dry mass basis and an area basis was significantly affected by [CO_2] in August 1995 ($p = 0.0076$ and 0.05, respectively) (Appendix 10, Tables 5.3 and 5.4). Chlorophyll *b* was not affected by [CO_2] ($p > 0.05$) (Appendix 10, Table 5.4). Thus there was a trend towards chlorophyll *a / b* being significantly affected by elevated [CO_2] ($p = 0.063$).

There was a significant nutrient effect on alder chlorophyll *a* and chlorophyll *b* on an area basis in September 1995 ($p = 0.03$ chl *a*, $p = 0.0033$ chl *b*) as well as on the ratio of *a / b* ($p = 0.03$) but not at any other time. An interaction effect of [CO_2] and nutrients was found in alder in May 1995 on chlorophyll *a* both on a dry mass and an area basis ($p = 0.027$ and 0.0032, respectively).

In Scots pine chlorophyll *a* and chlorophyll *b* (on a dry mass basis) were consistently lower in elevated [CO_2], except chlorophyll *b* in June 1995. There were few significant reductions in chlorophyll *a* or *b* in leaves of Scots pine (see Appendix 10, Table 5.5). The only nutrient effect on chlorophyll *a* or *b* or *a / b* in pine was a trend in September 1995 on *a / b* ($p = 0.06$). In the same month there was an interaction effect of [CO_2] and nutrients on *a / b* ($p = 0.004$) and a trend towards an effect on chlorophyll *b* (dry mass) ($p = 0.07$). In August 1994 there was also a trend towards an interaction effect on chlorophyll *b* (area basis) ($p = 0.09$).

The presence of chambers only affected chlorophyll *a* and *b* in alder in May 1995

(p = 0.03 chl *a*, area basis; p = 0.085 chl *a* mass basis), June 1995 (chlorophyll *a* dry mass basis p = 0.006). Thus, there doesn't seem to have been a clear chamber effect on chlorophyll *a* or *b* concentration in alder. In Scots pine there was very little chamber effect on chlorophyll *a* or chlorophyll *b*, notably in May 1995 (p = 0.03 chl *a* dry mass basis) and a trend towards a chamber effect in August 1995 (p = 0.09 chl *b* mass basis).

5.3.2 Soluble Sugars

In general total soluble sugars were not significantly affected by elevated [CO_2] although there were some significant effects on individual sugars (see section on *Partitioning of Soluble Sugars*). Changes in individual sugar concentrations were not sufficient to affect total soluble sugar concentration, however.

Total Soluble Sugars

In both common alder and Scots pine elevated [CO_2] had no significant effect on total soluble sugar concentration (dry mass) at any time from the middle of 1994 to the end of 1995 ($p > 0.05$) (see Appendix 11, Tables 5.6). There was no nutrient or interaction effect of [CO_2] and nutrients on total soluble sugar concentration in both species ($p > 0.05$).

Partitioning of Soluble Sugars

In common alder the main soluble sugars over the time period September 1994 to October 1995 were inositol, glucose, xylose and fructose, with the occasional appearance of galactose and sorbitol. The only months in which soluble sugars were significantly affected ($p < 0.05$) by elevated [CO_2] were September 1994 (xylose and sorbitol) and September 1995 (glucose and fructose, see Appendix 11, Table 5.7). These significant differences had disappeared by October 1995, however ($p > 0.05$).

There was only a significant nutrient effect in September 1994 on inositol (p = 0.045) and a trend towards a nutrient effect in September 1995 on glucose (p = 0.07). An interaction effect was found only in September 1995 on inositol (p = 0.058).

In Scots pine the main soluble sugars were pinitol, inositol, glucose and fructose, with occasional appearance of sorbitol and sucrose.

In pine elevated [CO_2] only had a significant effect in August 1995 on sorbitol, glucose and sucrose ($p < 0.05$) (see Table 5.7 in Appendix 11). There was no nutrient effect on soluble sugars at any time in 1994 or 1995 ($p > 0.05$). There were a number of interaction effects ($p < 0.05$) between nutrients and [CO_2] in September 1994 (inositol), in August (sucrose), in September 1995 (glucose), and in October 1995 (fructose).

There were several cases of a chamber effect on main soluble sugar concentration in both species. In alder there was a chamber effect on xylose and glucose in June, August and September 1995 ($p < 0.05$). In Scots pine, there was a chamber effect on pinitol and inositol in August 1995 ($p < 0.05$) and a trend towards a chamber effect on fructose in June 1995 ($p = 0.08$) and on sucrose in August 1995 ($p = 0.084$).

5.3.3 Starch

At the first Glendevon harvest in April 1994 there was no significant difference in starch between elevated and ambient [CO_2] treatments in either species ($p > 0.05$), although [starch] in pine needles was higher in elevated [CO_2] than ambient [CO_2] (mean of 20.85 mg g^{-1}, SE 2.28 versus a mean of 17.29 mg g^{-1}, SE 2.91, respectively). Some of the 1995 starch samples were unfortunately mouldy and thus not available for analysis. In the data which were available however (see Table 5.8) there were no significant environmental effects on [starch] ($p > 0.05$) in either species.

Table 5.8 Mean starch concentration (mg g^{-1} dry mass) in common alder and Scots pine in 1995. Values are the means (plus one standard error in brackets) of eight samples for the [CO_2] treatments and four samples for the [CO_2] and nutrient treatments.

Treatment	May	August
common alder		
Ambient	2.85 (0.35)	--
Elevated	3.59 (0.69)	--
A-N	3.09 (0.63)	--
A+N	2.61 (0.39)	--
E-N	2.50 (0.31)	--
E+N	4.67 (1.16)	--
Scots pine		
Ambient	12.08 (1.16)	4.25 (1.21)
Elevated	10.84 (0.65)	4.38 (1.41)
Control	--	6.41 (0.53)
A-N	13.63 (1.97)	4.64 (2.08)
A+N	10.53 (0.88)	3.67 (1.03)
E-N	9.90 (0.59)	5.74 (2.86)
E+N	11.77 (1.02)	3.03 (0.07)
C-N	--	6.68 (0.84)
C+N	--	6.23 (0.81)

In May and in August 1995 starch concentration in pine current year needles was not significantly affected by elevated [CO_2] ($p > 0.05$). In May 1995 the elevated [CO_2] needles had lower starch than the ambient (10.26 % lower), whereas in August 1995 the reverse was true (3.06 %, despite not being significant). Nutrient addition and the interaction between [CO_2] and nutrients also had no significant effect on starch concentration in the needles ($p > 0.05$). In young, fully expanded common alder

leaves in May 1995 there was also no significant effect of either [CO_2], nutrients or the interaction of the two ($p > 0.05$), although the elevated starch was 25.96 % higher than that in ambient [CO_2] (see Table 5.8).

In 1996 percentage increase in starch in common alder in the elevated [CO_2] treatment compared with the ambient [CO_2] treatment was 66.7 % in May, 30.8 % in June, 146.7 % in July, 60.4 % in August and 56.1 % in September. Starch in common alder was significantly affected by elevated [CO_2] in May and July ($p = 0.042$ and 0.0042, respectively) and there was a trend towards a [CO_2] effect in June 1996 ($p = 0.071$).The addition of nutrients had no significant effect on starch except in June when there was a tendency towards a nutrient effect ($p = 0.061$). There was no interaction effect between nutrients and [CO_2] on starch in alder at any time in 1996 ($p > 0.05$). The percentage increase in starch in elevated [CO_2] compared with ambient [CO_2] was smaller in Scots pine than in common alder, and starch levels even decreased in the elevated treatment in September 1996. Percentage increases in elevated [CO_2] were 2.5 % in May, 8.3 % in June, 97.6 % in July and - 22.2 % in September (see Table 5.9). In Scots pine elevated CO_2 had a significant effect on starch in July 1996 only ($p = 0.030$) but not in May, June or September ($p > 0.05$). Nutrient addition had a significant effect on starch in May ($p = 0.015$) but not at any other time in 1996 ($p > 0.05$). As in alder, there was no interaction effect of nutrients and [CO_2] on starch in Scots pine ($p > 0.05$).

In common alder there was a significant chamber effect on starch in all five months sampled ($p = 0.082$ in May, $p = 0.0053$ in June, $p = 0.026$ in July, $p = 0.018$ in August and $p = 0.0001$ in September), whereas in Scots pine there was a temporary chamber effect on starch in May and July ($p = 0.0001$ and 0.0033, respectively). alder, whereas in Scots pine the effect was only temporary.

Table 5.9 Mean starch concentration (mg g^{-1}) in common alder and Scots pine in 1996. Values are the means (plus one standard error in brackets) of eight samples for the [CO_2] treatments and four samples for the [CO_2] and nutrient treatments. Data provided by Gail Jackson and Diarmuid O'Neill. * = significant [CO_2] effect ($p < 0.05$) on starch; (*) = trend towards a significant [CO_2] effect on starch ($p < 0.10$).

Treatments	May	June	July	August	September
common alder					
Ambient	34.5 (8.7)	143.1 (21.3)	24.2 (4.9)	22.0 (3.9)	17.1 (4.7)
Elevated	57.5 (7.8) *	187.2 (25.4) (*)	59.7 (11.9) **	35.3 (6.2)	26.7 (5.9)
Control	15.6 (1.5)	64.8 (5.2)	49.0 (8.2)	44.8 (9.2)	53.3 (31.2)
A-N	37.0 (9.1)	166.4 (45.3)	17.7 (3.2)	20.5 (1.1)	16.6 (1.4)
A+N	32.0 (8.9)	119.8 (11.2)	30.7 (15.9)	23.4 (3.7)	17.6 (3.2)
E-N	68.1 (15.6)	205.2 (20.0)	67.5 (1.1.6)	34.4 (5.2)	23.3 (3.3)
E+N	46.8 (3.4)	169.1 (32.9)	51.9 (8.4)	36.2 (7.1)	30.1 (9.1)
C-N	18.2 (2.5)	79.6 (9.9)	44.6 (8.5)	49.2 (1.0)	44.6 (24.3)
C+N	12.9 (1.2)	49.9 (5.9)	53.3 (9.9)	40.3 (8.7)	61.9 (33.2)

Table 5.9 continued

Treatments	May	June	July	September
Scots pine				
Ambient	215.7 (10.1)	167.1 (27.8)	16.6 (4.2)	4.5 (0.22)
Elevated	221.0 (12.5)	180.9 (34.6)	32.8 (6.1) *	3.5 (0.56)
Control	96.3 (4.9)	--	41.9 (7.9)	5.7 (0.19)
A-N	203.9 (10.2)	180.7 (26.2)	14.8 (4.6)	2.1 (0.3)
A+N	227.4 (11.9)	153.5 (41.4)	18.3 (4.7)	6.9 (3.8)
E-N	203.6 (13.7)	184.0 (34.4)	39.5 (5.2)	2.4 (0.7)
E+N	238.4 (16.8)	177.7 (36.0)	26.0 (8.6)	4.6 (1.3)
C-N	81.1 (2.9)	--	36.1 (7.5)	6.2 (2.2)
C+N	111.5 (13.9)	--	47.7 (8.8)	5.1 (2.3)

5.3.4 Rubisco, Soluble Protein and Photosynthesis

Rubisco Concentration

Tables 5.10 and 5.11 in Appendix 12 show Rubisco concentration on a fresh mass basis in 1995. In common alder elevated [CO_2] had a significant effect on Rubisco in April 1995 and October 1995 (p = 0.012 and 0.051, respectively) (Table 5.10 Appendix 12). In April the elevated [CO_2] leaves had higher Rubisco concentrations than the ambient leaves but in October the effect was reversed, with elevated [CO_2] leaves having lower Rubisco than the ambient [CO_2] ones. Thus there was considerable seasonal fluctuation of Rubisco over the 1995 growing season. There were no nutrient or interaction effects on Rubisco in common alder ($p > 0.05$).

In Scots pine there was no significant [CO_2], nutrient or interaction effect on Rubisco at any time during 1995 ($p > 0.05$, see Table 5.11 in Appendix 12) when the data were log transformed (the variances were larger than the means). In the un-transformed data there was a nutrient effect on Rubisco expressed on a fresh and dry mass basis in September 1995 (p = 0.0076 and 0.045, respectively).

A chamber effect and a tendency towards a chamber effect on Rubisco was only found in Scots pine, and only in April and August 1995 (p = 0.0062 and 0.07, respectively).

It is difficult to draw any firm conclusion from these data, except that elevated [CO_2] and nutrient addition had no significant effect on [Rubisco] in Scots pine, and a temporary, varying effect on [Rubisco] in common alder.

Total Soluble Protein

The total soluble protein data were very variable, and very few statistically significant differences between treatments were found. It is not clear whether this reflected the true state of total protein concentration in the leaves and needles or if the data were too variable for a vigorous statistical test to be able to pick up any differences. Soluble protein concentrations in elevated [CO_2] fluctuated over the season in a similar way to Rubisco, with elevated [CO_2] occasionally being higher than ambient

[CO_2] means (e.g. alder in April) and vice versa. (e.g. alder in June). Elevated [CO_2] had no significant effect on total soluble protein concentration in common alder, although there was a slight trend towards an effect in April 1995 (p = 0.112) when protein at elevated [CO_2] was higher (see Table 5.12 in Appendix 12). There was an tendency to an interaction effect of [CO_2] and nutrients in September 1995 on a fresh mass and a dry mass basis (p = 0.08 and 0.09).

In Scots pine there was a significant [CO_2] effect on soluble protein expressed on a fresh mass and dry mass basis in April 1995 (p = 0.039 and 0.049, respectively). There was no other significant [CO_2] effect in 1995. In October 1995 there was a significant nutrient effect on protein (p = 0.042 fresh mass, p = 0.035 dry mass). No interaction effect of [CO_2] and nutrients on protein was found in Scots pine (p > 0.05).

There was only a tendency to a chamber effect on protein in Scots pine in August 1995 (p = 0.095); otherwise there was no chamber effect in either Scots pine or common alder.

Photosynthesis

Although photosynthesis was not measured directly in 1995, Dr Gail Jackson measured gas exchange at 350 µmol mol^{-1} versus 700 µmol mol^{-1} [CO_2] in current common alder and Scots pine leaves in 1996 and 1997 (see Figures 5.4, 5.5 and 5.6). Although *A / Ci* curves were not available, these data show a clear acclimation of photosynthesis in common alder grown with and without fertiliser in 1996 (see Figure 5.4). In Scots pine needles on the other hand, elevated [CO_2] needles measured at 350 µmol mol^{-1} [CO_2] showed an acclimation of photosynthesis in July 1996 but no clear acclimation in September 1996 or in 1997 (see Figures 5.5 and 5.6).

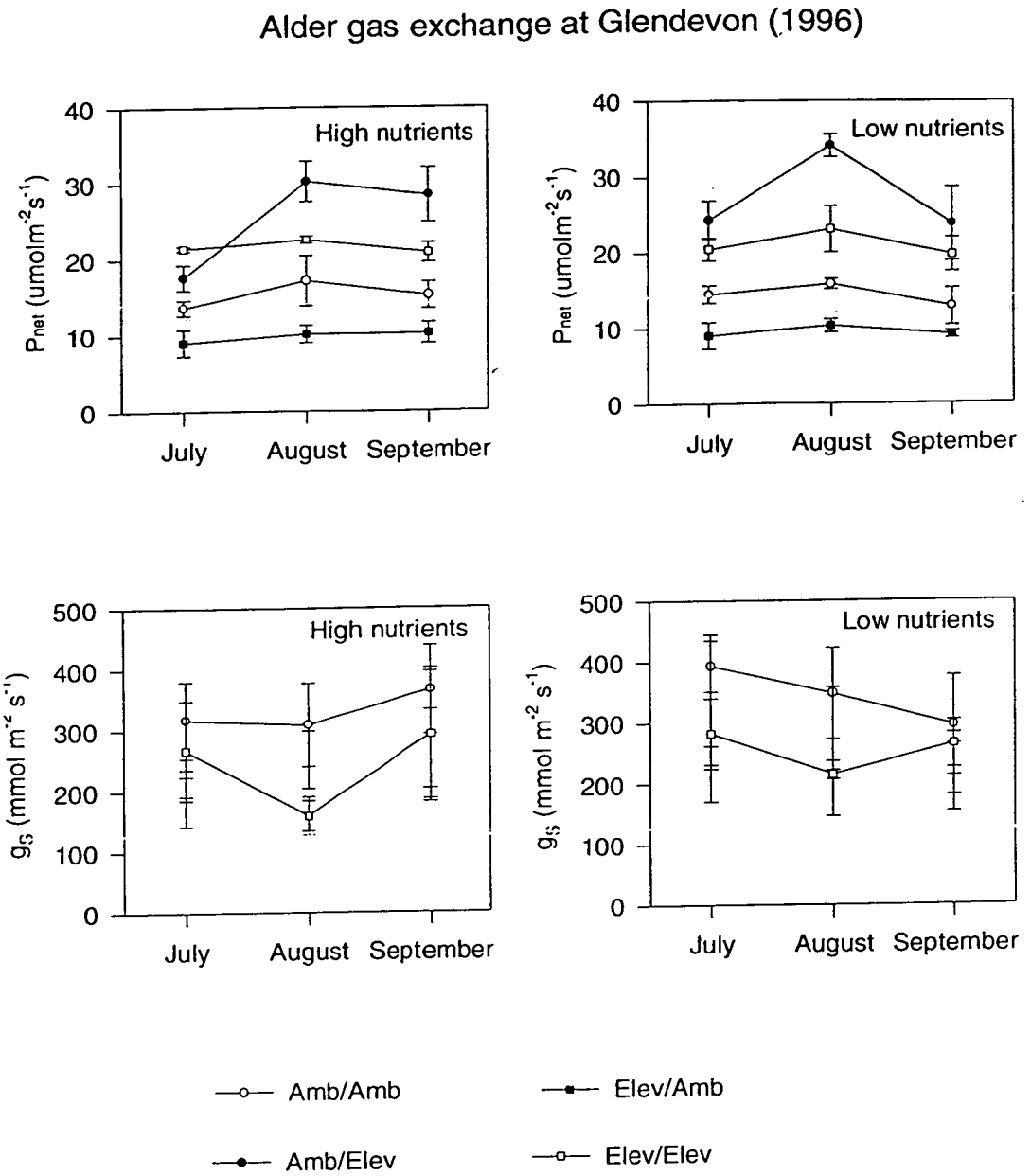


Figure 5.4 Net photosynthesis and stomatal conductance in common alder leaves grown in elevated or ambient [CO_2], with or without fertiliser at Glendevon in 1996 (c/o Gail Jackson). All leaves were measured at the [CO_2] in which they were growing and at the other [CO_2] in order to assess whether acclimation had occurred.

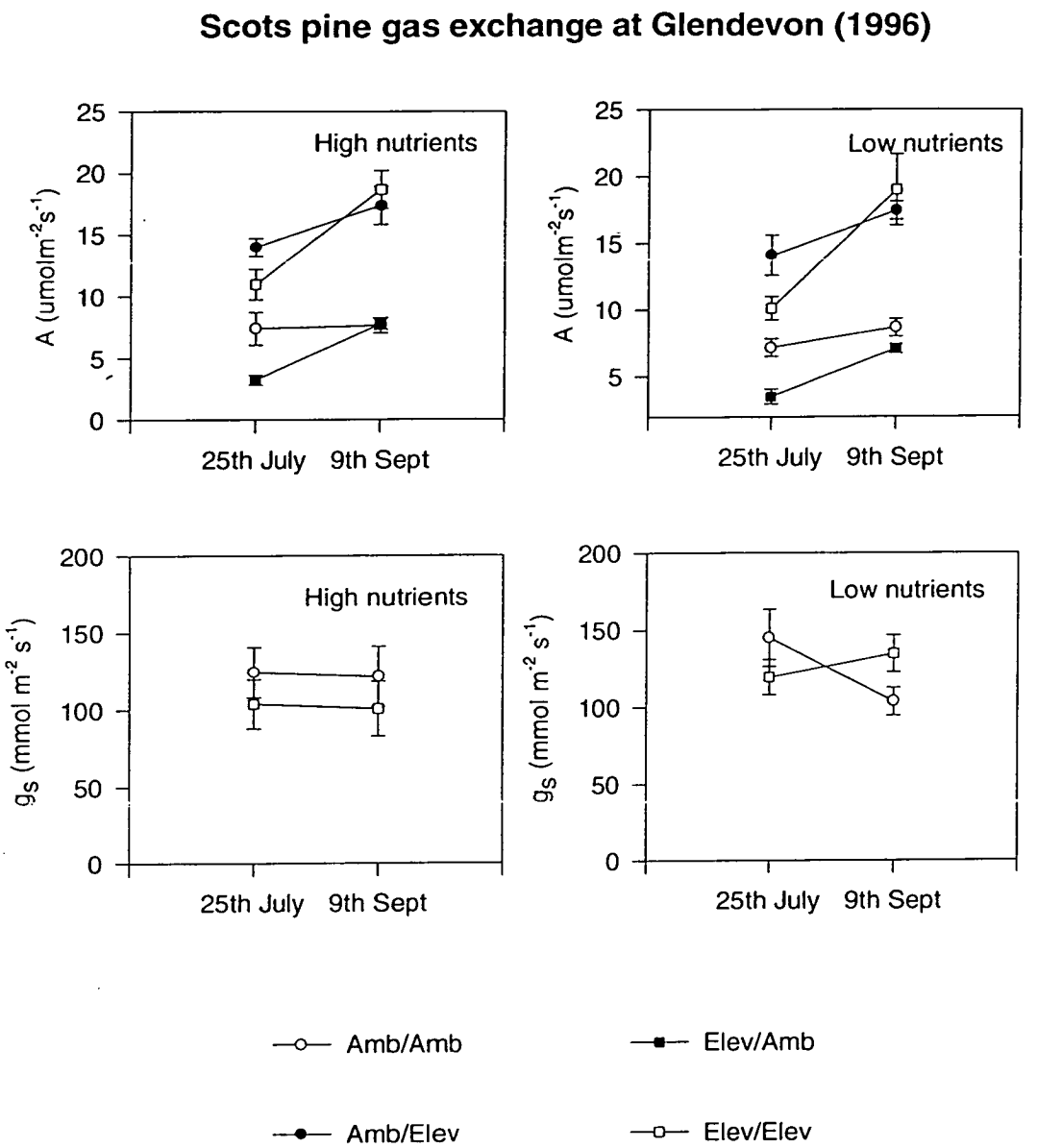


Figure 5.5 Net photosynthesis and stomatal conductance in new growth Scots pine needles grown in elevated or ambient [CO_2], with or without fertiliser at Glendevon in 1996 (c/o Gail Jackson).

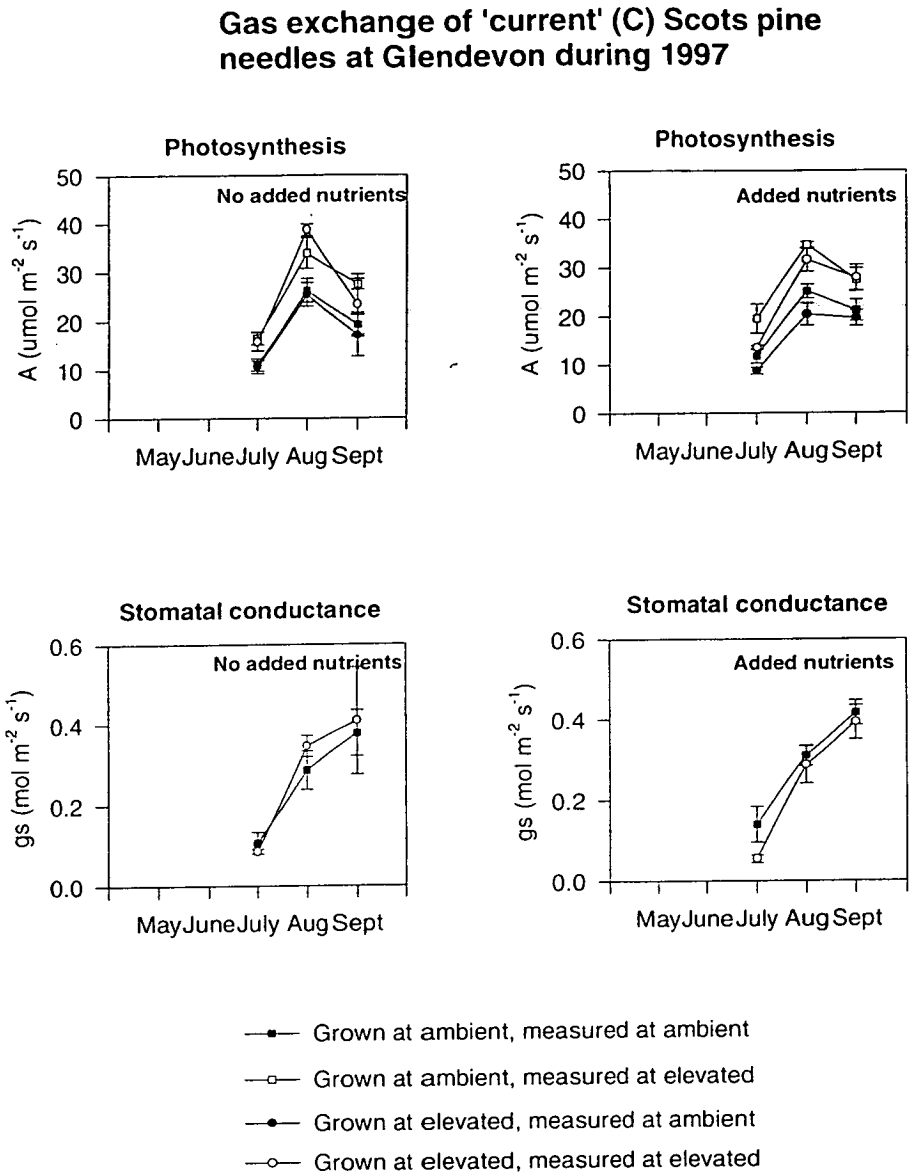


Figure 5.6 Net photosynthesis and stomatal conductance in new growth Scots pine needles grown in elevated or ambient [CO_2], with or without fertiliser at Glendevon in 1997 (c/o Gail Jackson).

5.4 Discussion and Conclusions

A summary of any changes in leaf composition in common alder and Scots pine grown in elevated [CO_2] compared with ambient [CO_2] is reported below in Table 5.14 and Table 5.15.

Table 5.14 Summary of main effects of growth in elevated [CO_2] on biochemistry of *common alder* in the two nutrient treatments, fertiliser supply, F and no supply, NF, combined and separately. The table shows significant statistical differences and how a variable in elevated [CO_2] compared with the ambient [CO_2] control. ++ large increase, + increase, (+) slight increase, ~ no change, (--) slight decrease, -- decrease, --- large decrease. * = statistically significant ($p < 0.05$) at least at some time during the experiment. (*) = trend towards significant difference between treatments ($p < 0.1$). It should be noted that any * against either the F or NF treatment is only indicative that there was a significant nutrient effect; it does not indicate between exactly which treatment combinations this effect was found.

Variable	F + NF together	F	NF
total [chlorophyll] (dry mass basis)	-- *	--	--
total [chlorophyll] (area basis)	-- *	-- *	-- *
[chlorophyll *a*] (dry mass basis)	-- *	--	--
[chlorophyll *b*] (dry mass basis)	(--) (*)	+	--
[chlorophyll *a*] (area basis)	-- *	--	--
[chlorophyll *b*] (area basis)	~	--	--
a/b	+ *	+	~
[total soluble sugars]	~	~	~
[sorbitol]	+ *	+	+
[xylose]	+ *	+	+
[glucose]	+ *	+	+
[fructose]	+ *	+	+
[starch] 1995	+	+	--
[starch] 1996	+ *	+	+
[Rubisco] (see Appendix 12)	+ / -- *	+ / --	+ / --
[soluble protein] (see Appendix 12)	~	~	~

Table 5.15 Summary of main effects of growth in elevated [CO_2] on biochemistry of *Scots pine* in the two nutrient treatments, fertiliser supply, F and no supply, NF, combined and separately. The table shows significant statistical differences and how a variable in elevated [CO_2] compared with the ambient [CO_2] control. ++ large increase, + increase, (+) slight increase, ~ no change, (--) slight decrease, -- decrease, --- large decrease, + /-- increase followed by decrease, ? not measured. Stars * indicate that the result was statistically significant ($p < 0.05$) at least at some time during the experiment. (*) = trend towards significant difference between treatments ($p < 0.1$).

Variable	F + NF together	F	NF
[total chlorophyll]	-- (*)	-- *	--
[chlorophyll *a*]	-- (*)	-- *	-- *
[chlorophyll *b*]	-- *	--	--
a/b	-- (*)	--	--
[total soluble sugars]	~	~	~
[glucose]	+ *	+	+
[fructose]	+ (*)	+	+
[sucrose]	+ *	+	+
[starch] 1995	-- / +	~	~
[starch] 1996	+ *	+	+
[Rubisco] (see Appendix 12)	~	~	~
[soluble protein]	+ *	+	+

5.4.1 Chlorophyll

A reduction in chlorophyll on a dry mass and area basis in leaves of plants exposed to elevated [CO_2] has been observed in many impact studies (Mousseau and Enoch 1989, Tissue *et al.* 1993, El Kohen and Mousseau 1994, Wilkins *et al.* 1994). Other investigators have found no significant [CO_2] effect on chlorophyll (Delgado *et al.* 1994). The results presented here for both common alder and Scots pine show a consistent reduction in chlorophyll (both total chlorophyll and chlorophyll *a* or *b*) over 1994 and 1995 and 1996, although mostly not a statistically significant reduction. Evans (1994) and Rey (1997) found similar results with birch grown in elevated [CO_2] (the former's trees grown in pots, the latter in the field) in which

chlorophyll was consistently reduced if not significantly or only occasionally significantly. Inadequate nitrogen supply is known to affect chlorophyll. It is interesting to note that nutrient addition did not in general significantly affect chlorophyll in either alder or pine. In August and September 1995 there was a significant nutrient effect on total chlorophyll in alder on an area basis only, and in August 1995 there was a significant nutrient effect on pine in which **E-N** and **E+N** trees had lower chlorophyll than **A-N** and **A+N** trees, respectively. This is different to the findings of El Kohen and Mousseau (1994) in which leaf chlorophyll was reduced in unfertilised sweet chestnut but not in fertilised trees. As with some other variables measured at Glendevon, there was no clear-cut response to nutrient addition.

Chlorophyll was only reduced by *c.* 9 - 14 % in alder and by *c.* 8 - 20 % in pine. Since chlorophyll is believed to be present in the leaf at concentrations higher than is required for maximum photosynthesis and as such is rarely limiting except in conditions of severe stress (discussed in Rey 1997), it is possible that the light-harvesting capacity in alder and pine was not inhibited or if there was a decline in light-harvesting capacity it was not the main controlling factor in any acclimation of photosynthesis.

5.4.2 Soluble Protein and Rubisco

Elevated [CO_2] affected soluble protein and Rubisco in different ways depending on the time of year. In common alder both soluble protein and Rubisco followed the same pattern of fluctuation over the 1995 growing season: increasing and declining or remaining the same together. The only exception to this was in October 1995 when soluble protein increased very slightly in elevated [CO_2] but Rubisco was significantly decreased by 19.96 %. In Scots pine soluble protein and Rubisco were decreased in elevated [CO_2] although not significantly ($p < 0.05$). Soluble protein was reduced by a larger proportion than chlorophyll. Since many soluble proteins play an important role in photosynthesis in the light reactions and in the PCR cycle, photorespiration, CO_2 assimilation, RuBP regeneration and sucrose and starch synthesis) a change in their concentration is likely to affect photosynthetic rates.

As mentioned in the Introduction, a very common response to elevated [CO_2] is a reduction in photosynthetic proteins, especially Rubisco and chlorophyll (Besford 1990, Stitt 1991, Van Oosten 1992, Mousseau and Saugier 1992, Sicher *et al.* 1994, Stitt and Schulze 1994, Wilkins *et al.* 1994, Besford 1998, Jacob *et al.* 1995). Sage *et al.* (1989) in their study of five C_3 species found acclimation to elevated [CO_2] in all five species although Rubisco content was lower in only two of the five. Rubisco activation state was reduced between 19 % and 48 % in all five species. There have been cases of increased Rubisco activity or content in trees grown in elevated [CO_2] although these are much less common than the generally found decline in the enzyme's activity and / or content (e.g. in Beaupre clones of poplar, Besford *et al.* in press). The Ecocraft survey of European trees (see Besford *et al.* 1998) also reported that *Quercus ilex* (Italy) grown in elevated [CO_2] did not show any reduction in activity, content or activation state of Rubisco.

There is considerable debate in the literature as to whether the long-term reduction in photosynthesis is mediated through a decline in activity and amount of Rubisco or accumulation of carbohydrates in the leaves, i.e. inhibition of sucrose synthesis (Bowes 1991). Stitt (1991) postulated that photosynthesis is inhibited in elevated [CO_2] because of inadequate demand for carbohydrate in the rest of the plant and that such an imbalance between sources and sinks is dependent on the species and may explain species-specific differences in responses to elevated [CO_2]. Stitt (1991) also hypothesised that direct inhibition of photosynthesis through accumulation of large starch grains or Pi limitation due to inhibition of sucrose synthesis was likely to only be a short-term response to elevated [CO_2]. The more likely long-term response would probably be indirect and hence adaptive (bringing all photosynthetic processes back into balance) and be mediated via a decline in key photosynthetic enzymes, including Rubisco. This has as an end effect that N is allocated away from the leaves and into the sinks thus readjusting the source-sink imbalance (Stitt 1991).

Tissue *et al.* (1993) found in their long-term study on loblolly pine, that proportionately less N was invested in Rubisco and N was reallocated elsewhere, to light reaction components not to any sinks outside of the leaf. In loblolly pine,

photosynthetic acclimation occurred in the third year of exposure to elevated [CO_2] with reduced Rubisco activity by 30 % which nearly balanced Rubisco ad RuBP regeneration regulation of photosynthesis (Lewis *et al.* 1996). There is mounting molecular evidence that carbohydrate accumulation can control photosynthesis. Other authors have hypothesised that changes in pH of the stroma as a result of PGA accumulation could cause the reduction in photosynthetic proteins (Sharkey 1985, cited in Rey 1997) although there is as yet little experimental evidence for this hypothesis.

The mechanism for the reduction in Rubisco activity or content commonly found in elevated [CO_2] has been studied with transformed tobacco plants and is attributed to a change in activation state of the enzyme (Sicher *et al.* 1994, Stitt and Schulze 1994). A larger proportion of the residual enzyme is converted into the active form *via* carbamylation by Rubisco activase (Stitt and Schulze 1994). Van Oosten *et al.* (1994b) reported that the abundance of transcripts coding for the SSU of Rubisco (rbcS), chlorophyll *a* / *b* binding proteins and for Rubisco activase were reduced in tomato plants exposed to elevated [CO_2]. In addition when sucrose or glucose was fed to leaf tissue grown in elevated [CO_2], the response of the transcripts was mimicked and, conversely, an up-regulation of enzymes involved in carbohydrate metabolism was found (Van Oosten 1994a). Camm (1993) found that Rubisco also functioned as a form of N storage in mature needles of Douglas fir, where total foliar protein and Rubisco concentration increased prior to and declined during shoot elongation. That Rubisco not only acts as a crucial enzyme in the PCR cycle but it may also act as a storage protein has been known since the seventies (Huffaker and Miller 1978). Rubisco exerts little or no control of photosynthesis at low light or high [CO_2] (Sage 1990, Bowes 1991). Thus, reductions in Rubisco content or activity may occur with no significant effect on photosynthesis. This would depend on the species and the environmental conditions in which the plant was growing, however, since Rubisco is highly responsive to environmental stresses (Huffaker and Miller 1978). Photosynthetic acclimation can also occur when no loss of Rubisco protein is found but the activity changes via reduced carbamylation (Socias et al. 1993).

It seems that in the present study Rubisco was not greatly affected by growth in elevated [CO_2], especially in Scots pine. It is likely that the proportional reduction in Rubisco was insufficient to be a main controlling factor of photosynthetic rate at high [CO_2]. Although not statistically significant, the reduction in Rubisco was more consistent in Scots pine than in alder, and starch accumulation occurred to a lesser extent in pine. Combined with inconclusive evidence for continuous photosynthetic acclimation to elevated [CO_2], it is possible that the pine trees were better able to restore the balance between Rubisco activity and regeneration of important components of the PCR cycle and / or sugar metabolic processes than the alder trees. The end result was that alder showed larger signs of photosynthetic acclimation. Starch did nevertheless accumulate in Scots pine needles and there were signs of photosynthetic acclimation.

The experiment of Sicher *et al.* (1994) in which acclimation to elevated [CO_2] occurred in tobacco plants irrespective of whether Rubisco levels were transgenically decreased or not, could shed some light on the situation at Glendevon. Conversely to many the findings of other authors in which the decline in Rubisco was attributed to a decrease in activation state, Sicher *et al.* (1994) found that reduced specific activity of Rubisco was responsible for acclimation. No measurement of Rubisco activity, activation state or specific activity or carbamylation was made in the present study but it is possible that acclimation occurred in both Scots pine and common alder irrespective of whether Rubisco amounts decreased or not, as found by Socias *et al.* (1993) in *Phaseolus vulgaris*. In their study acclimation was mediated via a reduction in carbamylation which in turn reduced Rubisco activity, with no loss of protein. It is possible, however, that in the present study there were no significant changes in either Rubisco, activity or activation state in the long-run. This leads us back to the idea that starch accumulation played an important role in the down-regulation of photosynthesis in alder (and pine) at Glendevon. It would have been very interesting also to have Rubisco data for 1996, to see what happened to Rubisco in the next growing season.

Another factor which could be playing a role in photosynthetic acclimation of trees in elevated [CO_2] is phosphate deficiency. Conroy *et al.* (1990c) found that phosphorus deficiency generally diminished the response to elevated [CO_2]. They found that when soil phosphorus (P) was low, higher P uptake occurred in elevated [CO_2] and hypothesised that this may have been related to changes in mycorrhizal competition (Conroy *et al.* 1990a). Higher foliar P concentrations were necessary for realisation of maximum growth potential in *Pinus radiata* and *Pinus caribaea*. Scots pine in the present experiment had more mycorrhizae in elevated [CO_2] than in ambient. Lewis *et al.* (1994) reported that mycorrhizal seedlings of loblolly pine supplied with low phosphorus in elevated [CO_2] experienced phosphorus limitation, and they related this to effects on Rubisco activity and RuBP regeneration rates. Extreme phosphate deficiency affected the specificity factor of Rubisco in favour of oxygenation (Jacob and Lawlor 1993). Phosphorus concentrations in Scots pine needles were optimum at Glendevon until about June 1995, after which concentrations were in an intermediate range (see Van den Burg 1985), whereas nitrogen was in the deficient to intermediate range in 1995. Although phosphate deficiency did not occur in either pine or alder in this experiment, it is possible that exposure to elevated [CO_2] induced changes in mycorrhizae and this in turn produced increased phosphorus uptake initially in Scots pine. How exactly this may have affected photosynthetic rates is not known.

5.4.3 Soluble Sugars and Starch

The most common response in trees and other plants to elevated [CO_2] is an accumulation of non-structural carbohydrate in the leaves (Stitt 1991, Mousseau and Saugier 1992, Ceulemans and Mousseau 1994). If growth at elevated [CO_2] induces more carbohydrate production, availability of the latter for the plant is increased (Luo *et al.* 1994). This additional sugar has three possible fates: it can be stored in leaves as starch and / or soluble sugars, it can be used for additional structural growth in the leaves, such as more layers of mesophyll cells, or it can be transported out of the leaf (Luo *et al.* 1994). If the carbohydrate accumulates as starch and / or sugars in the leaf and nitrogen dilution occurs, then photosynthetic capacity can be down-regulated via feedback inhibition (Stitt 1991). If the additional carbohydrate is used for mesophyll growth, this could compensate either completely or partially for nitrogen dilution. If

all the additional carbohydrate is exported out of the leaf then leaf composition and photosynthesis may hardly change (Luo *et al.* 1994).

As in many other studies, starch accumulated in the leaves of alder and pine when exposed in the long-term to elevated [CO_2]. Jacob *et al.* (1995) found increased non-structural carbohydrates (sugars and starch) in the sedge *Scirpus olneyi* which had been exposed to elevated [CO_2] for a period of eight years. Starch also accumulated in both fertilised and unfertilised sweet chestnut, although the accumulation lasted longer in the unfertilised trees (El Kohen and Mousseau 1994). Norby *et al.* (1996) found that [starch] increased significantly in two very different tree species, *Liriodendron tulipifera* and *Quercus alba*, grown in elevated CO_2. Amongst the findings of the Ecocraft group of researchers, all trees exposed to elevated [CO_2] showed starch accumulation in the leaves (Besford *et al.* 1998).

Demonstration that starch accumulation occurred in alder and pine leaves in 1995 was inconclusive although accumulation in the elevated [CO_2] treatment was higher than ambient [CO_2] in the two of the three sets of data available, although not significantly (May for alder and August for pine). Nevertheless in 1996 starch accumulated significantly in leaves of alder (in May, June and July) and pine (in July), with a larger accumulation occurring in alder. In both species the concentration dropped after the mid-season high and although starch in elevated [CO_2] was still higher than in ambient [CO_2] the difference was no longer significant. This drop in starch build-up could be the result of acclimation of photosynthesis to elevated [CO_2]. A similar accumulation and subsequent depletion of carbohydrate was found by Chomba *et al.* (1993) when *Picea engelmannii* was exposed to elevated [CO_2], indicating that elevated [CO_2] increased the rate of carbohydrate accumulation but not the final concentration before cold storage (i.e. dormancy). Gas exchange measurements on alder leaves made by Gail Jackson at Glendevon in July, August and September 1996 showed not only increased photosynthesis in elevated [CO_2], irrespective of fertiliser addition, but also clear down-regulation of photosynthesis (gas exchange in elevated [CO_2]-grown leaves measured in ambient was lower than ambient [CO_2]-grown leaves measured in ambient [CO_2]). Stomatal conductance was also reduced in elevated [CO_2] in July and

August. There seems to have been a time-lag between down-regulation of photosynthesis (which could have started to occur at any time before July 1996, and judging by the growth reponses of the alder, could have occurred around August 1995, see Chapter 4 Discussion and Chapter 6 Synthesis and Conclusions). In Scots pine there was a tendency towards down-regulation of photosynthetic capacity but the effect was not as clear-cut as in alder (Gail Jackson, *data not shown*).

Total soluble sugars were generally not affected by growth in elevated [CO_2] in both Scots pine and common alder. Generally sucrose was not found in the leaves of either alder or pine in 1995, which could have been indicating inhibition of sucrose synthesis in the leaves, which is associated with increased starch synthesis (Stitt and Quick 1989). Sucrose, a disaccharide, is the main transport form of carbohydrate within plant cells and as such is central to the distribution of photosynthate. Starch is synthesised in two distinct situations: firstly for short-term storage when the capacity of chloroplast to synthesise carbon exceeds the capacity to export it to the cytoplasm and secondly starch is synthesised in storage tissues such as seeds or tubers for long-term storage (Smith 1993).

Stitt and Quick (1989) reported from studies with mutants that the rate of starch synthesis can be increased in response to lowered rates of sucrose synthesis, but not vice-versa. Sucrose synthesis is inhibited by low Pi whereas starch synthesis is stimulated by low Pi. Thus if Pi levels in the cytoplasm fall below an optimum level, sucrose synthesis is inhibited and starch synthesis is set in motion. This has implications in leaves exposed to elevated [CO_2] with the common response of enhanced photosynthetic rate, because temporarily up-regulated photosynthetic rate could lead to Pi regeneration limitation which in turn would induce starch synthesis but not sucrose synthesis. Starch granules would accumulate in the chloroplast but this extra carbon would not be exported out of the leaf, in the form of sucrose. Lenz *et al.* (1995) also found no increase in sucrose concentration but starch accumulation in beech leaves grown at elevated [CO_2] for three years. They hypothesised that this could have been caused by unchanged sucrose transport from the leaf and concomitant starch accumulation, indicating that the starch pool could not be

removed to other parts of the plant fast enough to prevent accumulation. It is probable that this is what happened in alder and pine in this experiment, although the lack of sucrose in the leaves during several months's sampling is more indicative of sucrose synthesis inhibition. Often, only some individual trees had sucrose in their leaves at any one sampling time. Temporarily increased levels of glucose and fructose could be an indication of sucrose synthesis, however. SLA in common alder (data not available for pine) was not affected by elevated $[CO_2]$ in 1995, indicating that increased leaf thickness caused by starch accumulation or production of new mesophyll layers did not seem to have occurred.

Direct inhibition of photosynthesis through accumulation of large starch amounts could have also occurred in this experiment, particularly in alder. As a pioneer species and a nitrogen-fixing tree, alder is often considered to be source-limited (Bowes 1991). Growth of such trees in elevated $[CO_2]$ should be less sink-limited than in species such as Scots pine. It is strange, therefore, that starch accumulated more in alder leaves than in pine needles in this experiment.

It can be concluded that neither Scots pine nor alder were able to utilise all the extra carbon fixed in elevated $[CO_2]$, since sucrose synthesis and concomitant transport of carbon out of the leaf did not seem to have been taking place. Sucrose was found more often in pine needles than in alder leaves, starch accumulated more in alder and down-regulation of photosynthesis was more pronounced in alder. Canopy closure in common alder seems to have played a key role in photosynthetic acclimation. It is possible that Scots pine was able to export more sugar as sucrose out of the leaves and thus not show such marked down-regulation of photosynthesis as common alder.

The results from this study once again show that starch accumulation and photosynthetic acclimation are not restricted to pot studies, but occur with trees grown over the longer-term in field conditions. These finding agree with those of Ana Rey's with field grown birch (1997), although the extent of starch accumulation was less than in her study. Of particular interest is the finding that the nitrogen-fixing, fast-growing alder showed clearer signs of acclimation than the slower-growing,

mycorrhizal pine, despite the findings of other authors that fast-growing pioneer species and / or nitrogen-fixers are more stimulated by growth in elevated [CO_2] than other species (Bowes 1991, Poorter 1993).

Conclusion

Growth in elevated [CO_2] over four years did change leaf composition in both pine and alder and produced photosynthetic acclimation in common alder. The reponses in this study are similar to those in other experiments although the magnitude of the response was less. Probably as a result of growth in field conditions. The mechanism by which photosynthetic acclimation occurred in this experiment is not clear, although it seems likely that a complex interaction of effects was at play. Starch accumulation played an important role as well as reductions in Rubisco, soluble protein and chlorophyll. Yelle et al (1989) found that acclimation in two different tomato species could not be explained entirely by accumulation of starch and / or sugar. This may have also been the case at Glendevon. Particular conclusions are as follows:

1) declines in Rubisco, soluble protein and chlorophyll were found in both species, although mostly the declines were not statistically significant at $p < 0.05$;

2) changes in leaf composition were more pronounced in common alder, where more of a source-sink imbalance occurred than in pine: this surprising result is at variance with the hypothesis (see Chapter 4 Introduction) that alder, with its ability to fix nitrogen, would be able to keep additional carbon and nitrogen availability in balance;

3) nutrient addition did not generally affect leaf composition in either Scots pine or common alder in elevated [CO_2]; and

4) starch accumulation occurred in both common alder and Scots pine grown in elevated [CO_2], although the effect was more pronounced in common alder leaves.

CHAPTER 6

Synthesis and Conclusions

This chapter is a synthesis of the results from the short-term and long-term experiments. The observed differences in response to elevated [CO_2] and nutrient addition of common alder and Scots pine are interpreted with respect to different time scales and experimental conditions as well as the different functional groups of the species. The main conclusions from the experiments are presented and suggestions for future research given.

6.1 Schematic Representation of Results

Figures 6.1 and 6.2 provide an overview of the main changes in common alder and Scots pine in the long-term experiment.

6.2 Photosynthesis and Growth

As discussed in Chapter 5 photosynthetic rate in C_3 plants is almost always stimulated in response to elevated [CO_2] and this is related to the increased carboxylation efficiency of Rubisco. Photosynthesis as measured monthly in common alder and Scots pine at Glendevon by Gail Jackson in 1996 and 1997 was indeed significantly enhanced in elevated [CO_2] (see Figures 5.4, 5.5 and 5.6). Although not measured directly, photosynthetic enhancement in both the short-term experiment and the long-term experiment in 1994 and 1995 must have occurred, since biomass accumulation was stimulated but leaf area was not affected by elevated [CO_2]. I define photosynthetic 'acclimation' as a reduction in photosynthetic components and 'down-regulation' as a reduction in photosynthetic rate without a change in photosynthetic components (see Delgado *et al.* 1994).

Figure 6.1 Schematic representation of the proposed mechanism of photosynthetic acclimation at elevated [CO_2] in common alder in the long-term experiment.

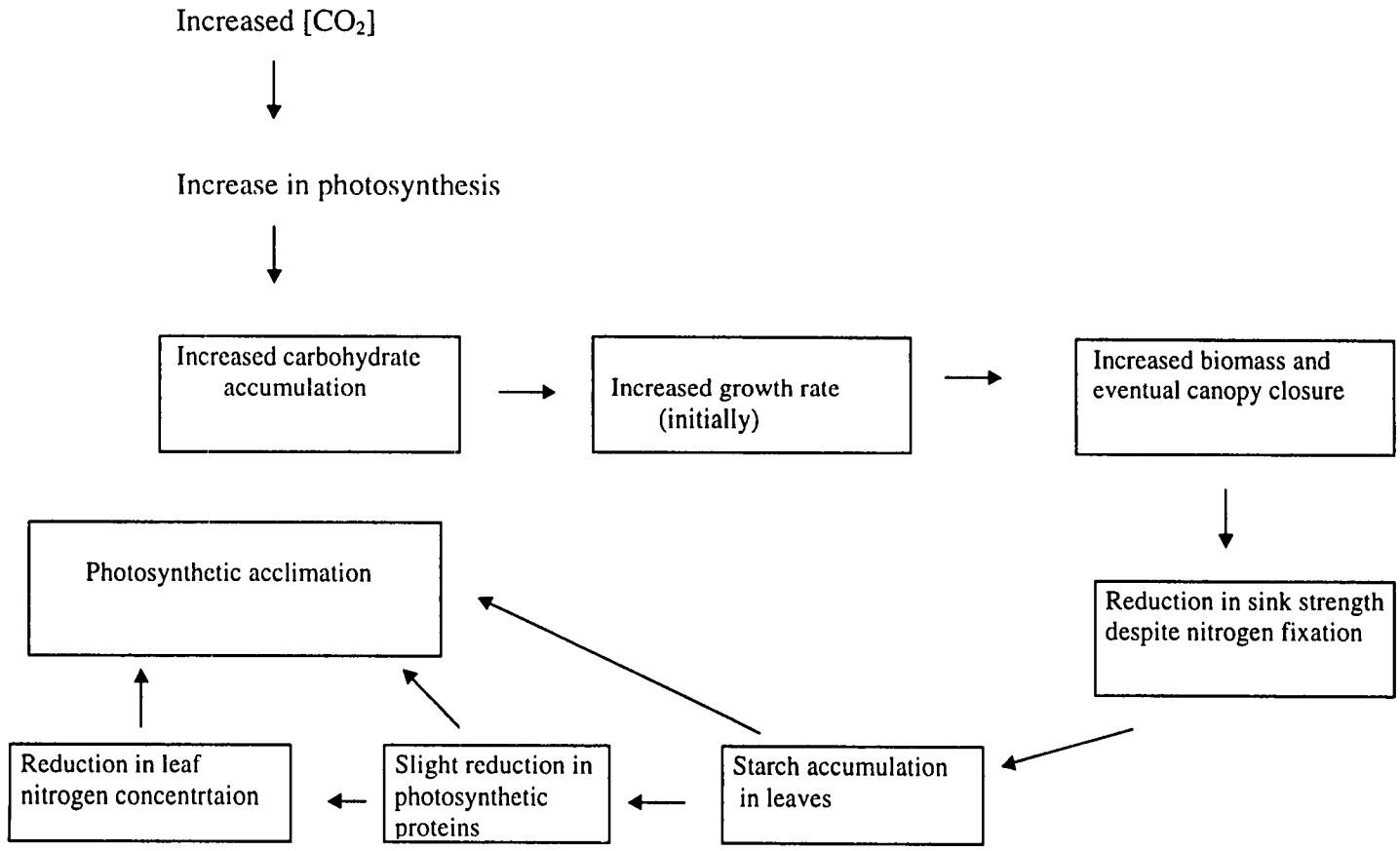
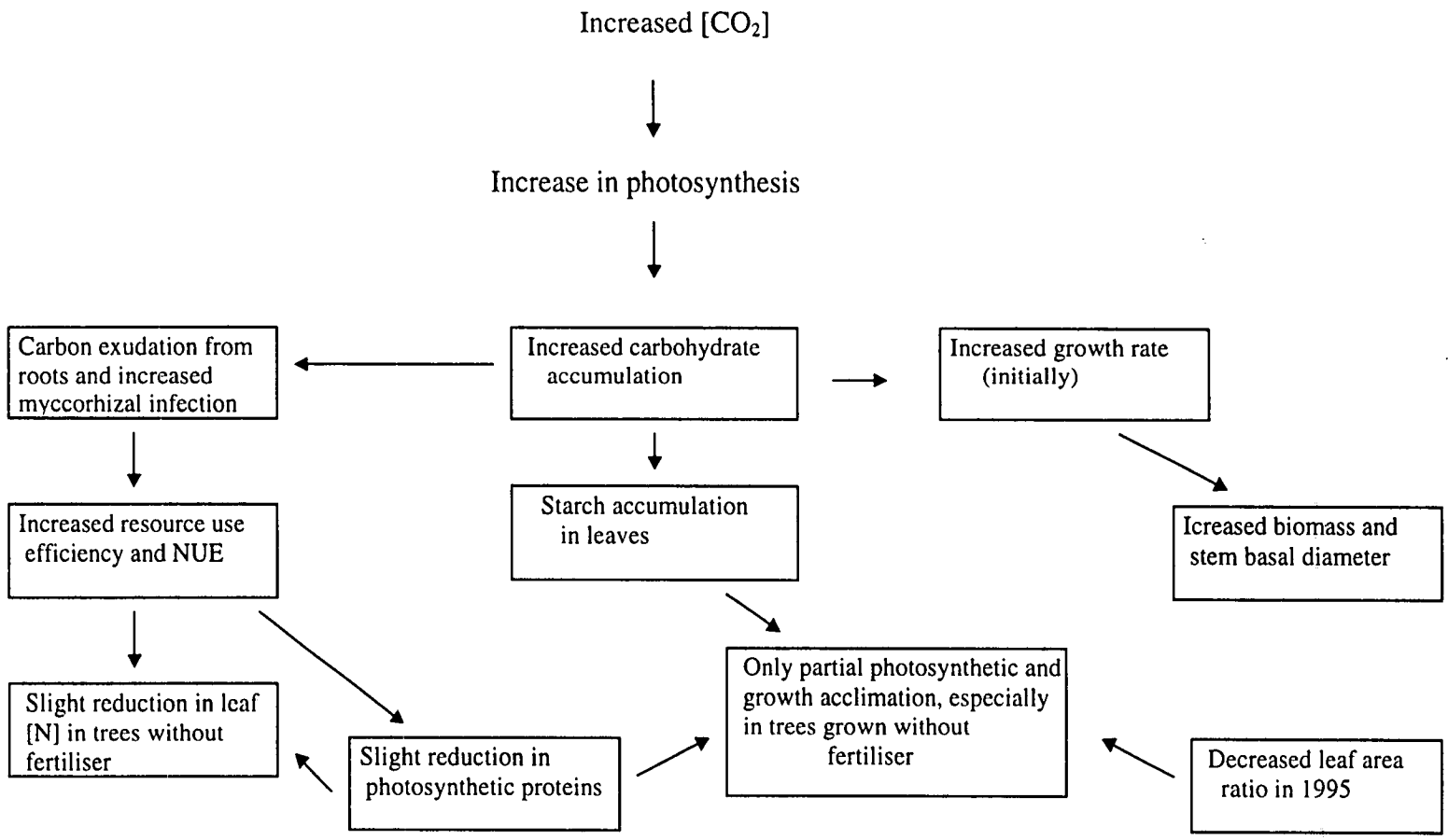


Figure 6.2 Schematic representation of the main responses of Scots pine in the long-term experiment to elevated [CO_2].



Photosynthetic acclimation is generally measured by comparing photosynthetic rates measured in ambient [CO_2] of elevated [CO_2] grown plants with those of ambient [CO_2] grown plants (Bowes 1991). This measure indicates that the photosynthetic apparatus in elevated [CO_2] plants has been reduced and can therefore not keep up the same photosynthetic rates as ambient-grown trees when measured in ambient [CO_2]. Nevertheless Pearcy and Bjoerkman (1983, cited in Bowes 1991) maintain that the most relevant comparison is between plants grown and measured at ambient [CO_2] and those grown and measured at elevated [CO_2]. Using the first measure, photosynthetic acclimation occurred in both common alder and Scots pine at Glendevon. Photosynthetic acclimation was more consistent and more pronounced in common alder than in Scots pine, especially in 1997. This acclimation was coincident with a larger starch accumulation in the leaves of alder than in those of pine in 1996. For further discussion of this result and its possible mechanisms see sections 6.4 to 6.6.

Despite evident photosynthetic enhancement, specific growth rate in both the short-term and long-term experiment and in both tree species was not found to be increased in elevated [CO_2] during the experimental period 1995-1996. Caution should be exercised when interpreting these results since it was not possible to measure the specific growth rate of the same individual trees over time. The graphical representation of natural log of biomass against time using mean biomass data (see Figure 4.8a and 4.8b in Chapter 4) indicates a potential increase in specific growth rate between April 1994 and September 1994 especially in Scots pine, however. Since total seedling biomass was not significantly different at the initial harvest in April 1994, I propose that there was a stimulation of growth rate in the long-term experiment in the 1994 growing season (difficult to measure for lack of harvests). In the short term experiment it seems that any stimulation in growth rate occurred during the first year of growth prior to the start of the experiment. This is in agreement with many impact studies in which photosynthesis and growth rate were found to be stimulated only during the first weeks to months of exposure to elevated [CO_2] (see Mousseau and Saugier 1992, Poorter 1993, Ceulemans and Mousseau 1994).

As in many other impact studies, biomass accumulation increased in both tree species in elevated [CO_2] (see Norby 1987, Arnone and Gordon 1990, Poorter 1993, Norby *et al.* 1996, Tissue *et al.* 1996). The relative accumulation of biomass was largest in common alder compared with Scots pine and in the long-term experiment compared with the short-term experiment. I attribute the difference between the experiments to root restriction in the pot-experiment as well as to specific growth rate, i.e. the larger the plant the more assimilatory material it has which in turn maintains its larger size. Biomass accumulation in common alder probably responded more to elevated [CO_2] than in Scots pine because of functional differences between the species. Common alder is a fast-growing, nitrogen-fixing pioneer species with an indeterminate growth pattern and thus was able to derive more of an advantage from elevated [CO_2] than Scots pine, a slow-growing species with a determinate growth pattern. Although second flushes of needle growth (lammas growth) were observed more frequently in individual elevated [CO_2] trees of Scots pine than in ambient [CO_2], the extra carbon fixed by such needles would have been small relative to the carbon fixed by the rest of the tree and second flushes of growth did not occur on all trees.

Interesting differences in the nature of biomass accumulation occurred between the two experiments. Stem height increased in elevated [CO_2] alder in the short-term but not in the long-term experiment, whereas the opposite pattern was found with respect to stem basal diameter. In Scots pine stem basal diameter was significantly increased in both experiments but stem height was only enhanced in the long-term experiment. These results show the plasticity of potential growth responses to elevated [CO_2]. The same species can show different biomass accumulation patterns depending on the length and nature of the experiment.

6.3 Allocation

Biomass allocation in Scots pine and common alder was affected differently by elevated [CO_2]. In the short-term experiment biomass allocation was generally not much affected by elevated [CO_2]. In contrast there were significant changes in biomass allocation in Scots pine such as increased root to shoot ratio in elevated [CO_2]. In Scots pine the needle mass fraction increased in response to fertiliser supply

but not to growth in elevated [CO_2]. As with biomass growth discussed in section 6.2, the pattern of species response was reversed in the long-term experiment with common alder experiencing more changes in allocation than Scots pine. I attribute the increased root to shoot ratio in Scots pine in the short-term experiment to a reallocation of resources to roots in order to increase nutrient acquisition. This was not necessary in the long-term experiment where the roots were not restricted by pots and increased nitrogen use efficiency enabled non-fertilised trees to be stimulated in growth as well as fertilised trees. The case of common alder is more complicated. I suggest that higher rates of carbon assimilation and biomass growth and increasing competition between plants (see section 6.4) in the long-term experiment led to more changes in resource allocation than in the short-term experiment. In the long-term experiment photosynthetic acclimation in alder occurred from about July-August 1995 onwards. This was coincident with shifts in biomass allocation away from the leaves and into the stem. Norby *et al.* (1992) also found no significant growth response in yellow-poplar grown in elevated [CO_2] and changed allocation of carbon, notably a decline in leaf production and an increase in fine root biomass. Although these changes reduced the stimulatory effect of growth in elevated [CO_2] they also represent a more efficient use of resources over the longer term.

It has been suggested that woody plant species will be able to respond to elevated [CO_2] more than herbaceous species, since woody plants can allocate excess carbon to the development of wood thus reducing photosynthetic acclimation (see Barton 1997). In the long-term experiment, leaf to shoot ratio in common alder was significantly reduced in June 1995 but not in August 1995. The proportion of wood to 'functional' tissue was thus temporarily increased in alder but not in pine. This change in allocation in August may have been related to the onset of canopy closure, since it has been suggested that allocation between foliage and stem-wood varies with canopy closure (see Lucas 1998). A potential increase in wood in mature trees may increase carbon sequestration in the medium to long-term. Nevertheless it is extremely difficult to predict the response of mature trees to climate change from seedlings. In addition common alder is not a dominant forest tree species but a typical high production,

short rotation 'energy forest' species, so that the length of any extra carbon sequestration is likely to be restricted compared to a dominant forest species.

6.4 Are photosynthetic and growth stimulation maintained in the long-term?

Photosynthetic acclimation occurred in both the short-term and the long-term experiment. In the former Scots pine showed more of a source-sink imbalance than common alder, as hypothesised. In the long-term experiment photosynthetic acclimation (in 1996 and 1997) occurred to a larger extent and more consistently in common alder than in Scots pine. Photosynthesis measured in September 1996 even showed no acclimation of photosynthesis in those trees grown with fertiliser in elevated [CO_2], although this pattern was not consistent throughout 1996 or 1997. This is a surprising result which does not support the hypothesis that common alder would be less sink-limited than Scots pine in elevated [CO_2].

Although photosynthetic acclimation to elevated [CO_2] clearly reduces photosynthetic capacity, the reduction in photosynthetic rate rarely completely compensates for the stimulation of the rate by elevated [CO_2] (Drake *et al.* 1997). This was the case at Glendevon where photosynthetic acclimation occurred but the rate of photosynthesis in the trees grown and measured in elevated [CO_2] still remained higher than in trees grown and measured in ambient [CO_2]. Although photosynthetic rate and net assimilation rate in alder were stimulated in 1995 these effects were offset by various other factors such as decreased leaf mass ratio throughout the 1995 growing season and reduced leaf area ratio in spring 1995. In Scots pine photosynthetic rates increased but net assimilation rate and leaf mass ratio were not affected by elevated [CO_2]. Leaf area ratio decreased significantly in April and August 1995, however, which again could have offset any photosynthetic stimulation. DeLucia *et al.* (1994) also found that decreased leaf area ratio in *Pinus ponderosa* counteracted the increase in photosynthetic and net assimilation rate.

I suggest that the tree-density within the chambers (there were initially twelve seedlings per quadrant) affected responses of the two species to a large extent. Scots pine, being a slow growing species did not grow to canopy closure over the three

years of the long-term experiment and thus there was little competition for light between individuals. Furthermore, the distance between the seedlings suggests little interaction between plants. Common alder seedlings, in contrast, grew much faster than the pine and formed a closed canopy by the summer months of 1995, when roots of one seedling were growing in the vicinity of roots of the neighbour. In addition the summer of 1995 was hotter and drier than that in 1994 (see Chapter 2 Materials and Methods section 2.3.1). The dry 1995 growing season probably caused the fungal infection (*Taphrina tosquinetti*) in June on the common alder leaves. Leaves wilted in August and were lost. I propose that these factors contributed significantly to the appearance of signs of photosynthetic and growth acclimation in elevated [CO_2] around July-August 1995. Although photosynthesis was not measured directly in 1995, signs of acclimation in alder in August 1995 were: increased root to shoot ratio, previously significant differences in root mass were no longer significant, previously reduced leaf to stem ratio readjusted, and leaf nitrogen concentrations went from being intermediate-optimum before August 1995 to being deficient (according to the criteria of Van den Burg 1985). There were few signs of acclimation of photosynthesis in Scots pine in summer 1995 although nitrogen concentrations previously intermediate also changed to being deficient in August 1995 and phosphorus concentrations dropped from optimum to intermediate in the same month.

Enhanced photosynthesis in elevated [CO_2] does not always translate into increased biomass (Körner 1995), although this was the case in both the short-term and the long-term experiment in this study. The largest increases in biomass occurred in 1995 in common alder, prior to canopy closure in July to August. By 1996 there was no significant difference in total biomass in alder in elevated [CO_2]. In Scots pine, in contrast, the largest increase in biomass was found in 1996. I attribute this continuing stimulation of biomass increment in Scots pine to a slower growth rate of pine and therefore lack of competition between trees, as well as the lack of consistent photosynthetic acclimation. It may be that Scots pine would have experienced appreciable photosynthetic acclimation in 1998 or later, or that the trees may have been physiologically able to continue accruing biomass in the longer term. This has implications for increased carbon sequestration in boreal forests where Scots pine is a

dominant tree species. There are no experiments of a decade or more with which to compare and predict the longer-term response of Scots pine to elevated [CO_2]. Haettenschwiler *et al.* (1997) found a 12 % larger final radial stem width of *Quercus ilex* growing for 30 years in the vicinity of natural CO_2 springs. Analysis of tree ring width showed that this increase in stem width occurred largely when the trees were young.

6.5 Did Nutrition Keep Up With Increased [CO_2]?

In the short-term experiment the nitrogen-fixing common alder seedlings did not show reduced leaf [N] whereas Scots pine did. The response of the latter is in agreement with many short-term experiments in which growth at elevated [CO_2] reduces leaf [N] (Mousseau and Saugier 1992, Ceulemans and Mousseau 1994) and I attribute the lack of change in [N] in alder to the ability to fix nitrogen. It is a commonly held view that plants grown in elevated [CO_2] with limited nutrient availability will experience a larger acclimation of photosynthetic rate than those provided with a high nutrient supply (Bazzaz 1990, Bowes 1993, El Kohen and Mousseau 1994). A more recent viewpoint is that plants grown in elevated [CO_2] have increased efficiency of nitrogen use (Drake *et al.* 1997). Could it be that the effect of increasing [CO_2] is not the removal of a limitation but increased efficiency of use? Lloyd and Farquhar (1996) analysed the available evidence and found that the relative stimulation of plant growth with low nitrogen supply was not any smaller than stimulation of plant growth with high nitrogen supply.

As hypothesised, common alder grown without fertiliser was able to fix nitrogen from the atmosphere and thus was not nitrogen-limited in elevated [CO_2]. Contrary to expectations, however, those trees grown without fertiliser did not show more growth stimulation in response to [CO_2] than those with fertiliser. It is not clear what the exact mechanisms of this nutrient response were. I suggest that nitrogen-fixing plants have an upper ceiling to nitrogen fixation, probably regulated via phosphorus limitation (see the Chapter 4 Discussion) and that this upper ceiling explains the lack of nutrient treatment effect in this study. Trees provided with fertiliser were not

nutrient limited and those without fertiliser fixed nitrogen from the atmosphere and were as enhanced in growth by elevated [CO_2] as the former.

Scots pine grown without fertiliser addition did not suffer photosynthetic acclimation as much as expected. Although plants provided with fertiliser in elevated [CO_2] temporarily had taller stems, larger stem basal area and total biomass than plants grown without fertiliser, this effect was not consistent and clear-cut. In addition changes in proteins and carbohydrates at the leaf scale were generally not affected by nutrient addition. Thus elevated [CO_2] probably facilitated the acquisition of additional nutrients from the soil even without fertilisation. Norby *et al.* (1986) found increased root exudation, mycorrhizal density and allocation of photosynthate to fine roots in *Pinus echinata* grown in elevated [CO_2]. This is in agreement with Gifford's viewpoint that the nitrogen cycle in the long-term follows the carbon cycle, rather than the reverse on a seasonal scale (1994). Increased root exudation of carbon compounds may lead to faster rates of nitrogen mineralisation. I suggest, therefore, that a combination of and increased carbon exudation from the roots in elevated [CO_2], higher mycorrhizal density and improved nutrient acquisition may have enabled Scots pine to respond positively despite low nutrient supply.

It is now generally accepted that photosynthetic acclimation in elevated [CO_2] can be a response to nutrient limitation (Mousseau and Saugier 1992). There is a real risk that differences in growth have been wrongly attributed to elevated [CO_2] alone, instead of to the interaction between elevated [CO_2] and nutrient limitation (Linder and McDonald 1993). In the long-term experiment reduced leaf nutrient concentrations in both species coincided with starch accumulation in 1996. However, initial signs of photosynthetic and growth acclimation in alder occurred in the summer of 1995 suggesting that the initial acclimation was related primarily to the effects elevated [CO_2]. Johnson *et al.* (1996) studied the effects of [CO_2] and nitrogen on growth and nitrogen dynamics in *Ponderosa pine* over two growing seasons and found increased NUE in the first 6 months but that this disappeared by 18 months.

There are few impact studies in which the effects of phosphorus (P) interaction with elevated [CO_2] have been investigated. Conroy *et al.* (1990a) found increased P uptake in *Pinus radiata* and *Pinus caribea* when soil P was low and attributed this to changes in mycorrhizal competition. Higher P concentrations were required in elevated [CO_2] to satisfy the increased potential for growth. Interestingly in the short-term experiment [P] in the needles of Scots pine was significantly reduced by the end of the experiment, whereas in the long-term experiment leaf [P] was not affected by elevated [CO_2] in either species.

6.6 Biochemical Response

In the short-term experiment there were classical signs of photosynthetic acclimation in Scots pine such as starch accumulation and reduced leaf nutrient concentrations, but not in common alder. Common alder in the long-term experiment experienced a time lag between signs of growth acclimation coincident with canopy closure and root restriction and biochemical changes at the leaf scale. In the early 1995 growing season there was no starch accumulation and changes in leaf proteins were variable but rarely significantly affected by elevated [CO_2]. Although by August 1995 chlorophyll concentrations in both species were significantly reduced in elevated [CO_2] other proteins showed only slight, non-significant reductions in response to elevated [CO_2]. A number of authors have reported changes in the kinetics of Rubisco, especially increases in activation state, in response to elevated [CO_2] even though concentrations of the enzyme did not change (Bowes 1991). It is possible that the kinetics of Rubisco were changed in the trees in this study despite the lack of consistent reduction in content of the enzyme, but it is beyond the scope of this work to ascertain whether this was the case or not.

It was only in 1996 that starch accumulated significantly in alder and pine leaves, and to a larger extent in the former. I propose therefore that although important photosynthetic proteins such as Rubisco were slightly reduced in elevated [CO_2] in 1995, photosynthetic acclimation in common alder in August 1995 was mainly caused by canopy closure, root restriction and reduced leaf area ratio as well as by reduced leaf chlorophyll concentration. Accumulation of starch in 1996 seems to have been a

consequence of the changes in 1995, not the direct cause of acclimation. Stitt and Quick (1989) found that activation of sucrose phosphate synthase (SPS), important in the synthesis of sucrose from glucose and fructose, was altered by wilting of leaves. It may be that the wilted leaves in common alder in August 1995 experienced changes in sucrose synthesis, perhaps inducing more starch synthesis. Such changes may have contributed to photosynthetic acclimation at this time.

Although photosynthetic acclimation in Scots pine was measured in certain months of 1996 and starch accumulation occurred, this accumulation was not as large as in common alder and leaf nitrogen concentrations remained similar in elevated [CO_2]. It can be concluded that neither species, but especially common alder, was able to utilise all the extra carbon fixed. This is a very commonly observed effect of long-term growth in elevated [CO_2] (Stitt 1991, Chomba *et al.* 1993, Ceulemans and Mousseau 1994). Starch concentrations in both species dropped after July 1996 which I attribute to photosynthetic acclimation in alder, although Scots pine may have been able to supply growth from starch as well as photosynthate production.

McMurtrie and Comins (1996) using the ecosystem model G'DAY found that plant nitrogen to carbon ratios (N:C) were particularly important in determining species responses to elevated [CO_2]. They showed that if the N:C ratio changes little the response to [CO_2] is much less than if the N:C ratio falls a lot. In this study, the N:C ratio decreased in association with extra carbon assimilation both in Scots pine and common alder, but the lack of corresponding significant reductions in leaf nutrients or proteins in Scots pine, in contrast to the decrease in Rubisco in common alder, led to a larger decrease in N:C. I propose that Scots pine was able to sustain its positive growth response under nutrient-limited conditions as a result of increased mycorrhizal exploration of the soil, increased N uptake, particularly increased NUE.

Additional evidence which supports the suggestion that changes at the leaf scale in Scots pine were not particularly pronounced, are the findings of Heyworth *et al.* (in press) on carbon based secondary compounds in the Scots pine seedlings at Glendevon in 1995. Of the condensed tannins and monoterpenes measured, only the

monoterpene α-pinene was significantly increased in elevated [CO_2]. This suggests that changes in litter decomposition or herbivory are not to be expected in Scots pine in a changing climate (see Woodward 1992, Penuelas and Estiarte 1998, Mooney *et al.* in press).

6.7 Nitrogen Fixation

Nodules on common alder roots grown in elevated [CO_2] were smaller, more numerous and of larger mass, although not significantly. Nitrogenase activity per unit dry mass has been found to increase with decrease in nodule size (Wilson and Coutts 1985). Nitrogenase activity was higher per mass of nodules and per plant per hour in elevated [CO_2] in 1995, although not significantly. It is unclear whether these trends towards increased nitrogen fixation observed in 1995 also occurred in 1996, since the differences in nodule mass were not statistically significant in 1996. Changes in nodule size and distribution (see Photos 4.4 to 4.6) were nevertheless apparent and showed that nitrogen fixation in common alder responded primarily to elevated [CO_2] via changes in nodule morphology. This is in agreement with the work of Norby (1987) on nitrogen-fixing trees, one of which was common alder, in which specific nitrogenase activity was not consistently or significantly increased by elevated [CO_2], despite increased nodule mass and total plant mass. This suggests that the nodules were fixing nitrogen more efficiently, at least in the first growing seasons of growth in elevated [CO_2]. Significant increases in nitrogenase activity seem to only occur when leaf area increases as well as total biomass (see Arnone and Gordon 1990).

As discussed Chapter 1, two key aspects of nitrogen-fixing plants are the need to keep the C:N ratio high in order to be successfully competitive and the importance of phosphorus for nodulation. Growth in elevated [CO_2] should supply ideal conditions for a high C:N ratio, whereas phosphorus supply in the long-term experiment could have been limiting in the non-fertilised plants. Although leaf [P] in 1996 was not significantly reduced by elevated [CO_2] in alder, it is possible that the continued stimulation of photosynthesis and growth required larger amounts of phosphorus than were available from the soil. Fertilised trees probably received sufficient phosphorus but nitrogen fixation may have been suppressed by the addition of nutrients. This

might explain why photosynthetic acclimation occurred irrespective of nutrient treatment. By 1996 above-ground biomass in common alder was larger in the fertilised trees than in the non-fertilised trees, although not significantly, suggesting that an upper ceiling to nitrogen fixation had been reached.

The above discussion of the effects of elevated [CO_2] and nutrient addition on the nitrogen-fixing alder highlights the fact that there are still many uncertainties in this area, particularly when more than two factors (i.e. [CO_2], nutrients, nitrogen fixation) are acting at once in an experiment. Mooney *et al.* (in press) found that one of the most important conclusions from the study of species-specific responses to elevated [CO_2] in mixed communities was that plants of traditional functional type (such as nitrogen fixers, C_3 versus C_4) did not respond consistently, rendering such grouping of individual species into functional types with respect to elevated [CO_2] of limited use. For example the species which responded the least in a highly diverse calcareous grassland exposed to elevated [CO_2] were five nitrogen-fixing species of *Trifolium*, whereas a slow growing sedge was most responsive.

6.8 Nitrogen Allocation to Different Tissues

The pattern of N storage in trees depends on whether trees are evergreen or deciduous. Allocation of nitrogen in common alder and Scots pine over the winter and its contribution to spring growth responded in a similar way to other species (e.g. Nambiar and Fife 1991, Millard 1996). Nitrogen was allocated to and remobilised for spring growth from the previous year's stem in alder, but from the previous year's needles in pine.

More N from the labelled fertiliser was taken up by alder in elevated [CO_2] than in ambient [CO_2] and this may explain how growth of the fertilised alder seedlings was able to be as stimulated as growth of the non-fertilised trees. N from the labelled fertiliser also contributed more to new leaf growth in elevated [CO_2]. Elevated [CO_2] did not affect N allocation to different tissues in Scots pine, but 1993 and 1994 common alder stems contained more labelled N both in autumn 1994 and spring 1995. Leaves of common alder also contained more labelled N in elevated [CO_2] in spring

1995. These results suggest increased sink capacity in alder grown in elevated [CO_2], at least in early 1995, before photosynthetic and growth acclimation set-in.

Rubisco has often been proposed as one of the main forms of N storage in plants over the winter in conifers (Camm 1993, reviewed in Millard 1996). Increases in Rubisco concentration in needles of Scots pine in autumn 1995 were not found, indicating that Rubisco may not have played a significant role in N storage over the winter. Rubisco concentration in common alder also did not increase before the onset of winter.

6.9 Species comparison

Common alder and Scots pine are taxonomically, structurally and physiologically different kinds of trees and they responded quite differently to growth in elevated [CO_2]. Although the response was largest in common alder, the length of the positive response was longest in Scots pine. I attribute the latter to the occurrence of canopy closure and root restriction in common alder and therefore to a lack of sinks for the extra carbon fixed. Although Scots pine showed a more prolonged response, it is possible that it too would experience more negative feedback on growth stimulation had it grown to canopy closure. Nevertheless there are also ecological characteristics of both species which could partially explain their different responses.

It has been found before that species with indeterminate growth respond faster to elevated [CO_2] than those showing determinate growth (see also Lucas 1998). According to the classical logistics of population growth, both common alder and Scots pine are *K*-selected organisms, being perennial and more growth-orientated than reproduction-orientated (*r*-selected) organisms (Fitter and Hay 1987). Grime (1977, 1979, cited in Fitter and Hay 1987) suggested a more complex functional classification with three rather than two poles. Plants can be considered as stress tolerators, ruderal plants and competitive plants depending on the favourability of the environment and the level of disturbance. Since trees are not annuals and are *K*-selected, most of them live in conditions of reasonable environmental favourability and minimum disturbance, putting them into Grime's competitive category. I propose that pioneer species which fix nitrogen from the atmosphere such as common alder,

are not only competitive organisms but are also stress tolerators, capable of growth on unfavourable sites with considerable disturbance such as disused opencast-coal-spoils. Common alder is a mid-successional species and not a dominant forest tree, although it is sometimes grown in association with conifers on reclamation sites (Hood 1993). By inter-planting alder with conifers, site and tree nutrition can be improved, although there is a tendency for alder to out-compete the conifer in the first years.

I suggest that as in such mixed-species planting scenarios, common alder has the biggest advantage during the first months to years of growth in an unfavourable environment. When soil nitrogen levels are high the competitive advantage is lost and carbon invested into growth gives more competitive ability than carbon invested into nitrogen fixation. One result of this is that nitrogen fixers are generally rare in mature communities (Fitter and Hay 1987). Not being a dominant forest species with characteristics such as longevity and large height and girth, alder may not be able to keep up stimulated rates of growth compared to a dominant forest species such as Scots pine in elevated [CO_2]. Alders are not as long-lived as pines and therefore invest more resources into rapid growth in the early stages of their life-cycle. The results of this long-term study seem to agree with these ecological attributes. Common alder was able to respond more positively at first but there was an upper ceiling to nitrogen fixation indicating that even in elevated [CO_2] nitrogen fixation was not able to become much more efficient and thus to avoid photosynthetic acclimation.

Scots pine on the other hand showed more signs of increased resource use efficiency, probably via enhanced mycorrhizal growth and nutrient acquisition, which in turn was most likely stimulated by carbon exudation out of the roots. Ectomycorrhizas can grow along the root surface, whereas nodules, although perennial, require separate point inoculations to be induced (Wilson and Coutts 1985). It may be that mycorrhizas are able to respond more flexibly than nodules to elevated [CO_2] and thus were able to improve the nutrient status of Scots pine more than the nodules did in common alder.

One implication for forests and their possible carbon sequestration is that Scots pine, if it shows similar response patterns to elevated [CO_2] when mature, may be a significant carbon sink. However, assessing the role of both common alder and Scots pine in potential global carbon sequestration is beyond the scope of this study.

6.10 Experimental Limitations and Contributions

Limitations

The use of pots in the short-term experiment led to different results than the long-term field experiment, indicating that the results from past experiments using pots could have been misleading. Results from the long-term experiment were used to assess these consequences.

In common with all other studies using OTC to expose plants to elevated [CO_2] the environmental conditions were affected to a certain degree. The main differences between the environments of the OTCs and the outside control plots were in temperature, radiation and wind turbulence. The presence of chambers may affect growth and physiology, often to the same extent as the effect of [CO_2], but such effects are not universal or consistent (Leadley and Drake 1993).. The design of the experiment with outside control plots enabled the magnitude of the chamber effect to be separated from the effect of [CO_2], however.

In nature atmospheric [CO_2] is rising gradually whereas in this and other impact studies the trees were subjected to a doubling of current atmospheric [CO_2] or discrete increases.

In nature atmospheric temperatures are predicted to rise gradually whereas in the OTCs the trees experienced a mean increase in temperature compared to the outside of 2.8 °C.

Cost and logistics limited the number of replicates possible and this may have precluded the results from reaching the statistical significance that might be found with a larger sample size. Replication is still usually higher in experiments using OTCs

than in controlled environment chambers, however, and the OTCs provide a more natural environment.

More generally, it is difficult and expensive to measure directly the response of forest ecosystems to elevated [CO_2] because trees are long-lived, large and form very complex systems. Even with FACE, now being used in loblolly pine plantations and elsewhere, only a snapshot in time can be investigated. Recent evidence from ecosystem experiments on grasses and shrubs show that the outcome of many experiments can be drastically changed by competition.

Predicted effects of global warming include increased climatic perturbations and uncertainty. Increases in storms and natural disasters cannot be taken into account when assessing the effects of climate change on trees and forests.

Contributions

Assessment of the response of two very different tree species in the same experiment enabled any differences in response to be attributed to real species responses and not to differences in methodology.

The characterisation of the response to elevated [CO_2] of an evergreen tree species widely distributed throughout the North temperate and boreal forest zone and an important mid-successional, deciduous, nitrogen-fixing species often used in land reclamation, has provided a useful contrast.

This study is one of the longer field studies so far using trees planted directly in the ground in conditions as close to natural as possible. The length of the study enabled the examination of long-term responses including acclimation.

The split plot experimental design enabled the effect of nutrient addition to be incorporated into the experiment, allowing assessment of the interactive effect of the former with elevated [CO_2].

This study involved the investigation of a wide variety of responses from the whole tree scale to the biochemical scale, including a study of nitrogen cycling within the trees, giving a good overall picture of the growth responses of the trees.

6.11 Conclusions

Exposure to elevated [CO_2] led to significant increases in growth and biomass in both species (although total biomass was not statistically significant in the short-term experiment). Specific growth rate was enhanced in both species in the first year of growth in the short-term experiment (before the start of the experiment) and in the early stages of the second year of growth in the long-term experiment.

Nutrient addition had some unexpected effects on plant response to elevated [CO_2] in both species. Nitrogen fixation in common alder grown without fertiliser had a very similar growth response to common alder grown with fertiliser, indicating that nitrogen fixation has an upper ceiling. This upper limit to nitrogen fixation capacity may be related to phosphorus limitation or may be genetically controlled. In Scots pine trees grown without fertiliser were generally not as stimulated in growth and biomass as those with fertiliser. There was evidence, nevertheless, of increased resource use efficiency and increased nutrient availability via increased mycorrhizal infection. Thus a stimulation of growth in Scots pine grown in nutrient-limited forest soils cannot be ruled out.

There were generally few cases of an interaction effect of elevated [CO_2] and nutrient addition on growth, biomass or leaf composition.

The species which experienced more photosynthetic and growth acclimation in each experiment showed more changes in biomass allocation than the species which was less down-regulated. In the short-term experiment this was Scots pine, in the long-term experiment common alder.

Starch accumulated in the leaves of all species as a response to growth in elevated [CO_2], especially in the long-term experiment in 1996 in common alder. Chlorophyll was reduced more in common alder than Scots pine in both experiments.

There were clear species-specific responses in both experiments. Whereas photosynthetic acclimation occurred more in Scots pine in the short-term experiment, the opposite was true in the long-term experiment. In fact Scots pine only showed consistent photosynthetic acclimation in 1996 and 1997 in trees grown without fertiliser. An important factor in the acclimation of common alder was the advent of canopy closure and root competition between individual trees. This highlights the importance of distinguishing between responses of trees grown in relative isolation and those grown to canopy closure. It is not known whether Scots pine would have shown similar photosynthetic acclimation to that in common alder had canopy closure occurred.

The differing results of the short-term and long-term experiment show very clearly that the impacts of elevated [CO_2] on growth are often different depending on the length and nature of the experiment. Short-term experiments in pots should be avoided if possible since they can provide misleading results. Although the long-term experiment lasted three years, a longer period would have yielded more interesting and conclusive results.

Nitrogen fixation seemed to become more efficient in common alder, at least in 1995. Trees grown without fertiliser addition did not grow more than trees with fertiliser, however, suggesting and upper ceiling to nitrogen fixation.

Elevated [CO_2] did not affect the allocation of labelled nitrogen to different tissues in Scots pine, but in common alder there was evidence for increased allocation of nitrogen over the autumn and winter to stems.

Nitrogen was allocated to the previous year's needles in Scots pine and possibly to stems in common alder over the winter and withdrawn from these tissues

in the spring. Growth in elevated [CO_2] increased the allocation of labelled N to the stems and leaves in alder. Alder grown in elevated [CO_2] took up more labelled fertiliser than alder in ambient [CO_2] which may have enabled fertilised alder trees to grow as well as nitrogen-fixing, unfertilised trees.

Changes in biochemical leaf composition were of a lesser magnitude than often found in [CO_2] impact studies. In keeping with the relative lack of effect of elevated [CO_2] on nitrogen allocation, protein concentrations were generally not affected by elevated [CO_2].

6.12 Suggestions for Future Research

The interactive effects of elevated [CO_2] and nitrogen are better understood than the effect of phosphorus and other nutrients on plant growth in elevated [CO_2]. There is a need for more experiments with different phosphorus supply rates to examine how phosphorus limitation affects tree responses.

The complexity of the interaction between nitrogen-fixation, fertilisation and elevated [CO_2] in this study highlights the need for more extensive information on the interactive effects of elevated [CO_2] and fertilisation on nitrogen fixation.

There is a need for experiments designed to investigate photosynthetic acclimation mechanisms in the long-term. In particular interaction between the kinetics of Rubisco and the accumulation of carbohydrates in leaves needs more attention.

Allocation to hidden components such as fine root turnover, mycorrhizal growth and root exudation needs to be quantified since it is clear from this study and others that such changes often play a larger role than growth of biomass.

Further tracer experiments with labelled nitrogen are required to elucidate the effects of elevated [CO_2] and nutrient addition on internal nitrogen cycling. An improvement on the design used in this study would be the application of a tracer signal over a longer period and using high and low nitrogen fertilisation.

There is a need for impact studies on different tree species grown together, as in mixed deciduous forests, to investigate how competition between plants affects a species response to elevated [CO_2].

More longer-term experiments of at least three years with plants grown in soil in the ground in as natural conditions as possible are needed to assess the possible long-term effects of climate change on forests. These, in conjunction with controlled chamber experiments to assess the mechanisms of photosynthetic acclimation, should provide a clearer picture of possible future tree response to climate change.

Investigations at more than two CO_2 concentrations are necessary. The response of photosynthesis to [CO_2] is saturated, with no further stimulation of photosynthesis, at a certain, species-specific [CO_2]. Experiments which use an elevated [CO_2] beyond a plant's saturation point can have very different results to experiments with an elevated [CO_2] below the saturation point (see Körner 1995).

The interaction of elevated [CO_2] and temperature on plants has mostly not been investigated in climate change impact studies. Such experiments are crucial if the response of trees grown in OTCs are to be scaled-up to the response of natural ecosystems living in a 'greenhouse' world with probable temperature rises associated with rising atmospheric[CO_2].

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Appendix 1

Nutrient solution used for plants in OTCs at the Institute of Ecology - after the Ingestad and Lund solution for *Betula* sp. (1986)

For optimum growth the following ratios of nutrients by mass are recommended:

N	100 ;	K	65 ;	P	13 ;	Ca	7 ;
Mg	8.5 ;	S	9 ;	NH_4	38.8 ;	NO_3	61.2

Nutrient Solution Contents

The following was made up as two separate solutions and only mixed when diluted :

Solution 1 (mol dm^{-3})

chemical compound	final desired conc (mg / dm^3)	stock solution (g / dm^3)
K_2SO_4	48.97	0.00490
K_2HPO_4	33.62	0.00336
KH_2PO_4	30.89	0.00310
KNO_3	49.24	0.00492
NH_4NO_3 (note- this was left out for the low nutrient treatment)	221.6	0.00222

Solution 2 (mol dm^{-3})

	final desired conc. (mg / dm^3)	stock solution (g / dm^3)
$Ca(NO_3)_2$	28.72	0.00287
$Mg(NO_3)_2$	51.92	0.00519

Micronutrients (mmol dm^{-3}) mg / dm^3

chemical compounds	desired final conc ($\mu g / dm^3$)	stock soln. (mg / dm^3)
$Fe(NO_3)_2$	302.4	0.0003024
$Mn(NO_3)_2$	130.3	0.001303
H_3BO_3	114.4	0.000114
$Zn(NO_3)_2$	17.4	0.0000174
$CuCl_2$	6.35	0.0000064
Na_2MoO_4	1.50	0.0000015
HNO_3	106.6	0.00107

Addition was varied depending on rate of growth, half strength solutions were added weekly in May and June, three-quarter strength in July (full strength would have been used in August). Thus in the field the stock solution was diluted 100 times, and each plant received a certain volume of nutrient solution. which varied over the season.

Appendix 2

Nutrient solution used for plants in OTCs at the Glendevon Field Site for both common alder and Scots pine

The following Wallco Plant Nutrition (Sweden) solution was applied on 85 m^2 (*c.* 7 m^2 per chamber) of the site corresponding to half of the ground area in the chambers and control plots via a computer-controlled fertigation system (see Chapter 2 Materials and Methods for details).

element	grammes
N	40 (ammonium)
	60 (nitrate)
K	65
S	9
Ca	-
Mg	4
Fe	0.7
Mn	0.4
B	0.2
Zn	0.06
Cu	0.03
Mo	0.007

pH 3.5 ; density (per dm^3) 1.223

This solution was diluted 1: 10 when applied to the site, giving 10 g of nitrogen per dm^3. 20 dm^3 of solution were diluted at a time in a 25 dm^3 tank. On the 30 August 1995, 240 g of calcium and magnesium were added to each quadrant in each chamber and control plot, to account for the lack of calcium and magnesium in the Wallco nutrient solution.

Appendix 3

ANOVA probability values (p) relating to the effect of $[CO_2]$ and nutrients on stem hieght of common alder and Scots pine in the short-term experiment. Data are p values for the effects of $[CO_2]$, nutrient addition, $[CO_2]$ × nutrient interaction and chamber. ** = significant at $p < 0.01$; * = significant at $p < 0.05$, (*) = significant at $p < 0.10$.

common alder	$[CO_2]$	nutrient	$CO_2]$ × nutrient	Chamber
week 1	0.015 *	0.499	0.047 *	--
week 2	0.021 *	0.133	0.354	--
week 3	0.012 *	0.052 (*)	0.381	--
week 5	0.064 (*)	0.881	0.025 *	0.035 *
week 7	0.140	0.272	0.301	0.140
week 8	0.082 (*)	0.820	0.082 (*)	0.001 **
week 9	0.185	0.823	0.195	0.001 **
week 12	0.242	0.394	0.273	0.001 **
week 15	0.117	0.322	0.501	0.001 **

Scots pine	$[CO_2]$	nutrient	$[CO_2]$ × nutrient	Chamber
week 1	0.514	0.225	0.271	0.514
week 2	0.604	0.224	0.582	0.604
week 3	0.995	0.242	0.419	0.995
week 5	0.803	0.522	0.203	0.248
week 7	0.933	0.366	0.709	0.560
week 8	0.956	0.499	0.168	0.115
week 9	0.832	0.170	0.974	0.636
week 12	0.583	0.428	0.067 (*)	0.146
week 15	0.649	0.530	0.474	0.707

Appendix 4

Mean dry mass (g) of tissues of common alder and Scots pine at the final harvest in the short-term experiment. Data are means of 25 for ambient / elevated / control treatments, and means of 12 for [CO_2] and nutrient treatments; * = [CO_2] effect significant at $p < 0.05$; (*) = [CO_2] effect significant at $p < 0.1$.

Treatment	Roots	Branches	Stems	Leaves
common alder				
Ambient	22.32	39.67	65.35	83.00
Elevated	30.49 (*)	41.18	66.93	97.63 (*)
Control	16.15	24.86	41.8	56.78
A-N	23.45	47.09	72.18	98.40
A+N	21.19	32.24	58.51	67.60
E-N	33.47	37.74	64.22	83.58
E+N	27.51	44.61	69.65	111.68
C-N	18.18	28.6	45.73	63.09
C+N	14.13	21.12	37.88	50.46

Treatment	Roots	Branches	Stems	Leaves
Scots pine				
Ambient	2.52	2.95	5.74	13.32
Elevated	3.40	3.13	6.16	14.56
Control	2.47	2.97	6.00	11.05
A-N	2.56	3.10	6.12	13.02
A+N	2.48	2.79	5.37	13.63
E-N	3.67	3.08	6.58	15.11
E+N	3.13	3.18	5.73	14.01
C-N	2.20	2.82	5.01	10.31
C+N	2.75	3.13	7.01	11.8

Appendix 5

Integration parameters used for the calculation of soluble carbohydrate peak area on the Dionex hpae / pad machine

Peak Width	2 seconds
Peak Threshold (baseline)	35
Area Reject	10,000 counts
Reference :	Area Reject 1000 area counts

Appendix 6

ANOVA probability values (p) relating to the effect of $[CO_2]$ and nutrients on nitrogen concentration (mg g^{-1}) in leaves of common alder and Scots pine in the long-term experiment. Data show p values for the effects of $[CO_2]$, nutrient addition, $[CO_2]$ × nutrient interaction and chamber. ** = significant at $p < 0.01$; * = significant at $p < 0.05$, (*) = significant at $p < 0.10$.

Treatment	$[CO_2]$	nutrient	$[CO_2]$ × nutrient	Chamber
common alder				
Sep. 1994	0.612	0.178	0.112	0.433
April 1995	0.980	0.288	0.261	0.269
August 1995	0.249	0.493	0.169	0.249
December 1995	0.348	0.195	0.421	0.278

Treatment	$[CO_2]$	nutrient	$[CO_2]$ × nutrient	Chamber
Scots pine				
Sep. 1994	0.382	0.100	0.905	0.006 **
April 1995	0.601	0.350	0.594	0.141
August 1995	0.183	0.655	0.281	0.797
October 1995	0.159	0.693	0.751	0.159

ANOVA probability values (p) relating to the effect of $[CO_2]$ and nutrients on phosphorus concentration (mg g^{-1}) in leaves of common alder and Scots pine over time. Data show p values for the effect of $[CO_2]$, nutrient and $[CO_2]$, $[CO_2]$ × nutrient interaction and chamber. ** = significant at $p < 0.01$; * = significant at $p < 0.05$, (*) = significant at $p < 0.10$.

Treatment	$[CO_2]$	nutrient	$[CO_2]$ × nutrient	Chamber
common alder				
Sep. 1994	0.668	0.758	0.915	0.365
April 1995	0.419	0.188	0.0538 (*)	0.0202 *
August 1995	0.559	0.992	0.573	---
December 1995	0.211	0.247	0.705	---

Treatment	$[CO_2]$	nutrient	$[CO_2]$ × nutrient	Chamber
Scots pine				
Sep. 1994	0.0767 (*)	0.227	0.0178 *	0.0581 (*)
April 1995	0.542	0.359	0.900	0.278
August 1995	0.4503	0.413	0.786	0.843
October 1995	0.640	0.787	0.735	0.641

Appendix 7

Results of one-way ANOVA of labelled N in common alder and Scots pine tissues grown at elevated and ambient [CO_2]. Each [CO_2] treatment had four replicates (occasionally three). *R* = root; *S* = shoot; *L* = leaves; *N* = needles. * = significant at $p < 0.05$.

Tissue	1994 Sep.	1995 April	June		Aug.	Dec.	1996 April
common alder							
R	0.30	0.23	0.27	*R*	0.71	0.59	0.07
1993*S*	0.01 *	0.017 *	0.40	**1994***S*	0.95	0.14	0.18
1994*S*	0.022 *	0.024 *	0.20	**1995***S*	0.94	0.04 *	0.11
1995*S*	--	0.31	0.72	*L*	0.46	0.48	--
L	0.14	0.03 *	0.64				

Tissue	1994 Sep.	1995 April	June	Aug.	Oct.	1996 April
Scots pine						
R	0.69	0.28	0.82	0.50	0.61	0.66
1993*S*	0.31	0.35	0.73	0.40	0.86	0.37
1994*S*	0.23	0.42	0.99	0.32	0.57	0.43
1995*S*	--	--	0.46	0.55	0.60	0.52
1993*N*	0.63	0.16	0.35	0.44	0.55	0.34
1994*N*	0.65	0.26	0.73	0.38	0.35	0.35
1995*N*	--	0.51	0.78	0.78	0.76	0.79

Appendix 8

The SAS 6.1 general linear model used for analysis of variance of variables measured

```
data one;
infile 'c:\user\datafile.csv' firstobs=4 lrecl=300 dlm=',';
input unit co2 cpres nut dw;
trt=co2+cpres;
;
proc glm data=one;
class trt unit nut;
model dw= trt unit(trt) nut trt*nut  /ss3 p;
random unit(trt)/test;
lsmeans unit (trt) / tdiff pdiff e= unit(trt);
lsmeans nut /tdiff pdiff e= nut;
contrast 'no chamber v. chamber no CO2' trt -1 1 0 / e=unit(trt);
contrast 'CO2 v.no CO2' trt 0 -1 1 / e=unit(trt);
output out=dw p=pdw;
title FIRST ANALYSIS;
;
proc sort;
by co2 nut cpres;
;
proc means mean stderr;
var dw;
by co2 nut cpres;
;
data two;
set one;
if cpres=0 then delete;
;
proc glm data=two;
class co2 unit nut;
model dw=co2 unit(co2) nut co2*nut/ss3;
```

```
random unit(co2)/test;
lsmeans co2*nut/tdiff pdiff;
contrast 'co2 v no co2' co2 -1 1/e=unit(co2);
title "ANALYSIS EXCLUDING OUTSIDE PLOTS";
;
run;
```

Appendix 9

Additional information for determining Rubisco concentration

Rubisco Standard Purification for Calibration Use

In order to determine Rubisco by enzyme-linked immunosorbtion assay (ELISA) one Rubisco standard of known concentration is required, preferably purified from the same or a similar species of plant. Since the large sub-units of Rubisco which are recognised by the primary antibody show very little inter-species variation (Catt and Millard 1988, Afif *et al.* 1993), a deciduous tree species, *Prunus avium x pseudocerasus* Colt, was chosen for the extraction as half the study-trees were deciduous, and some of the standard was used by Dr. Renate Wendler at the Macaulay Land Use Research Institute, also working on deciduous trees. The bulk purification of Rubisco standard was made in April 1995 in the laboratories of Horticulture Research International (Littlehampton) using the deciduous tree species *Prunus avium* and the methodology of Besford (1990) adapted from Maechler, Keys and Cornelius (1980, cited in Besford 1990). The whole procedure was carried out at 4 °C and involved an extraction phase, a centrifugation stage and dialysis and took five days. Day 1 and 2: Rubisco was extracted from 3 leaf discs or 3 needles ground in liquid nitrogen using 50 cm^3 of extraction buffer containing 50 mmol m^{-3} HEPES-KOH (pH 8.0), 25 mmol m^{-3} $KHCO_3$, 5 mmol m^{-3} $MgCl_2$, 0.2 mmol m^{-3} Na_2EDTA and 10 mmol m^{-3} 2-mercaptoethanol) and 5 g of PolyclarAT and then homogenised in a coffee blender for one minute.

Using a Beckmann L8-80M ultracentrifuge, with a six-well, 70 Ti rotor (Beckmann Instruments Inc., Palo Alto, CA, USA) the homogenate was centrifuged at 37 500 rpm (equivalent to 100 000 g) for 35 minutes at 4 °C under vacuum. The supernatant was pipetted onto each of six tubes of sucrose gradient (0.9 mol dm^{-3} and 0.23 mol dm^{-3} sucrose in extraction buffer without PolyclarAT, mixed together and then passed through sucrose density gradient apparatus) and the tubes were spun overnight for approx. 20 hours at 100 000 g . Day 3: the tubes were re-spun for 22.5 hours at

37 500 rpm. Day 4: fractions were collected from the bottom of each gradient using a fraction collector (Retriever II fraction collector, ISCO, Lincoln, USA) and passed through a UV detector (ISCO Type 6 optical unit, UA-5 absorbance / fluorescence detector) in order to monitor the absorbance at A_{280}. Day 5 (dialysis): combined fractions from the first optically dense peak were dialysed against 5 mmol dm^{-3} HEPES pH 8.0 and 1 mmol dm^{-3} DTT (reducing agent) overnight and subsequently a number of times for one or two hours. The Dialysis / viscing tubing (18/32) separated the larger molecules (molecular mass of 30 000) from the smaller molecules, which passed through it. The purified protein was then retrieved, kept at well below freezing temperatures, and subsequently freeze-dried in aliquots.

Immunodetection of Rubisco Protein by Sodium Dodecyl Sulphate Polyacrylamide (SDS-PAGE) Gel Electrophoresis

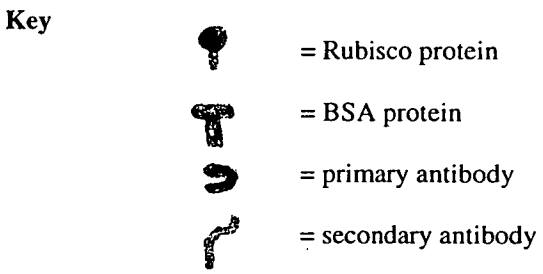
Supernatant (SN) from the Rubisco standard extraction process was diluted with SDS buffer (1:2, 1:5 and 1:10) and bromophenol blue. The standard used was 2 mm^3 of known proteins and 38 mm^3 of SDS and mercaptoethanol. All dilutions were placed in a water bath at 95 °C for 5 minutes to denature the proteins. The buffers used were an SDS buffer (25 % (v / v) glycerol, 5 % SDS (w / v) , 1 % (v / v) mercaptoethanol, 0.5 mol dm^{-3} Tris-HCl) and an electrode buffer containing a Tris base (15 g dm^{-3}) and glycine (72 g dm^{-3}). The silver reagent and the oxidiser concentrate were diluted ten times. The SDS-PAGE was carried out at 100 V for 10 minutes through a 3 % (w/v) acrylamide stacking gel, and then at 150 V for 50 minutes through a 10 % (w/v) acrylamide resolving gel. The gel was then developed in the same way as a black and white photograph (see Photo 5.1 in Chapter 5).

Accuracy and Choice of ELISA Assay

Determining Rubisco using the ELISA assay gives direct and unambiguous information on the amount of protein present, although care should be taken since ELISAs show larger variability between replicate determinations than enzyme activity measurements (Stitt and Schulze 1994). Although the best approach is to use both activity assays and immunological tests and to compare them, logistical considerations did not allow for this in the present research. Since any Rubisco protein present in an

extract is activated using the activity assay, the two tests should be compatible. Indeed, Stitt and Schulze (1994) found a strict linear relationship between immunological Rubisco amounts and Rubisco activity using antisense plants with decreased Rubisco content. Given the focus of this PhD on nitrogen and on concentrations of photosynthetic components, it was considered of more importance to investigate Rubisco content via ELISA assay than to measure activity (which would be more appropriate with detailed gas exchange research) (R.T. Besford, *pers. comm.*). Partial proteolytic degradation during extraction can also cause less accuracy, although the extraction buffer used contained an array of protective chemicals such as DTT (which protects sulphur bonds in protein) and polyvinylpolypropyrrolidone (PVPP; protects enzymes against phenolics and tannins).

Schematic Representation of Elisa Assay



Day 1

The diluted samples and Rubisco standards are pipetted into the wells on the Elisa plates and incubated overnight for 18 hours at 4 °C.

Day 2

Invert and shake all plates to remove all that is not attached to the wells, and wash *three times* with Tris / NaCl buffer. Then fill all wells with Tris buffer containing bovine serum albumin (BSA) and Tween-20 and leave for one hour at room temperature in order to block any unused sites in the wells.

Wash again as above but using the Tris buffer with added BSA and Tween-20 in order to remove any unattached protein. Add the diluted primary antibody diluted in the Tris buffer/BSA/Tween-20 to all the wells and incubate for two hours at room temperature.

Wash again as above and then add the secondary antibody diluted in Tris buffer/BSA/Tween-20 and leave for one hour at room temperature.

Wash again three times and add carbonate / bicarbonate buffer containing p-nitrophenolphosphate and leave to develop colour. The latter removes the tail on the secondary antibody producing a yellow colour which is read spectrophotometrically. The reaction is stopped using strong sodium hydroxide.

Appendix 10

Table 5.3 Mean chlorophyll *a* and chlorophyll *b* concentration in common alder leaves on a dry mass basis (mg g^{-1}) and the *a* / *b* ratio of young fully expanded leaves from August 1994 to September 1995. Values are the means (with one standard error in brackets) of eight samples for the [CO_2] and four samples for the [CO_2] and nutrient treatment. * = significant [CO_2] effect at $p < 0.05$; (*) = trend towards a [CO_2] effect at $p < 0.10$.

Treatment	Aug. 94	May 95	June 95	Aug. 95	Sep. 95
chlorophyll *a*					
Ambient	9.02 (1.08)	3.81 (0.37)	4.60 (0.41)	7.43 (0.71)	5.63 (0.25)
Elevated	7.78 (0.83)	3.57 (0.11)	4.11 (0.29)	4.14 (0.60) *	4.56 (0.30)
Control	11.25 (2.34)	3.39 (0.20)	6.38 (0.41)	7.80 (1.96)	5.31 (0.19)
A-N	10.86 (3.44)	4.51 (0.42)	4.45 (0.63)	6.44 (0.95)	5.82 (0.43)
A+N	7.19 (0.13)	3.13 (3.13)	4.71 (0.50)	8.42 (0.57)	5.45 (0.16)
E-N	8.70 (1.09)	3.34 (0.09)	4.18 (0.13)	3.78 (0.41)	4.32 (0.17)
E+N	6.86 (3.00)	3.80 (0.16)	4.05 (0.60)	6.02 (0.65)	4.81 (0.53)
C-N	12.02 (3.00)	3.27 (0.32)	6.79 (0.63)	6.02 (0.65)	4.94 (1.81)
C+N	10.48 (2.44)	3.50 (0.10)	5.97 (0.54)	9.58 (3.87)	5.68 (2.64)

Table 5.3 continued

Treatment	Aug. 94	May 95	June 95	Aug. 95	Sep. 95
chlorophyll *b*					
Ambient	4.22 (0.96)	1.63 (0.21)	2.00 (0.40)	2.90 (0.90)	2.72 (0.51)
Elevated	4.04 (0.85)	1.34 (0.076) (*)	1.60 (0.20)	2.42 (0.40)	1.96 (0.20)
Control	4.18 (0.89)	1.36 (0.11)	2.80 (0.22)	2.32 (0.70)	1.98 (0.77)
A-N	4.06 (1.28)	2.06 (0.28)	2.05 (0.42)	2.51 (0.42)	2.55 (0.24)
A+N	4.38 (0.94)	1.21 (0.078)	1.9 (0.40)	3.30 (1.25)	2.88 (0.84)
E-N	5.97 (1.71)	1.19 (0.027)	1.38 (0.080)	2.06 (0.41)	1.50 (0.065)
E+N	2.1 (0.16)	1.50 (0.10)	1.82 (0.36)	2.78 (0.42)	2.42 (0.43)
C-N	4.75 (0.80)	1.37 (0.21)	3.01 (0.49)	2.26 (0.43)	1.93 (0.77)
C+N	3.62 (1.21)	1.35 (0.12)	2.58 (0.54)	2.39 (1.00)	2.01 (0.86)
a / b					
Ambient	2.24 (0.29)	2.40 (0.10)	2.39 (0.19)	3.72 (1.56)	2.19 (0.20)
Elevated	2.52 (0.34)	2.69 (0.11) *	2.71 (0.22)	1.85 (0.25) (*)	2.51 (0.21)
Control	3.13 (0.89)	2.60 (0.21)	2.34 (0.25)	2.61 (0.28)	2.61 (0.28)

Table 5.4 Mean chlorophyll *a* and chlorophyll *b* concentration of young fully expanded leaves in common alder on an area basis (μg cm^{-2}) from August 1994 to September 1995. Values are the means (with one standard error in brackets) of eight samples for the [CO_2] treatment and four samples for the [CO_2] and nutrient treatment. * = significant [CO_2] effect at $p < 0.05$; (*) = trend towards a [CO_2] effect at $p < 0.10$.

Treatment	Aug. 94	May 95	June 95	Aug. 95	Sep. 95
chlorophyll *a*					
Ambient	31.43 (3.93)	22.28 (0.36)	86.81 (6.15)	40.14 (7.98)	39.84 (5.81)
Elevated	31.51 (3.64)	23.94 (0.72)	82.19 (5.64)	24.51 (1.87) *	27.94 (2.54) *
Control	42.96 (6.98)	25.16 (0.68)	90.66 (6.46)	29.75 (4.06)	30.04 (4.29)
A-N	31.91 (4.37)	25.64 (0.64)	85.26 (7.74)	32.96 (5.14)	29.87 (2.26)
A+N	30.94 (3.73)	18.92 (2.13)	88.36 (4.53)	47.32 (9.77)	49.81 99.94)
E-N	32.50 (4.78)	22.59 (0.70)	78.34 (4.82)	23.46 (1.32)	27.23 (0.86)
E+N	30.52 (1.06)	25.29 (0.74)	86.04 (6.64)	25.86 (2.36)	28.64 (3.07)
C-N	48.81 (8.95)	25.17 (0.71)	85.87 (9.99)	24.50 (2.11)	25.11 (4.17)
C+N	37.11 (5.24)	25.14 (0.56)	95.45 (2.93)	35.0 (5.68)	34.97 (4.42)

Table 5.4 continued

Treatment	Aug. 94	May 95	June 95	Aug. 95	Sep. 95
chlorophyll *b*					
Ambient	15.25 (2.05)	9.47 (0.68)	37.26 (4.56)	14.95 (4.98)	18.52 (1.99)
Elevated	15.02 (2.38)	9.05 (0.76)	32.19 (4.05)	14.81 (2.76)	11.92 (1.46) *
Control	16.13 (3.01)	10.25 (1.42)	40.51 (4.25)	10.76 (0.92)	12.08 (2.45)
A-N	12.00 (1.29)	11.62 (0.87)	38.80 (4.48)	13.33 (3.15)	13.25 (1.69)
A+N	18.49 (3.62)	7.31 (0.58)	35.72 (4.33)	16.56 (5.50)	23.80 (2.82)
E-N	20.52 (0.97)	8.09 (0.50)	25.75 (1.36)	12.66 (2.30)	9.52 (0.80)
E+N	9.51 (0.97)	10.00 (0.75)	38.63 (6.36)	16.96 (3.69)	14.31 (2.60)
C-N	19.68 (2.83)	10.70 (1.82)	38.91 (7.32)	9.22 (1.77)	10.01 (2.31)
C+N	12.57 (3.38)	9.80 (1.29)	42.10 (3.86)	12.29 (0.78)	14.15 (2.26)

Table 5.5 Mean chlorophyll *a* and chlorophyll *b* in Scots pine on a dry mass basis (mg g-1) and the *a* / *b* ratio of young needles from August 1994 to September 1995. Values are the means (with one standard error in brackets) of eight samples for the [CO_2] and four samples for the [CO_2] and nutrient treatment. * = significant [CO_2] effect at $p < 0.05$; (*) = trend towards a [CO_2] effect at $p < 0.10$.

Treatment	Aug. 94	May 95	June 95	Aug. 95	Sep. 95
chlorophyll *a*					
Ambient	3.92 (1.20)	2.59 (0.36)	2.21 (0.33)	5.64 (0.54)	4.30 (1.51)
Elevated	3.33 (0.58)	2.44 (0.26)	1.71 (0.42)	4.18 (0.30)	2.91 (0.38)
Control	4.78 (0.30)	6.50 (0.59)	1.86 (0.29)	4.96 (0.30)	3.63 (0.56)
A-N	5.45 (1.22)	2.80 (0.55)	2.49 (0.16)	5.50 (0.44)	4.68 (1.83)
A+N	2.39 (1.20)	2.38 (0.091)	1.92 (0.42)	5.77 (1.00)	3.93 (0.92)
E-N	2.62 (0.46)	2.94 (0.37)	1.75 (0.36)	3.86 (0.45)	2.64 (0.42)
E+N	4.05 (0.73)	1.94 (0.17)	1.67 (0.59)	4.51 (0.30)	3.18 (0.16)
C-N	7.27 (3.66)	3.83 (0.72)	1.45 (0.24)	4.61 (0.59)	3.24 (0.52)
C+N	2.30 (1.04)	3.16 (0.26)	2.26 (0.37)	5.31 (0.13)	4.03 (0.64)

Table 5.5 continued

Treatment	Aug. 94	May 95	June 95	Aug. 95	Sep. 95
chlorophyll *b*					
Ambient	2.61 (0.73)	1.77 (0.16)	1.12 (0.66)	3.36 (0.61)	3.41 (0.17)
Elevated	1.20 (0.062) *	1.71 (0.29)	1.33 (0.42)	2.28 (0.43) (*)	2.32 (0.16)
Control	1.92 (0.85)	1.57 (0.21)	0.91 (1.05)	2.17 (0.16)	1.74 (0.53)
A-N	2.47 (0.65)	1.74 (0.21)	1.36 (0.66)	2.83 (0.61)	3.41 (1.73)
A+N	2.75 (0.90)	1.79 (0.52)	0.87 (0.24)	3.88 (0.81)	1.66 (0.55)
E-N	1.02 (0.076)	1.76 (0.49)	1.19 (0.32)	2.31 (0.29)	1.81 (0.36)
E+N	1.38 (0.050)	1.67 (0.38)	1.46 (0.54)	3.31 (0.68)	2.83 (0.091)
C-N	6.15 (1.47)	1.35 (0.30)	0.51 (0.17)	2.25 (0.40)	1.06 (0.26)
C+N	0.69 (0.21)	1.79 (0.28)	1.31 (0.61)	2.09 (0.089)	2.42 (0.80)
a / b					
Ambient	1.68 (0.56)	1.51 (0.13)	2.99 (1.10)	1.90 (0.34)	2.10 (0.32)
Elevated	2.76 (0.51)	1.99 (0.70)	1.40 (0.22)	1.62 (0.230	1.35 (0.19)
Control	3.99 (0.22)	2.39 (0.23)	5.66 (3.56)	2.36 (0.26)	2.60 (0.34)

Appendix 11

Table 5.6 Mean total soluble sugar concentration (mg g^{-1}) in common alder and Scots pine from September 1994 to October 1995. Values are the means (and on standard error in brackets) of eight samples for the [CO_2] treatments and four samples for the [CO_2] and nutrient treatments, unless otherwise stated. In August 1995 there were only 13 alder samples analysed out of a potential 24, with only three elevated [CO_2] replicates; in the same month there were 14 pine samples. There was no significant [CO_2] effect ($p > 0.05$) in either species in any month on total soluble sugar concentration.

Treatment	Sep. 94	June 95	Aug. 95	Sep. 95	Oct. 95
common alder					
Ambient	62.9 (2.9)	73.1 (5.4)	80.6 (7.6)	103.3 (1.02)	53.8 (3.0)
Elevated	79.5 (13.2)	68.8 (8.5)	76.8 (1.1)	125.2 (0.66)	54.1 (5.9)
Control	76.7 (3.8)	81.1 (6.3)	79.6 (6.3)	95.7 (0.70)	--
A-N	55.4 (1.0)	78.0 (2.3)	72.8 (3.7)	93.4 (5.5)	56.1 (4.0)
A+N	70.3 (5.6)	68.1 (8.7)	88.3 (14.4)	113.1 (15.9)	51.4 (2.6)
E-N	77.0 (24.5)	76.1 (14.0)	53.6 (one sample)	124.9 (7.6)	51.0 (5.8)
E+N	81.9 (4.4)	61.5 (2.9)	100.0 (14.1)	125.4 (5.9)	57.1 (6.0)
C-N	75.7 (3.5)	83.7 (8.7)	82.6 (12.7)	95.4 (7.2)	--
C+N	77.7 (5.5)	78.4 (4.5)	76.6 (3.2)	95.9 (6.0)	--

Table 5.6 continued

Treatment	Sep. 94	June 95	Aug. 95	Sep. 95	Oct. 95
Scots pine					
Ambient	41.8 (5.1)	65.8 (8.8)	60.3 (8.2)	54.8 (6.1)	53.8 (3.0)
Elevated	49.0 (4.0)	68.0 (8.2)	62.5 (8.8)	65.5 (2.7)	40.6 (2.9)
Control	39.2 (7.2)	78.9 (4.0)	66.5 (10.1)	61.3 (7.3)	--
A-N	48.2 (2.0)	69.4 (7.7)	60.2 (7.2)	49.7 (7.9)	38.1 (4.4)
A+N	35.3 (7.1)	62.1 (9.5)	60.3 (9.9)	59.8 (4.4)	37.5 (2.9)
E-N	50.0 (3.8)	73.3 (13.0)	68.9 (7.8)	65.1 (2.8)	36.2 (2.1)
E+N	47.9 (4.5)	62.6 (2.7)	56.1 (10.2)	65.9 (2.8)	44.9 (3.8)
C-N	37.2 (7.5)	83.4 (3.9)	64.2 (9.3)	59.7 (8.4)	--
C+N	41.2 (7.0)	74.4 (4.0)	68.7 (10.4)	62.9 (7.7)	--

Table 5.7 Main soluble sugar concentrations (mg g^{-1}) in common alder and Scots pine in September 1995. Values are the means (plus one standard error in brackets) of eight samples for the [CO_2] treatments and four samples for the [CO_2] and nutrient treatments. * = significant [CO_2] effect at $p < 0.05$.

Treatment	glucose	xylose	fructose
common alder			
Ambient	29.5 (2.9)	11.6 (3.4)	45.6 (2.9)
Elevated	47.3 (3.8) *	8.3 (2.7)	56.0 (4.3) *
Control	36.4 (2.9)	5.2 (1.4)	38.5 (3.1)
A-N	24.8 (3.8)	10.6 (3.2)	44.2 (1.6)
A+N	34.1 (2.5)	12.5 (4.5)	47.0 (1.6)
E-N	45.0 (4.4)	11.2 (2.7)	52.1 (4.2)
E+N	49.5 (3.5)	5.4 (2.8)	59.9 (6.6)
C-N	36.9 (3.4)	7.0 (1.5)	37.3 (2.7)
C+N	35.9 (2.7)	3.3 (1.3)	39.7 (3.7)

Table 5.7 continued

Scots pine	inositol	pinitol	glucose	fructose	sucrose
Ambient	19.8 (0.5)	2.9 (5.5)	14.1 (2.7)	17.1 (1.9)	10.8 (3.9)
Elevated	13.6 (0.5)	3.1 (5.9)	21.6 (1.8) *	20.3 (1.9)	10.0 (1.2)
Control	10.8 (7.9)	4.1 (3.3)	17.0 (2.0)	17.3 (2.4)	13.8 (5.9)
A-N	13.4 (0.6)	2.7 (0.50)	9.4 (3.0)	16.4 (1.1)	14.6 (6.8)
A+N	12.7 (0.6)	3.0 (0.63)	18.8 (2.6)	17.7 (2.2)	7.0 (1.4)
E-N	13.5 (0.6)	3.0 (0.73)	20.7 (1.5)	20.6 (1.1)	9.0 (1.6)
E+N	13.7 (0.5)	3.2 (0.40)	22.5 (1.9)	20.0 (2.3)	11.0 (1.0)
C-N	11.0 (0.71)	4.2 (0.23)	19.0 (0.2)	17.5 (3.0)	12.5 (0.63)
C+N	10.6 (1.0)	4.0 (0.59)	17.2 (2.3)	17.2 (2.3)	15.0 (0.52)

Appendix 12

Leaf Rubisco and soluble protein in common alder and Scots pine in the Glendevon experiment

Table 5.10 Mean Rubisco concentration (mg g^{-1} fresh mass) in fully expanded leaves of common alder from April to October 1995. Values are the means of eight samples per [CO_2] treatment and four samples per [CO_2] and nutrient treatment. * = significant [CO_2] effect at $p < 0.05$.

Treatment	April	June	August	September	October
Ambient	22.14 (2.76)	23.21 (5.410	34.41 (5.28)	34.01 (4.39)	43.48 (6.54)
Elevated	31.04 (3.63) *	16.84 (3.52)	34.25 (4.92)	45.51 (10.19)	34.80 (2.54) *
Control	--	--	27.42 (1.30)	51.87 (6.20)	--
A-N	17.07 (3.32)	26.19 (10.53)	50.23 (18.77)	31.37 (7.28)	57.96 (one #)
A+N	25.95 (3.15)	21.22 (7.46)	18.59 (4.97)	36.64 (6.97)	39.87 (7.02)
E-N	26.34 (4.09)	21.25 (3.64)	52.32 (12.56)	38.64 (4.98)	36.18 (3.67)
E+N	35.73 (5.47)	10.23 (3.36)	16.18 (4.33)	52.39 (22.45)	32.15 (2.31)
C-N	--	--	23.83 (1.33)	35.84 (2.68)	--
C+N	--	--	31.01 (4.64)	67.89 (17.81)	--

Table 5.11 Mean Rubisco concentration (mg g^{-1} fresh mass) in young needles of Scots pine from April to October 1995. Values are the means (one standard error in brackets) of eight samples per [CO_2] treatment and four samples per [CO_2] and nutrient treatment. There was no significant [CO_2] effect on Rubisco in Scots pine ($p < 0.05$) at any time in 1995.

Treatment	April	August	September	October
Ambient	4.23 (1.10)	5.02 (0.65)	4.74 (1.22)	4.67 (0.94)
Elevated	3.06 (0.80)	5.27 (0.64)	3.87 (0.68)	3.93 (0.88)
Control	1.14 (0.15)	7.94 (1.41)	5.53 (0.23)	5.53 (0.23)
A-N	6.36 (1.18)	5.62 (0.91)	4.91 (1.81)	4.76 (2.04)
A+N	2.10 (1.14)	4.29 (0.89)	4.57 (1.39)	4.61 (0.88)
E-N	3.37 (1.29)	5.27 (0.82)	3.40 (0.71)	3.96 (1.38)
E+N	2.74 (1.10)	5.27 (1.11)	4.34 (1.21)	3.91 (1.32)
C-N	0.98 (0.29)	7.25 (1.70)	4.12 (0.15)	--
C+N	1.30 (0.09)	8.64 (2.47)	6.94 (0.92)	--

Table 5.12 Mean total soluble protein (mg g^{-1} fresh mass) in young leaves of common alder in 1995. Values are the means (one standard error in brackets) of eight samples for the [CO_2] treatment and four samples for the [CO_2] and nutrient treatment. * = significant [CO_2] effect at $p < 0.05$.

Treatment	April	June	August	September	October
Ambient	17.85 (4.98)	16.10 (4.25)	24.56 (12.93)	42.05 (11.59)	25.08 (8.72)
Elevated	29.00 (5.02)	9.93 (1.36)	23.66 (5.82)	47.89 (6.97)	25.64 (4.99)
Control	--	--	16.66 (3.36)	58.63 (9.37)	--
A-N	14.61 (5.69)	21.33 (3.85)	38.24 (17.93)	44.45 (19.94)	20.58 (9.37)
A+N	21.08 (7.96)	10.86 (7.28)	4.02 (2.36)	38.83 (11.65)	29.58 (10.74)
E-N	30.75 (8.85)	6.60 (0.75)	15.67 (8.02)	38.16 (9.37)	28.59 (5.32)
E+N	27.24 (4.79)	13.26 (3.74)	27.65 (7.62)	60.86 (4.15)	22.70 (6.08)
C-N	--	--	12.26 (2.73)	43.35 (7.40)	--
C+N	--	--	18.53 (8.77)	73.90 (14.12)	--

Table 5.13 Mean total soluble protein (mg g^{-1} fresh mass) in young needles of Scots pine in 1995. Values are the means (one standard error in brackets) of eight samples for the [CO_2] treatment and four samples for the [CO_2] and nutrient treatment. * = significant [CO_2] effect at $p < 0.05$.

Treatment	April	August	September	October
Ambient	2.69 (6.18)	17.27 (4.45)	7.63 (4.99)	3.36 (0.98)
Elevated	24.63 (13.92) *	2.91 (8.34)	4.89 (2.88)	1.79 (0.32)
Control	0.53 (4.08)	20.34 (6.77)	13.44 (3.12)	--
A-N	3.78 (7.45)	10.37 (1.86)	10.08 (2.49)	2.11 (1.35)
A+N	1.60 (2.25)	13.80 (6.10)	5.17 (9.12)	4.60 (0.91)
E-N	9.47 (8.51)	0.59 (10.40)	4.80 (2.97)	0.08 (0.38)
E+N	39.75 (20.29)	5.23 (7.58)	4.98 (3.34)	3.49 (0.31)
C-N	0.32 (3.71)	14.47 (13.69)	14.86 (6.34)	--
C+N	0.74 (8.89)	26.20 (1.04)	12.01 (2.39)	--