The effect of an elevated atmospheric CO(_2) concentration on secondary metabolism and resource allocation in plantago maritima and armeria maritima

Davey, Matthew Paul

The effect of an elevated atmospheric CO_2 concentration on secondary metabolism and resource allocation in *Plantago maritima* and *Armeria maritima*

Matthew Paul Davey

Submitted to the University of Durham for the degree of Doctor of Philosophy

School of Biological and Biomedical Sciences
University of Durham
2003

Plantago maritima (foreground) and *Armeria maritima* (midground in flower) on a coastal cliff-top in Shetland, UK (Photo. C. Gray)

Candidate's declaration

I declare that the material contained in this thesis has not been previously submitted for a degree in this or any other university. All the work recorded in this thesis is entirely my own, unless otherwise stated.

Statement of Copyright

Davey M. P. (2003) The effect of an elevated atmospheric CO_2 concentration on secondary metabolism and resource allocation in *Plantago maritima* and *Armeria maritima* Ph.D. Thesis. University of Durham, UK.

Abstract

There are relatively few reports concerning carbon and nitrogen partitioning into secondary metabolism in plants grown in elevated atmospheric CO_2. This thesis investigates the effects of changes in atmospheric CO_2 and soil nitrogen availability on compatible solute accumulation and resource allocation in *Plantago maritima* and *Armeria maritima*. It was hypothesised that contrasting metabolic responses to abiotic stress cause a species-specific response in *P. maritima* and *A. maritima* in resource allocation. In response to drought, *P. maritima* accumulates the carbon based compatible solute sorbitol, whilst *A. maritima* accumulates the nitrogen based solutes betaine and proline. Over ten weeks, *A. maritima* was more responsive to changes in C and N availability than *P. maritima*, especially in the amount of C fixed into leaf matter. After elevated CO_2 exposure for one year, the biomass of *P. maritima* increased and of *A. maritima* decreased, compared to the respective plants at ambient CO_2. Compatible solute concentrations were increased by drought at ambient CO_2 but decreased in droughted plants at elevated CO_2. This was hypothesised to be due to lower transpiration rates. Rates of transpiration in *P. maritima* decreased in response to elevated CO_2 but *A. maritima* did not. Tissue water-potential was also lowered in response to elevated CO_2. This thesis also examines resource allocation to other major C and N sinks. In *P. maritima* grown at elevated CO_2 for one year, total soluble phenolic concentrations increased and the concentration of soluble protein decreased. In *A. maritima*, phenolic concentrations decreased and protein concentrations increased. The effect of enhanced CO_2 on the secondary metabolism of *P. maritima* was investigated in greater detail. Six phenolics were identified using metabolite profiling, namely *p*-coumaric acid, ferulic acid, caffeic acid, verbascoside, plantamajoside and luteolin. *P. maritima* exposed to elevated CO_2 had higher concentrations of some of these individual phenolic compounds and histochemical analysis identified increases in the number of lignified vessels and a decrease of lignified vessel-wall thickness.

Acknowledgements

I would like to express my thanks to my supervisors Dr. Bob Baxter, Prof. Robert Edwards and Prof. Trevor Ashenden for discussion, ideas, support and most of all for giving me the freedom to explore my subject throughout the PhD. The studentship was funded by the Natural Environment Research Council (NERC) and the Centre for Ecology and Hydrology (CEH) (NERC-CASE grant award GT04/99/54/TS).

First, I would like to thank people at the University of Durham. Members of the 'old' lab 9; Neil Ellwood, Hannah Drewitt, Ben Turner, Dim Giantzoudis, Dr. Dave Balbi and Prof. Brian Whitton provided help, advice and made the work enjoyable. Members of Lab 2; Dr. Ian Cummins, Dave Bryant, Dr. David Dixon, Dr. Mark Skipsey, Melissa Brazier and Caroline Loutre provided extensive help, advice and patience with biochemical techniques, especially on the HPLC and mass spectrometer. Prof. Brian Huntley, Dr. Judy Allen, Dr. Steve Willis, Trish Ranner, and Jon Bennie gave advice and computer support. Dr. Phil Gates and Christine Richardson helped with microscopy. 'Little' Vicky Wilkins helped with sample collection and road navigation around the Northumberland coast. Eric Henderson, Michael Bone, Gordon Bainbridge, Margaret Snow and members of the teaching staff provided practical help throughout the PhD. Dr. Michael Jones at the Department of Chemistry gave technical help and advice with the mass spectrometry. Mike Hughes and staff at the University botanical gardens looked after the plants. Dan Maltman gave advice on techniques, scientific and climbing. Thanks to Jackie Hay, Ruth Cox, Sue Lewis, Andy Dean, Claire McSorley and John Hamilton, to name but a few, for friendship over the years, also, to Dave Bryant and Kerrie Farrar for friendship and lifts to Bangor. Thanks to other residents at Shincliffe hall, in particular Yuka Tajiri and Ute Dreher for being the most friendly and eccentric people I have ever lived with.

Secondly, I would like to thank people at CEH Bangor. In particular, Dr. Harry Harmens provided extensive advice on plant physiological techniques, especially with the IRGA. Phil Williams looked after my plants. Peter Hadfield maintained the domes and provided crash courses in fixing scientific equipment. Dr. Bridget Emmett gave support and opportunities for me to present my research at CEH and conferences. Thanks to Alwyn Sowerby for friendship, advice and accommodation, and Cathy Shields for sharing the pleasure and pain of working in the solardomes.

Thanks also to staff at Bangor University; Prof. John Farrar and members of his lab for help with the oxygen electrode and carbohydrate analysis, Dr. John Gorham for advice on compatible solute analysis, psychrometry and for the loan of a psychrometer, and Gareth Williams for technical help and advice, particularly on the CO_2 growth cabinets. Thanks to Jane Stott and Alison Johnston for friendship, help and accommodation.

And finally, a huge thanks to staff at the University Hospital, Durham who scooped me off the road one evening and fixed a rather broken leg; to my family for providing great support and encouragement throughout the whole of my PhD and especially to Catherine Gray for interesting discussions, editorial comments and love and support.

This thesis is dedicated to the memory of my best mate, Steve Lawrence.

Table of Contents

List of figures

List of tables

Abbreviations

A	Photosynthetic rate
ANOVA	Analysis of Variance
CEH	Centre for Ecology and Hydrology
CNB	Carbon-Nutrient Balance model
CO_2	Carbon dioxide
C	Carbon
c_i	Calculated intercellular CO_2 concentration
DTT	Dithiothreitol
dwt	Dry weight
E	(Evapo)transpiration
ESI	Electrospray ionisation
FC	Field capacity
Fig.	Figure
fwt	Fresh weight
g	Gravity
g_s	Stomatal conductance
GDB	Growth-Differential Balance model
GLM	General linear model
H	Hydrogen
H_2O	Water
HCl	Hydrochloric acid
HPLC	High performance liquid chromatography
IRGA	Infra Red Gas Exchange
J_{max}	Electron transport-limited rate of regeneration of RuBP
kPa	kiloPascals
LAR	Leaf area ratio
LC	Liquid chromatography
LN	Natural log transformed
LWR	Leaf weight ratio
m/z	mass/charge ratio
m/z^-	mass/charge ratio (negative mode)
Mpa	megapascals
MC	Maritime cliff
MS	Mass Spectrometer

N	Nitrogen
n.d.	no difference
nm	Nanometres
NaCl	Sodium chloride
NAD	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced)
NH_4NO_3	Ammonium Nitrate
NVC	National Vegetation Classification
PAL	Phenylalanine Ammonium Lyase
PAR	Photosynthetically Active Radiation
PCM	Protein Competition Model
PDA	Photodiode array
PHE	Phenylalanine
PTLC	Preparative thin layer chromatography
PVPP	Polyvinylpolypyrrolidone
QACs	Quaternary Ammonium Compounds
R_f	Ratio of distance of solute to the distance of the solvent front
R_t	Retention time
Rubisco	Ribulose 1,5-bisphosphate carboylase/oxygenase
RuBP	Ribulose 1,5-bisphosphate
r:s	Root:shoot ratio
SDH	Sorbitol dehydrogenase
SE	Standard error
SLA	Specific leaf area
SM	Saltmarsh
TLC	Thin layer chromatography
TNC	Total Non-structural Carbohydrates
TOF	Time-of-flight
UK	United Kingdom
UV	Ultra-Violet
V	Volts
$V_{c,max}$	Maximum velocity of carboxylation by Rubisco
WUE	Water Use Efficiency
λ_{max}	Absorbance maximum
Ψ	Plant water potential

Chapter 1: General introduction

The first part of this introduction is a review that outlines key aspects of stress tolerance and secondary metabolism and their relevance to plant resource allocation under environmental change. Firstly, the habitats in which secondary metabolism is important for plant survival, are explored. Secondly, the metabolic components of 'compatible solutes' are explained in terms of drought and sodium chloride (NaCl) tolerance. Thirdly, an overview is given of the physiological and biochemical response of plants to altered resource supply, in particular elevated atmospheric CO_2 concentrations.

The second part of this chapter introduces the two study species that are used to examine compatible solute and phenolic allocation under altered resource availability, and sets out the research aims and outline of the thesis.

1.1 Coastal cliff-top habitats

This section will describe briefly the cliff-top habitat, predominantly those in the British Isles, and the abiotic factors that affect plant community composition and growth in such habitats.

Coastal cliff top habitats are subject to generally dry and saline soils. The low moisture content is a result of shallow sandy soils and strong coastal winds, and salinity is derived from sea spray (Goldsmith 1973; Rodwell 2000). The extent to which these abiotic factors affect the floristic composition is determined by the severity of soil conditions. The flora and habitats of coastal cliff top communities were relatively poorly understood until the late 1960s, when comprehensive descriptions were made of such sites (Goldsmith 1967; Malloch 1971). This and other similar work has now been collated in the British plant communities National Vegetation Classification (NVC) series, Maritime Communities and Vegetation of Open Habitats Edition (Rodwell 2000; Fig. 1.1). Typical maritime species common to UK sea cliff habitats include *Armeria maritima* (Mill.) Willd, *Plantago maritima* (L.), *Beta maritima* (L.), *Crithmum maritimum* (L.), *Festuca rubra* (L.), *Suaeda maritima* (L.) and *Agrostis stolonifera* (L.).

To survive in sea-cliff habitats, seedlings must be able to tolerate dry, shallow and saline soils until long roots have developed (Hepburn 1943). Such an environment provides a unique ecological niche, devoid of non stress-tolerant glycophytes, in which maritime-adapted plants can flourish.

Away from cliff ledges and crevices, cliff tops receive less sea spray, but the soils can still be very shallow and prone to drought. Goldsmith (1967) examined the floristic composition of the sea-cliff communities and found that changes in the soil status away from the cliff edge are accompanied by an alteration in the species composition of the community. Goldsmith found that on the exposed sea cliffs at South Stack, Anglesey, UK, the vegetation at the cliff face consisted of maritime and stress-tolerant flora such as *Armeria maritima*, *Festuca rubra*, *Cochlearia officinalis* and *Plantago maritima*. The former two species were more common on drier situations than the latter two. As the soils became deeper and moister away from the cliff there was a tendency for the species composition to shift towards grasses and associated species such as *Agrostis stolonifera, Plantago major*, *Trifolium repens* and *Hieracium pilosella* rather than the hardier maritime species. This shift in vegetation composition away from the cliff edge was probably due to the inland species being more susceptible to drought and salt. This would cause a reduction in plant competition on the cliff-edge by the inland species, allowing maritime plant species to dominate the dry positions (Goldsmith 1973a,b).

The soil-water content has an effect on the species distribution as described by Goldsmith (1967). The drier south side of the cliff at South Stack, Anglesey, had a higher coverage of *Armeria maritima* than *Plantago maritima*, with the latter being more frequent on the slightly moister north side. *Armeria maritima*, although dominant in the dry situations on a cliff is susceptible to prolonged drought, and studies by Goldsmith (1967) found that the biomass of *A. maritima* was significantly reduced after a drought treatment for one year, compared to well-watered species.

The ability of maritime plants to withstand drought and out-compete other species suggests that maritime plants may have a physiological advantage over inland species in the maritime environment. Goldsmith (1967) concluded that the cause of a select few species being able to survive in such drought and saline conditions was due to the inability of neighbouring glycophytic species to tolerate drought and salinity. He also concluded that these maritime species must posses a cell-sap with a high osmotic potential. He reached this conclusion after finding that *P. maritima* was still able to

withstand saline sea-spray even after a wetting agent was added to its thick waxy cuticle. However, it was not until the late 1970s, when researchers such as Stewart & Lee (1974) and Stewart *et al.* (1979) reported that hyperaccumulation of solutes within the cell cytoplasm in maritime species could act in an osmoregulatory capacity, that the biochemical processes within such species to tolerate such stresses were better understood. The following sections describe such biochemical processes.

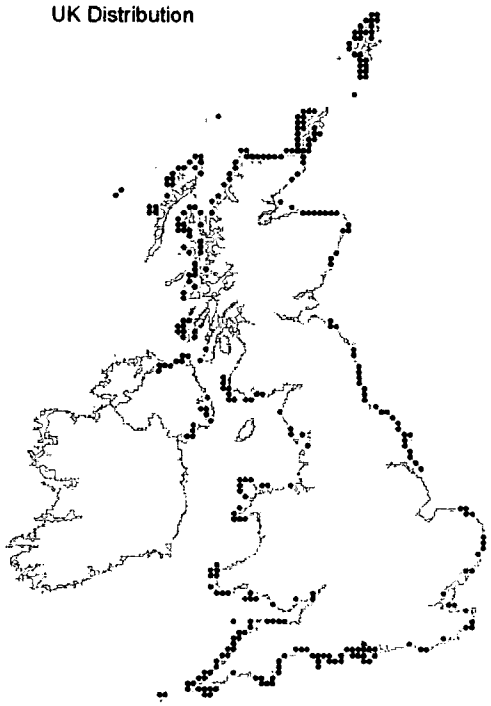


Figure 1.1. Distribution of vegetated cliff tops (dark circles) in the British Isles according to National Vegetation Classification descriptions (NVC). Jackson & McLeod (2002). Map reproduced with kind permission from the Joint Nature Conservation Committee (JNCC), Peterborough, UK.

1.2 Compatible solute metabolism and drought tolerance

This section describes the biochemical mechanisms involved in plant tolerance to osmotic stress and how such responses are relevant to maritime plants

Coastal plants utilise primary and secondary metabolism to survive in habitats where drought and hypersaline soil conditions occur (Ryttari & Lahti 1992). As cell desiccation during drought (such as during plant growth on cliff tops) and an imbalance in the NaCl concentrations of the cytoplasm and hyperaccumulation of NaCl in the vacuole occurs (such as during plant growth in salt marshes), some mechanism must

exist to equalise the water potential of cytoplasm and vacuole and between the exterior and cytoplasm. One mechanism that achieves this equilibrium is an accumulation of organic solutes within the cytoplasm (Stewart *et al.* 1979).

The term 'compatible solute' (also termed osmoprotectants or cytoplasmic osmotica) was introduced by Brown & Simpson (1972) to describe non-inhibitory organic solutes that accumulate in the cytoplasm of cells at low external water potentials. These solutes can accumulate concentrations of up to 2 molar without affecting enzymatic functions within the cytoplasm (Wyn Jones & Gorham 1983). They can therefore play important roles in the adaptation of cells to various adverse environmental conditions. There are three main types of compatible solutes: quaternary ammonium compounds (QACs) (eg. betaines), polyhydric alcohols (e.g. sorbitol) and amino acids (eg. the imino acid proline). Such solutes are produced in the cells of xerophytic and halophytic plants like those found in maritime habitats. In drought situations, such solutes regulate cytoplasmic water activity so preventing cytoplasmic dehydration, protecting macromolecules and maintaining turgor pressure in the face of low external water potentials (Smirnoff & Stewart 1985; Gzik 1996). The concentrations of compatible solutes typically rise during exposure to stresses such as water deficit and salinity. Such solutes are largely confined to the cytoplasm and are almost absent from the vacuole, which generally occupies about 90 % of the cell volume. For example, the halophyte *Atriplex gmelini* was found to have concentrations of 320 mM of glycine betaine in the cytoplasm, but only 0.24 mM in the vacuole (McNeil *et al.* 1999).

Stewart *et al.* (1979) identified four groups of coastal plants that produce compatible solutes (Table 1.1). The first and second groups accumulate only QACs and proline, respectively. The third group accumulates both QACs and proline and the final group contains neither of the above solutes. However, within the final group, *Plantago maritima* accumulates the polyhydric alcohol, sorbitol. The regulation of these three compatible solutes is described below.

Table 1.1. Proline and methylated quaternary ammonium compounds in coastal plants. Table replicated from Stewart *et al.* (1979). Plants were collected from different coastal sites in North Wales and North-West Lancashire, UK. Values are the average of several determinations made over two growing seasons. Analyses were made on shoot or leaf tissue. * Unless otherwise indicated these are glycine betaine. † Major compound in these species is β-alanine-betaine. ‡ Includes glycine betaine and dimethyl propiothetin.

	μmoles g fwt.$^{-1}$	
	Proline	Methylated quaternary ammonium compounds *
Group I. Methylated quaternary ammonium accumulators		
Agropyron junceiforme	< 5	23
Agropyron pungens	< 5	80
Ammophila arenaria	< 5	70
Atriplex hastata	< 5	30
Atriplex patula	< 5	25
Beta maritima	< 5	40
Elymus arenaria	< 5	77
Halimione portulacoides	< 5	50
Salicornia europaea	< 5	45
Salsola kali	< 5	62
Suaeda maritima	< 5	63
Group II. Proline accumulaors		
Cochleria officinalis	35	< 5
Glaux maritima	31	< 5
Puccinella distans	33	< 5
Puccinella maritima	60	< 5
Spergularia marina	26	< 5
Spergularia media	43	< 5
Triglochin maritima	72	< 5
Group III. Proline and Methylated quaternary ammonium accumulators		
Agrostis stolonifera	40	15
Armeria maritima	38	32 †
Aster tripolium	25	29
Festuca rubra	30	19
Limonium vulgare	60	40 †
Spartina anglica	16	80 ‡
Group IV. Coastal plants not accumulating either proline or methylated quaternary ammonium compounds		
Carex arenaria	< 5	< 5
Eryngium maritimim	< 5	< 5
Juncus gerardii	< 5	< 5
Juncus maritimus	< 5	< 5
Plantago coronopus	< 5	< 5
Plantago maritima	< 5	< 5
Scirpus maritimus	< 5	< 5

Proline (imino acid)

The imino acid, proline (Fig. 1.2 A), is one of the most common and rapidly accumulating compatible solutes produced in drought-stressed plants. Although other amino acids increase during drought-stress, the degree of change is not comparable to that of proline (Irigoyen *et al.* 1992; Petrusa & Winicov 1997). The accumulation of proline in drought-stressed plants is caused by the activation of the biosynthesis of proline and by the inactivation of proline degradation (Fig. 1.2 B). L-proline (L-Pro) is synthesized from L-glutamic acid (L-Glu) via two intermediates, glutamic-γ-semialdehyde (GSA) and Δ (1)-pyrroline-5-carboxylate (P5C) and two enzymes, P5C synthetase (P5CS) and P5C reductase (P5CR). The L-Pro is then metabolised to L-Glu via P5C by two enzymes, proline dehydrogenase (oxidase) (ProDH) and P5C dehydrogenase (P5CDH). During drought, the expression of the gene for P5C is enhanced, whilst the gene for ProDH is inhibited. The rate-limiting factors for proline biosynthesis and proline metabolism are P5CS and ProDH respectively (Delauney & Verma 1993; Yoshiba *et al.* 1997). Once the osmotic stress is reduced or removed the oxidation of proline may then provide an important energy source for ADP phosphorylation (Hare & Cress 1997).

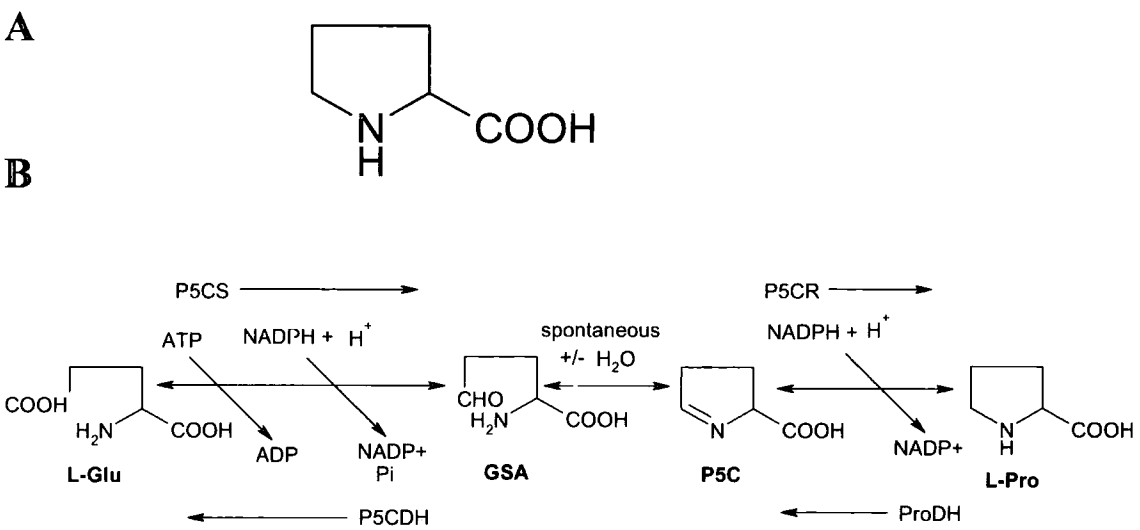


Figure. 1.2. Structure (A) and metabolism (B) of the imino acid, proline. L-glutamic acid (L-Glu); glutamic-γ-semialdehyde (GSA); Δ (1)-pyrroline-5-carboxylate (P5C); L-proline (L-Pro); P5C synthetase (P5CS); P5C reductase (P5CR); proline dehydrogenase (ProDH) and P5C dehydrogenase (P5CDH) (Yoshiba *et al.* 1997).

Betaine (quaternary ammonium compounds)

Betaines are amino acid derivatives in which the N atom is fully methylated; *i.e.*, they are quaternary ammonium compounds. Many coastal plants accumulate glycine betaine (Table 1.1), however, in the family Plumbaginaceae the main QAC is β-alanine betaine (Fig. 1.3 B; Hanson *et al.* 1994). β-alanine betaine is synthesised via a three-step methylation of β-alanine (Fig. 1.3 A). The two intermediate compounds are *N*-Methyl β-alanine and *N*,*N*-dimethyl β-alanine that are formed via *N*-methyltransferase. (Rathinasabapathi *et al.* 2000, 2001).

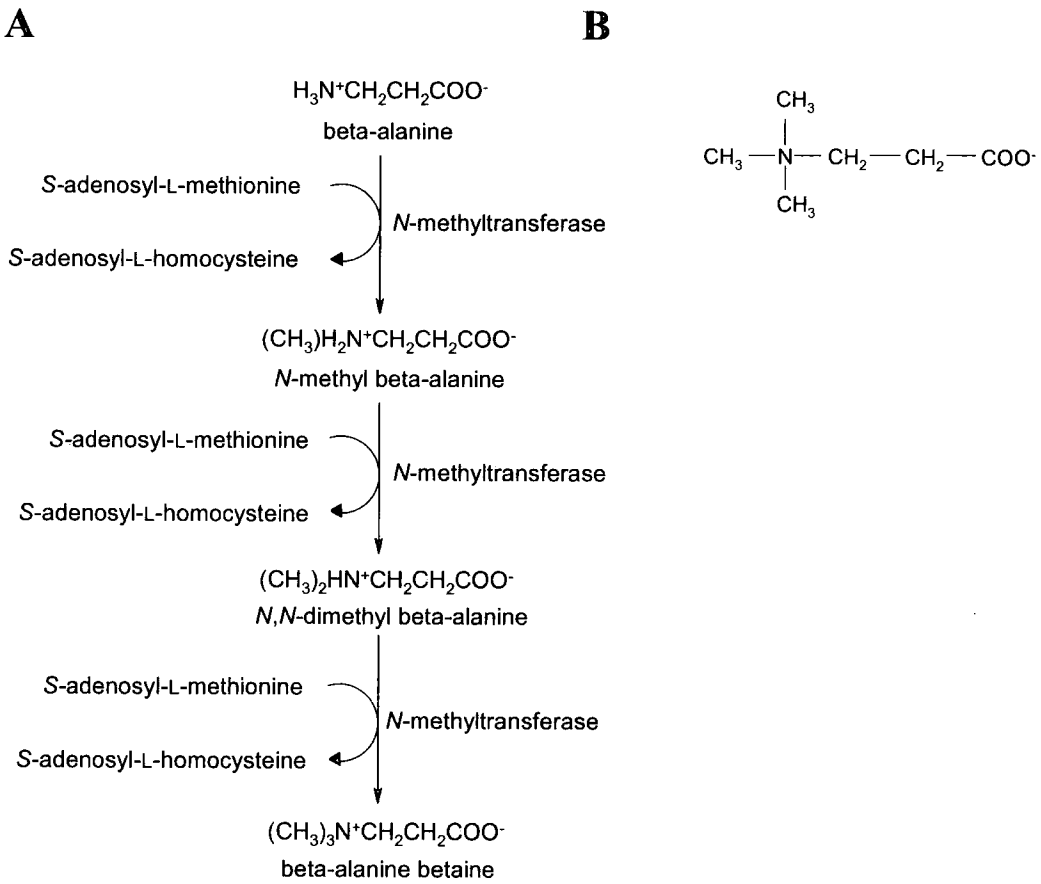


Figure 1.3. Biosynthetic pathway (A) and structure (B) of β-alanine betaine (Rathinasabapathi *et al.* 2000, 2001).

Sorbitol (polyhydric alcohol)

Sorbitol (Fig. 1.4 B) is one of 17 sugar alcohols (polyhydric alcohol), produced in higher plants. It is a major final product of photosynthesis and, together with sucrose, represents the main form of carbon (C) translocated in many species of the Rosaceae and Plantaginaceae families (Gutierrez & Gaudillere 1996). Biosynthesis of sorbitol is confined mainly to source leaves, whereas metabolic utilisation is restricted to sink tissues. Sorbitol biosynthesis is accomplished by initially converting fructose-6-phosphate to glucose-6-phosphate by phosphoglucose isomerase (Fig. 1.4 A). A NADPH-dependent aldose-6-phosphate reductase catalyses the conversion of glucose-6-phosphate to sorbitol-6-phosphate. This is then converted to sorbitol by sorbitol-6-phosphate phosphatase (Gutierrez & Gaudillere 1996).

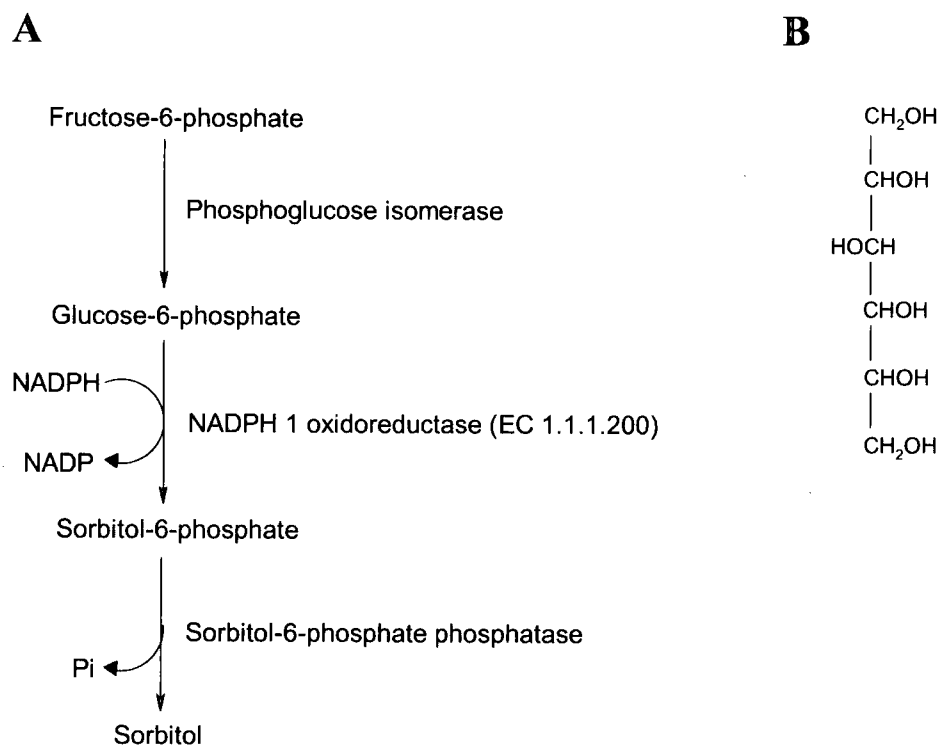


Figure 1.4. Biosynthetic pathway (A) and structure (B) of sorbitol (Gutierrez & Gaudillere 1996; Harborne 1997).

1.3 Compatible solute production and resource availability

To date, no study has comprehensively investigated the physiological and biochemical response of coastal cliff-top plants to drought. However, there has been considerable research on compatible solute accumulation and partitioning on the salt-marsh eco-types of certain coastal plants (Table 1.1; Stewart *et al.* 1979). Although the focus of this thesis is on the cliff-top ecotype, the physiological and biochemical response to NaCl also provides a valuable insight into possible drought response of these cliff-top maritime species. This section outlines how compatible solutes and plant growth responds to NaCl and drought stress, particularly in coastal plants, and how changes in resource availability can affect such a response.

The two maritime plants *Armeria maritima* and *Plantago maritima* were chosen as model species to test hypotheses set out in this thesis because they have contrasting responses to osmotic stress (Table 1.1). The structure, ecology and distribution of these species are described in section 1.5. *Armeria maritima* accumulates both of the nitrogenous compatible solutes, proline and betaine, while *P. maritima* accumulates a C-based compatible solute, sorbitol. For example, in *P. maritima*, sorbitol concentrations increased eight-fold in the shoot and 100-fold in the root tissue in response to an increase (0 to 400 mM) in the external NaCl concentration (Ahmed *et al.* 1979). In *A. maritima*, the nitrogenous compatible solutes were found to almost double in concentration when the soil salinity was increased from 40 to 200 mol m^{-3} (Köhl 1997). Köhl (1996) also studied traits of drought resistance in *A. maritima* where the accumulation of proline and betaine increased during water stress, implying that this is a general stress response in this species. There has been no specific study on the overall biochemical drought responses in the cliff-top variety of *P. maritima*, though a study by Walsh (2000) identified high concentrations of sorbitol in such plants. The diversion of C and nitrogen (N) to these compatible solutes to maintain cellular water potentials could be of an order of magnitude that would compete with the allocation for growth (Jefferies *et al.* 1979; Yeo 1983). For example, nitrogenous compatible solutes can account for over 20 % of total plant N and sorbitol can account for up to 50 % of the C allocated to non-structural carbohydrates, which relates to about 16 % of the total C pool (Stewart & Rhodes 1978; Poorter *et al.* 1997; Escobar-Gutierrez *et al.* 1998; Walsh 2000).

An example of how growth is affected by osmotic stress is presented in a study by Sheehy-Skeffington & Jeffrey (1988). They carried out a series of experiments on *P. maritima* and *A. maritima*, altering external NaCl and N concentrations. Plants grown at high salinity attained lower biomass, than plants grown at low salinity. This response was exacerbated at low N availability, particularly in *A. maritima*. In a high NaCl growth medium, the biomass of *A. maritima* was lower than plants grown at low NaCl, even when N availability was high. This implies that as a large proportion of N is allocated to osmoregulation, greatly affecting the biomass of *A. maritima*. *Plantago maritima* maintained a steady increase in shoot size even in at high NaCl stress. This could be partly due to the non-nitrogenous sorbitol accumulation in *P. maritima* with increasing salinity.

The fact that the biochemical response to osmotic stress can be altered by external resource supply gives rise to many interesting questions concerning the allocation of resources to growth and the survival mechanisms that involve C and N metabolism. For example, as well as changes in N and H_2O, C is also a limiting resource in the growth and survival of C_3 plants. An alteration of C supply in the form of atmospheric CO_2 can alter significantly C and N allocation and growth (Bazzaz 1990; Poorter *et al.* 1997). Therefore, this leads to the important question of how the allocation of C and N to compatible solutes will be affected by increased atmospheric CO_2 and whether such a response will alter other plant processes such as photosynthesis and growth. This will be discussed in the following section.

Plant responses to elevated atmospheric CO_2

Atmospheric CO_2 concentrations are predicted to rise from 360 to between 550-1000 μmol CO_2 mol^{-1} over the next 100 years (IPCC 2001). There is still much debate about the impact of elevated CO_2 on plant productivity and the functioning of ecosystems. It is also argued that the long-term ecosystem responses to elevated CO_2 may ultimately depend on N availability to plants and on the ability of plants to use N more efficiently under conditions of elevated CO_2 (Cotrufo *et al.* 1998). One way to predict C and N partitioning is by using models. The two main 'source-balance' models for predicting the allocation of C in plants exposed to elevated CO_2 are the carbon-nutrient balance model (CBM) (Bryant *et al.* 1983) and the growth-differentiation balance (GDB) model

(Herms & Mattson 1992). These source-balance models are summarised in Figure 1.5 (Peñuelas & Estiarte 1998). The basic prediction made in these models is that plants exposed to elevated CO_2 will acquire more C though increased photosynthesis. Therefore, a greater proportion of C is allocated to non-structural carbohydrates and secondary metabolism. This allocation of C to these processes in plants grown under elevated CO_2 is usually enhanced when another factor, such as N availability, is limited (Baxter *et al.* 1995). Biomass and N-based compounds are expected to be lower in relation to the C-based compounds. Nitrogen within the plant can be re-allocated from source growth to relieve constraints on sink growth (Stitt & Krapp 1999). These models work on the assumption that plant sinks are occasionally incapable of utilising carbohydrates at the rate at which it is being produced (*i.e.* photosynthetic production exceeds export from the leaf). If the plant, as a whole, cannot continue to import the carbohydrate indefinitely, it will accumulate in source leaves (Farrar 1999) and this accumulation of carbohydrate usually occurs in plants growing at elevated atmospheric CO_2. Therefore, these models predict that the concentration of C-based compounds, such as sorbitol would increase and that N-based compounds such as proline and betaine would decrease in plants exposed to elevated CO_2, especially when N availability is low, compared to plants grown at ambient CO_2. Changes in the assimilation and partitioning of compatible solutes could seriously affect plant growth, photosynthesis and water relations of these coastal plants exposed to elevated CO_2 (Wullschleger *et al.* 2002). To date there has been no detailed study on the effects of elevated CO_2 on compatible solute accumulation. However, in apple (*Malus domestica*) leaves, a four-fold increase in atmospheric CO_2 increased sorbitol by 50 % in leaves, this provides some evidence that elevated CO_2 can affect concentrations of compatible solutes (Wang *et al.* 1999).

Changes in C and N allocated to compatible solutes may affect growth as discussed above, but it is still unknown how the allocation of resources to such compounds will affect other major C-based plant compounds, for example soluble phenolics. Phenolic compounds are an important component of secondary metabolism (Dixon & Paiva 1995), with much of the literature reporting increases in the concentration of phenolic compounds (about 14 %) in plants exposed to elevated CO_2 (Peñuelas *et al.* 1997). Therefore, carbon-based phenolic compounds can potentially provide a valuable C sink within the plant. This could have significant consequences for ecosystem functioning

caused by altered decomposition rates and plant-herbivore interactions, as reduced digestibility, growth rates and body size and increased consumption rates, development times and mortality have all been observed in some herbivores feeding on plants grown at elevated CO_2 (Coviella & Trumble 1999; Peñuelas 2002). As maritime plants have different metabolic strategies to withstand osmotic stress, i.e. accumulating large pools of either C- or N-based compatible solutes, it is unclear how other C and N sinks are affected in these species.

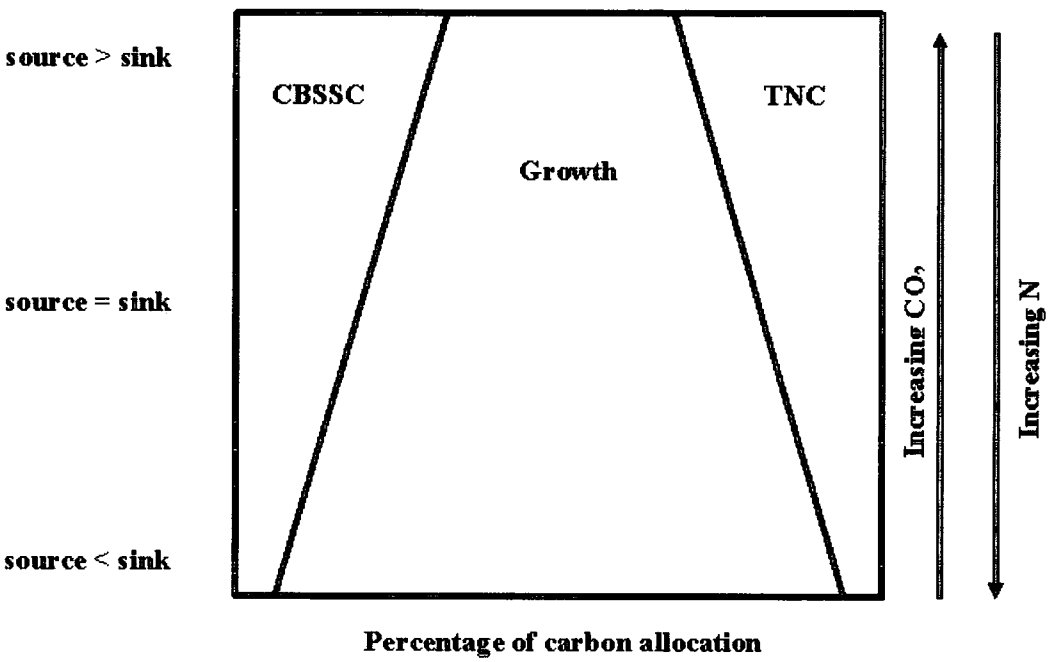


Figure 1.5. The carbon–nutrient balance hypothesis and the growth–differentiation balance hypothesis predict that changes in source–sink relationships are accompanied by variations in the relative partitioning of C to growth, Total Non-structural Carbohydrates (TNC) and C-based secondary or structural compounds (CBSSC). Shifts in this relative partitioning due to increased CO_2 and decreased N availability are summarized in the diagram. The *y*-axis represents a gradient of C source–sink relationships while the *x*-axis represents the percentage allocation of C to growth, TNC and CBSSC. When C source is higher than C sink, TNC and CBSSC would be relatively over-invested. CO_2 has greater positive impact on the strength of the C source whereas nutrient stress has greater negative impact on the strength of the C sink, but both are expected to increase plant tissue concentrations of CBSSC (diagram and text taken from Peñuelas & Estiarte 1998).

1.4 Study species: *Plantago maritima* and *Armeria maritima*

Plantago maritima L. (Sea plantain) is a member of the Plantain family, Plantaginaceae. Plantains are described as perennial herbaceous land plants with hairless, fleshy, 3-5 veined leaves in basal rosettes; narrow linear inflorescences in 2 – 6 cm spikes on long leafless stalks (Fig. 1.6; Ross-Craig 1968; Rose 1981). The morphology of *P. maritima* can alter according to the environmental conditions it is growing in, so indicating high phenotypic plasticity within the species (Gregor 1946). For example, *Plantago maritima* growing on coastal cliff-tops are smaller and flatter in structure than populations on near-by grassy slopes (Gregor 1930).
Populations of *P. maritima* within the British Isles have been recorded on coastlines, usually cliffs and salt-marshes and inland populations are usually located on rocky outcrops in mountainous areas.

Armeria maritima (Mill.) Willd. (Thrift) is a member of the sea lavender family, the Plumbaginaceae. These are described by Rose (1981) and Woodell & Dale (1993) as being perennial herbs with a woody root stock, alternate and simple, 1 – 3 veined leaves (10-150 mm x 0.4 – 2.5 mm) in basal rosettes only; inflorescences are dense with a round head (Fig. 1.7). The basal rosettes can grow individually or many rosettes from the same taproot can grow close together in large numbers (sometimes several hundred) to form a single cushion. The taproot branches can reach 1.5 m in length and can have many small divaricate roots in the upper 20-30 cm of soil. Individual plants can live for as long as 30 years and can grow actively throughout the year. They can grow actively throughout the year but do not always flower each year and they can take up to 6 years to reach full adult rosette size (Woodell & Dale 1993).

Tolerance to drought and salinity enables both plants to be found on sea cliffs and most salt marshes of the British Isles and mountains in North Wales, North England and Scotland (Fig. 1.8A & B). The two species have also colonised inland road verges, especially in Scotland, which have been salt-treated during winter months (Preston *et al.* 2002). Both species are described under the British National Vegetation Classification (NVC) as occurring on maritime cliff communities, predominantly *Festuca rubra – Armeria maritima* maritime grassland (MC8 NVC) and *Festuca rubra – Holcus lanatus* maritime grassland (MC9 NVC). They also occur in salt marsh communities ranging

from transitional low-marsh vegetation (SM10 NVC) to closed and species-poor salt marsh (SM13 NVC) (Rodwell 2000). Under *Armeria maritima – Cerastium diffusum* ssp. *diffusum* maritime therophyte communities and MC5 communities the soil is very shallow and well-drained in the rocky crevices in which the plants live. Under MC8 communities, the habitat is usually on steep to moderate slopes about 50 metres above sea level. The soils are usually brown rankers, rocky and with a neutral pH. The soil drainage can become excessive and plants can experience a soil-water deficit. Where shallow dry soils are eroded, especially on cliff edges, there is a tendency for *Armeria*-dominated communities to exist since both *A. maritima* and *P. maritima*, can maintain their position by extending tap roots deep along the soil and crevices. MC9 maritime grasslands are more sheltered and distant from the cliff edge, they have deeper soils and are moist but always free draining.

A classic example of a cliff-top habitat that exhibits dry, shallow soils is the Northumberland coast, UK (UK Ordnance survey reference: NU 264 174). It was at this site where plants were obtained for the experimental work described in this thesis. Figure 1.9A shows a classic example of the dry, compact soil from this site where the first 5 cm of the soil had on average 17 % water content (July 2001). However, the gravimetric soil water content rapidly drops to less than 2 % below a soil depth of 5 cm. The dominance of *Plantago maritima* and *Armeria maritima* in this habitat is highlighted in Fig. 1.9B.

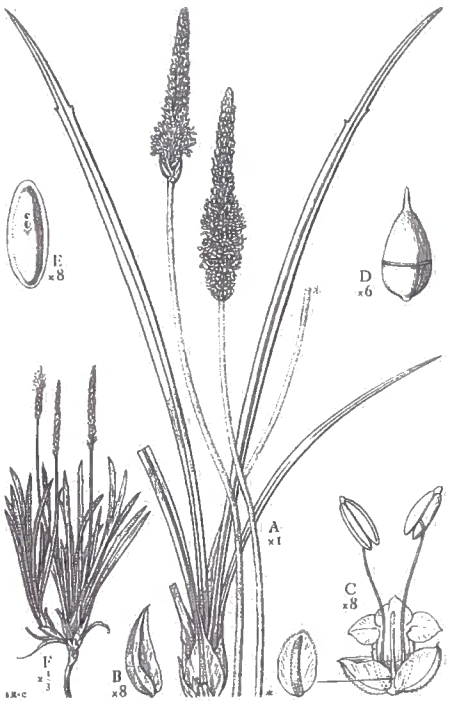


Figure 1.6. Drawing of *Plantago maritima* L.. A, part of a plant; B, floral bract; C, flower – part of a corolla and two stamens cut away and one sepal detached; D, capsule; E, seed; F, plant. (Ross-Craig 1968).

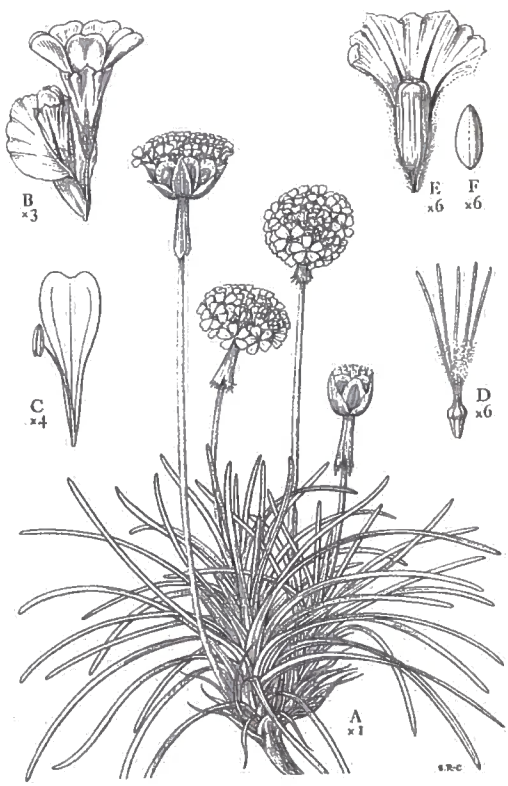


Figure 1.7. Drawing of *Armeria maritima* (Mill.) Willd. A, plant; B, floral bract and two flowers; C, petal and stamen; D, gynoecium; E, fruit – part of calyx cut away; F, seed. (Ross-Craig 1964).

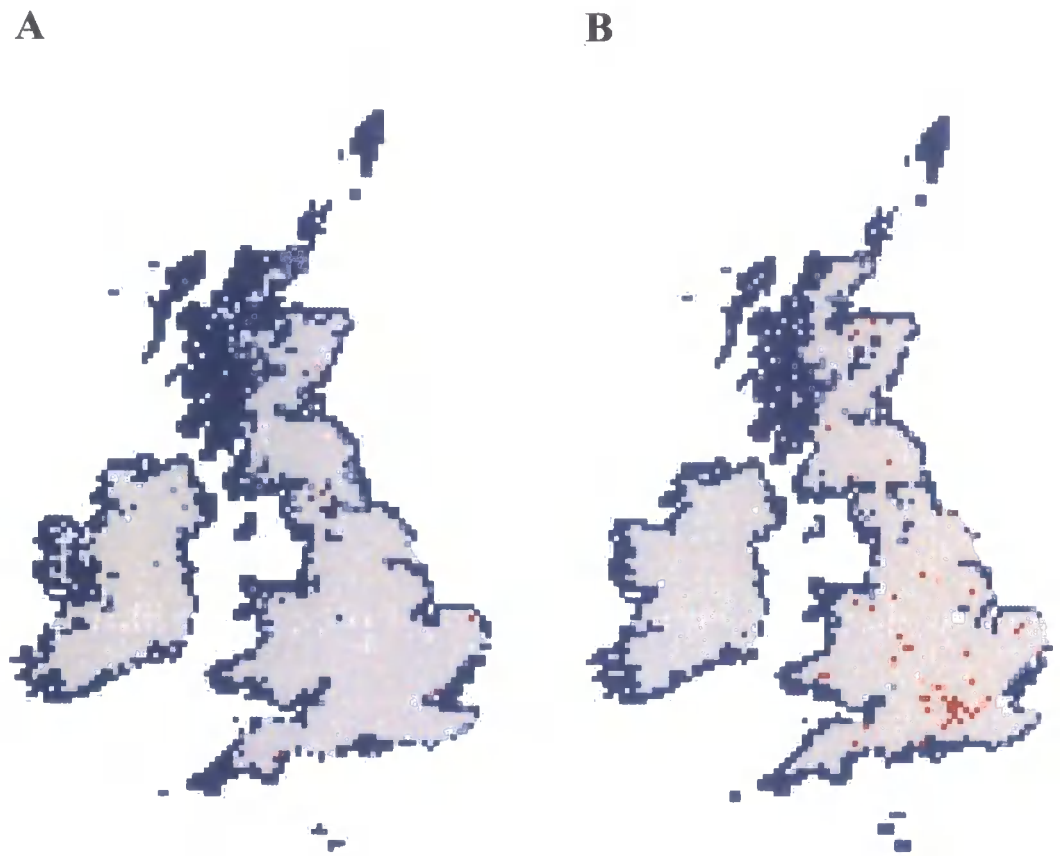


Figure 1.8. The distribution of (A) *Plantago maritima* and (B) *Armeria maritima* in Great Britain and Ireland. Dark blue spots are native populations and red spots are alien populations that are established continental or horticultural varieties (Preston *et al.* 2002).

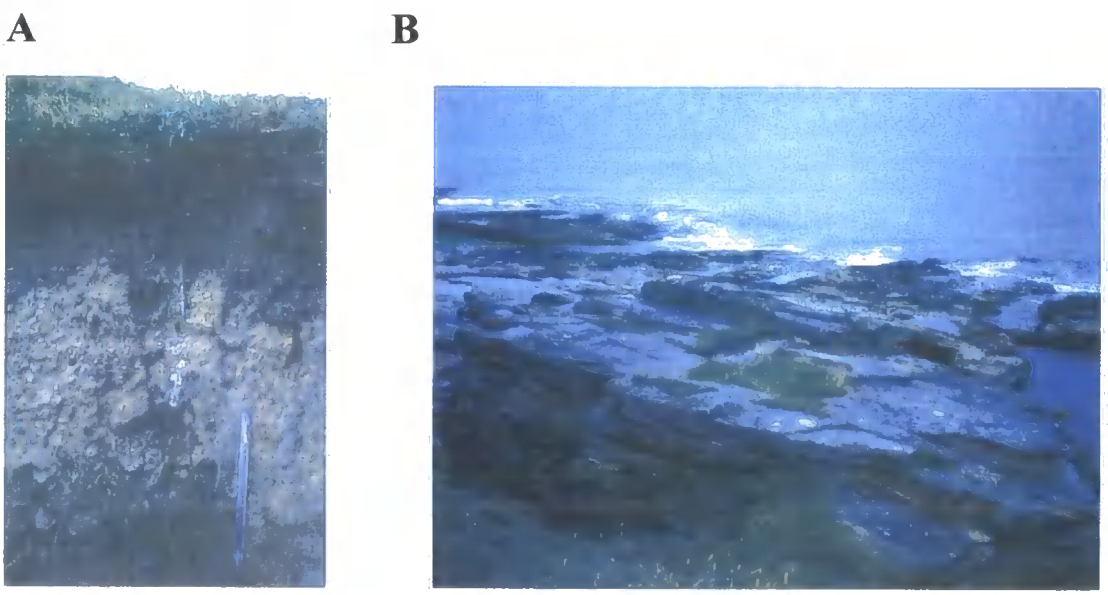


Figure 1.9. (A), Soil profile of coastal cliff-top in Northumberland, UK during July 2001. Note pen for scale next to a sample of *Plantago maritima*. (B), Cliff-top outcrop showing a single patch of vegetation consisting mainly of *Plantago maritima* and *Armeria maritima*.

1.5 Research aims and outline of thesis

The principle aim of this thesis is to reach a better understanding of how cliff-top ecotypes of *Plantago maritima* and *Armeria maritima* respond to changes in resource availability, in particular to atmospheric CO_2. It was hypothesised that contrasting metabolic responses to abiotic stress cause a species-specific response in *P. maritima* and *A. maritima* in resource allocation. A comprehensive introduction and discussion, together with more specific hypotheses, are provided within each results chapter. The thesis concludes with a general discussion in chapter 8.

Chapters 3 and 4 examine how the growth, photosynthesis and compatible solute status of the study species are affected by drought and whether these responses are modified by elevated CO_2 and reduced N availability. The mechanisms involved in the regulation of compatible solute synthesis are still unclear, particularly in plants grown under an elevated atmospheric CO_2 concentration (Wullschleger 2002) as outlined in this introduction. Therefore, chapter 5 explores how photosynthesis, transpiration and stomatal conductance may affect changes in the concentration of compatible solutes, particularly in plants exposed to a combination of drought and elevated CO_2. The effect on cellular water potentials are also explored in chapter 5.

Chapters 6 and 7 explore how changes in resource allocation within the study species affects other major sinks of C and N. Chapter 6 tests a literature-based model by using the level of photosynthetic acclimation and growth response to elevated CO_2 to predict how C and N is allocated to phenolics and proteins. A comprehensive study on the identification and fluctuation of individual phenolic metabolites and histochemical analysis of *P. maritima* is presented in chapter 7.

Chapter 2: General materials and methods

2.1 Plant and soil material

Whole plants of *Plantago maritima* and *Armeria maritima* were obtained from the coastal cliff-top area of Howick, Northumberland, UK (UK Ordnance Survey reference: NU 264 174). To minimise genetic variation, whole cushions of *A. maritima* and clumps of *P. maritima* were collected within close proximity to each other, roughly within 10 metres, where both species co-existed in large populations. Plants were placed in plastic bags and immediately transported to the School of Biological and Biomedical Sciences, University of Durham where clonal shoots were cut from the samples and transferred into seed trays (section 2.3.1).

Soil samples were taken (at 0.5 and 5-10 cm depths) from the Howick site, air dried for 48 hours and sieved through a 2 mm wire sieve. Sieved soil was added to distilled water (1:2 w/v) and the pH of the solution was measured after ten minutes using a calibrated pH probe (WTW pH 196 and SenTix electrode 41-3). Soil was dried at 110° C for at least 48 hours prior to dry weight determination.

2.2 Experimental growth conditions

Experiments were carried out in one of three growth units. These were; 1, Controlled environment growth room, School of Biological and Biomedical Sciences, University of Durham; 2, CEH-Bangor plant growth cabinets and 3, CEH-Bangor Solardomes:

2.2.1 Growth room facility at the University of Durham

Potted plants were grown under a light regime of 16 hours light and 8 hours dark provided by a rack of 12 fluorescent strip lamps (Sylvania standard F65W154-RS Daylight, Germany) (Photosynthetically Active Radiation (PAR) 150 $\mu mol\ m^{-2}\ s^{-1}$ at the leaf surface) and room temperature was regulated between 20-22 °C.

2.2.2 CEH-Bangor plant growth cabinets

Potted plants were placed within one of two growth cabinets (Sanyo Gallenkamp, Meridian Business park, Leicestershire, UK) at the CEH-Bangor Abergwyngregyn field station (Fig. 2.1) (UK Ordnance Survey reference: SH 653 733). A site description is provided in appendix 3.2. The plants were exposed to day/night temperature regimes of 20 °C/ 15°C and a 14 hours light (PAR 550 μmol m^{-2} s^{-1} at leaf surface) to 10 hours dark photoperiod was provided by two 60 watt incandescent (Osram tungsten, B&Q stores, Bangor, UK) and three metal halide bulbs (Osram powerstar HQI-TS 250W/NDL UVS, Germany) (Flanagan & Jefferies 1989 a, b, Fajer *et al.* 1992). Reduced light intensities to simulate sunrise and sunset were achieved by having only incandescent bulbs on for the first and last 30 minutes of the light period. Relative humidity was set at 60 % (0.93 kiloPascals, kPa). Cabinets were set to contain either an atmosphere of ambient CO_2 (360 μmol CO_2 mol^{-1}) or elevated CO_2 (600 μmol CO_2 mol^{-1}) concentrations. The elevated CO_2 concentration within the cabinets was achieved by pumping pure CO_2 gas (BOC Gases Ltd. Worsley, UK) into the cabinets under the control of an infrared gas analyser (IRGA, PP-systems WMA-2) and a Eurotherm controller unit (PP systems, Glovers Court, Hitchin, Hertfordshire, UK). CO_2 concentrations within the cabinets were checked at least five times a week with an ADC 225-Mk3 IRGA (ADC Bioscientific Ltd., Hoddesdon, Hertforshire, UK) and the CO_2 concentration recorded by a pen chart-recorder. Both Infra Red Gas Analysers (IRGAs) were calibrated at 700 μmol mol^{-1} CO_2 using CO_2 span gas (Cryoservice, Worcester, UK). Twice each week, plants and CO_2 concentrations were rotated between growth cabinets.

2.2.3 Solardome facility at CEH-Bangor

Potted plants were equally divided among four Solardomes (Solardome Industries Ltd., Southampton, Hampshire, UK) within which the atmospheres were regulated to either ambient (360 μmol mol^{-1}) in two replicate Solardomes or elevated (600 μmol mol^{-1}) CO_2 in two other replicate Solardomes (Fig. 2.2). The domes are 4.4 metres in diameter and constructed of an aluminium framework and glazed with 3-4 mm thick Sanalux® glass (Schott Glass Ltd., Staffordshire), which allows light in the ultra-violet (UV) range to pass through the glass. Dome temperature and relative humidity were monitored within the domes and Solardome temperatures were regulated by heat exchangers outside the domes to ensure that they remained the same as outside ambient

air temperatures. Elevated atmospheric CO_2 concentrations within selected Solardomes were achieved by adding CO_2 from a six ton bulk CO_2 supply tank, via an electrical vapouriser, directly into the Solardome air intake system. This was regulated by a mass-flow controller, which analysed CO_2 concentrations within and outside the Solardomes, to allow a constant elevated atmospheric CO_2 concentration (Rafarel *et al.* 1995; Stirling *et al.* 1997). Plants were watered each day at 18.00 hours, for 10 minutes, by an automated sprinkler system within each Solardome.

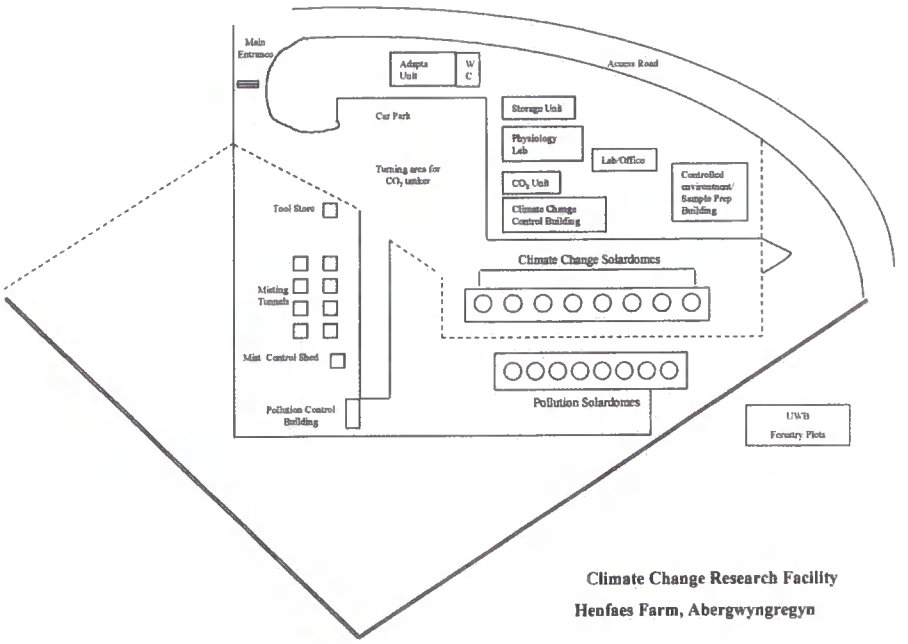


Figure 2.1. Overview of the climate change research facility at CEH-Bangor, UK.

Figure 2.2. Solardome facility at CEH-Bangor, UK.

2.3 General growth conditions

2.3.1 Plant cultivation

Plants collected from the field site were split into clonal shoots and transferred into seed trays (36 x 22 cm, 4 l) containing acid-washed silica sand (Silvaperl Silver Sand, Horticultural grade lime-free silica sand, William Sinclair Horticulture Ltd., Lincoln, UK) either at the University of Durham Botanical Gardens glasshouse facility or the CEH-Bangor Abergwyngregyn field station. True clonal replication for each experiment was not assured as clonal shoots from up to five cushions or clumps were used for each individual experiment. Plants were watered daily with distilled water and with 100 ml of quarter strength Long-Ashton solution containing 2.8 mg l^{-1} N-ammonium nitrate (NH_4NO_3) (Table 2.1) or commercial fertiliser (J. Arthur Bower's liquid fertiliser N-P-K; 5-5-10 William Sinclair Horticulture Ltd., Lincoln, UK) once per week.

Once established (new shoot and root growth), similar-sized plants were placed individually in either 9 cm diameter (250 cm^3) pots filled with 600 g of dry silica sand; 9 cm diameter (200 cm^3) pots filled with 370 g of dry silica sand or 13 cm diameter (500 cm^3) deep pots filled with 800 g of dry silica sand, depending on experimental requirements, and watered to saturation point. As both species are slow-growing, root growth was not restricted by pot size so minimising any likelihood of pot-effects on the experiments.

2.3.2 Manipulation of nitrogen availability

The range of N available to the plants was based upon Sheehy-Skeffington & Jeffrey (1985) and Sheehy-Skeffington & Jeffrey (1988). For N manipulation experiments, the quarter-strength Long Ashton solution was made up with either 2 ml or 10 ml ammonium nitrate (NH_4NO_3) stock solution (Table 2.1) to produce a 2.8 mg l^{-1} N or 28 mg l^{-1} N solution, respectively. The pH of the solution was pH 5.5. Soil pH of the study site (section 2.1) varied from pH 5.1 to pH 6.5.

Table 2.1. Long Ashton nutrient solution (one-quarter strength) modified to give a final concentration of 28 or 2.8 mg l^{-1} N-NH_4NO_3. Modified from Hewitt (1966).

Salt	Stock solution (g/l)	Volume of stock (ml) for 10 l of quarter strength Long Ashton solution
NH_4NO_3 Ammonium nitrate (for 28 mg l^{-1} N treatment)	224	10
NH_4NO_3 Ammonium nitrate (for 2.8 mg l^{-1} N treatment)	224	2
$NaH_2PO_4 . 2H_2O$ sodium di-hydrogen orthophosphate	208	2.5
Fe EDTA Ethylenediaminetetra-acetic acid ferric monosodium salt	37.3	2.5
$MgSO_4 . 7H_20$ Magnesium sulphate 7-hydrate	184	2.5
K_2SO_4 Potassium sulphate	87	2.5
$CaCl_2 . 6H_2O$ Calcium chloride 6-hydrate	82	10
Micronutrient complex		
$MnSO_4 . 4H_20$ Manganous sulphate 4-hydrate	22.3	0.2
$CuSO_4 . 5H_2O$ Copper (II) sulphate 5-Hydrate	2.5	
$ZnSO_4 . 7H_2O$ Zinc sulphate 7-hydrate	2.9	
H_3BO_3 Boric acid	31	
NaCl Sodium chloride	58.5	
$Na_2MoO_4 . 2H_2O$ Sodium molybdate 2-hydrate	1.2	

2.3.3 Manipulation of water availability

The availability of water to the plant was manipulated by altering the amount of water held within the sand-filled pot. Pots were watered to saturation point which was termed 100 % field capacity (FC), defined by the weight of the sand (including pot and retainer cloth weight) 48 hours after the sand was saturated with water (Hillel 1971). Once the weight of 100 % FC was known, other levels of watering can be maintained as a percent of field capacity, by weighing regularly whole pots on a balance and watering them to weights calculated to be 5 % over the desired percent of FC. Weighing 5 % over the desired percent of FC and allowing the sand within the pots to dry to a weight 5 % below the desired percent of FC meant that a mean percent of FC was effectively maintained over the duration of the experiment (Köhl 1996). The true term 'field capacity' equates to 100 %. However, although the term 'percent of field capacity' should be used for watering regimes below 100 % FC, the use of 'FC' will be used in this thesis for conciseness, eg 30 % FC instead of 30 % of FC. The weight of the desired FC was adjusted to take into account increases in plant biomass over time. This was achieved by adding the increase in fresh weight of the plant (as measured after a destructive harvest), if the increase in weight was over 1 g, of the original weight of the whole pot. The percent water content of the sand was determined by weighing a sample of the wet sand and reweighing after drying in an oven (120 °C). Due to automatic spraying of water inside the Solardomes, large wire frames partially covered with transparent polythene were placed over the plants during the night. This successfully avoided automated watering during experiments with drought treatments. Humidity was not considered a problem for drought treatments within the domes as watering was carried out in the evenings and any excess moisture in the atmosphere would have been quickly replaced due to the high turnover of air entering and leaving the Solardomes.

2.4 Gas exchange measurements

2.4.1 Photosynthetic capacity and rate of shoot tissue

Photosynthetic capacity was determined by placing 50 - 200 mg of young fully expanded leaves of each species into a LD2/2 gas phase oxygen electrode (Hansatech Instruments Ltd. Kings Lynn, Norfolk, UK). Before leaves were placed within the chamber, leaves were weighted and the leaf area was determined by using a Delta-T

Scan leaf area imager and software (Delta-T devices Ltd., Cambridge, UK). The chamber was maintained at 20 °C by a thermostat-controlled water circulator and all measurements were taken in a temperature-controlled laboratory (at 20°C) at the CEH-Bangor Abergwyngregyn field station. Light intensity was set at 417 μmol m^{-2} s^{-1} PAR and a 5 % CO_2 atmosphere within the chamber was achieved by the addition of five drops of saturated sodium bicarbonate solution (Walker 1993). The electrical output of the O_2 electrode was first set to zero by flushing the chamber with pure N_2 gas. The electrical output is produced when oxygen diffuses through a membrane within the chamber and is reduced at a platinum cathode surface so that a current flows, via a potassium chloride, to a silver anode. The current generated bears a direct relationship to the amount of oxygen reduced. The electrical output was calibrated by injecting 1 ml of air into the chamber and determining the increase of the electrical output from the O_2 electrode (Walker 1993). The rate of leaf O_2 evolution was measured from the change of electrical output from the O_2 electrode over a period of 5 minutes. At the conclusion of the study, leaves were immediately frozen in liquid N_2 and stored at -40 °C until they were analysed for photosynthetic pigment content.

Photosynthetic rate (A; μmol CO_2 m^{-2} s^{-1}); stomatal conductance (g_s mmol H_2O m^{-2} s^{-1}); calculated intercellular CO_2 concentration of the sub-stomatal cavity (c_i); μmol mol^{-1} CO_2 (von Caemmerer & Farquhar 1981) and evapotransporation rate (E) mmol H_2O m^{-2} s^{-1} were determined by either a CIRAS-1 v1.2 IRGA attached to a Parkinson PLC-5 (Broad) automatic leaf cuvette (PP systems, Glovers Court, Hitchin, Hertfordshire, UK; Parkinson *et al.* 1980) or an ADC IRGA (LCA4) attached to a broad leaf cuvette (ADC PLC4/B software v. 1.02) (ADC Bioscientific Ltd., Hoddesdon, Hertforshire, UK). Before intact leaves were placed within the leaf chamber the length and width of the leaves were measured with a pair of callipers and the total leaf area for analyses was calculated and programmed into the IRGA control unit. This method of leaf area determination was suitable as only the straight-edged mid-sections of the leaves were placed inside the chamber thus ensuring a uniform rectangular leaf shape.

Measurements using the CIRAS-1 were taken at a photosynthetic photon flux density (PPFD) of 1150 μmol m^{-2} s^{-1} provided by a Halogen lamp. This light intensity was used as it was the maximum rate of photosynthesis at light saturation, as determined by light response (AQ) curves produced from both species. The cuvette relative humidity was 65-70 % with the cuvette temperature set at 20 °C and an internal air flow rate of

200 ml min^{-1}. The system was calibrated for H_2O measurements every week with ferrous sulphate (PP systems, Hitchin, Hertfordshire, UK) and CO_2 span gas at 700 μmol mol^{-1} (Cryoservice, Worcester, UK) respectively. CO_2 concentration within the leaf cuvette was regulated by a CO_2 source within the CIRAS-1 unit.
Measurements using the ADC were taken at a photosynthetic photon flux density (PPFD) of 1150 μmol m^{-1} s^{-1} as provided by a Halogen lamp (Osram 12V 20W Dichroic 'white light', Germany) with a 0.2 iconel-coated glass neutral density filter to reduce light transmission, with the internal air flow rate set at 140 ml min^{-1}. CO_2 concentration within the leaf chamber was regulated by a CO_2 source within the ADC LCA4 unit or external ambient air source and was calibrated for CO_2 measurements with CO_2 span gas at a concentration of 700 μmol mol^{-1}. Both species produced similar gas exchange measurements between all systems and together with constant calibration of the systems and measurements on fully formed Oak leaves allowed confidence in the reproducibility and validation of the systems. Apart from the occasional outlier, standard error of each system for each harvest was below 20 % (n = 6) for both species. Reproducibility between the ADC and the CIRAS-1 systems was below 20 % for *Armeria maritima* and below 5 % for *Plantago maritima*.

2.4.2 A/c_i response curves

Data for A/c_i response curves of leaves of *Plantago maritima* and *Armeria maritima* were acquired by measuring A and c_i from either the CIRAS-1 at eight or ten leaf chamber CO_2 concentrations (approximately 50, 80, 110, 150, 200, 260, 460, 570, 700, 1000 and 1300 μmol mol^{-1} CO_2 acquired by using an internal CO_2 source within the CIRAS-1) and at light saturating conditions (1150 μmol m^{-2} s^{-1}). Readings of A and c_i were taken at least 15 minutes after the new CO_2 concentration was applied. The response of net photosynthesis (A) to intercellular CO_2 (c_i) was modelled by mechanistic and empirical A/c_i curve analysis, based upon equations described by Harley *et al.* (1992) and Olsson & Leverenz (1994), using the "Photosyn assistant program" (Dundee Scientific, Dundee, UK).

2.5 Plant water potential (thermocouple psychrometry)

Leaf and root water potential was determined using screen-caged peltier thermocouple psychrometers connected to a digital thermocouple meter (model 85) (Brown & Bartos

1982; J.R.D. Merrill speciality equipment, 1105 West 2200 South, Logan, Utah, USA). Psychrometers were calibrated with small pieces of Whatman number 1 filter paper soaked with 80 µl NaCl (at 0.2 M, 0.5 M or 1.0 M) and correction coefficients for each psychrometer calculated (Brown & Bartos 1982; Wyn Jones & Gorham 1983).
At approximately 8 hours into the photoperiod, a single freshly-excised leaf or root section was placed in a stainless steel sample chamber, which was then securely screwed onto a screen-caged psychrometer. Up to 20 psychrometers were available for use at any one time. The psychrometers were placed in a heated water bath set at 25 °C overnight to allow the water potential within the sample to come to equilibrium with the air in the chamber. The electrical output (microvolts µV) of each psychrometer was measured on the meter under the following conditions; 30-second cool time; cool current 5 mA and a delay time of 2 seconds. Equation 2.1 is from a predictive model which explains the relationships between psychrometer outputs in µV and water potentials in bars (Brown & Bartos 1982). The final µV output was converted to water potential in bars and then to megapascals (MPa) by dividing bars by 10 [eq. 2.1].

$$\text{MPa} = \frac{\left(\frac{(\text{microvolts} - 0.406)}{-0.429} \times 1.039\right)}{10} \qquad [2.1].$$

Psychrometers were cleaned after each measurement with distilled water, followed by a non-residue solvent (solvent cleaner plus, RS-197-5488, RS components Ltd. Corby, Northants, UK), and rinsed with copious amounts of distilled water and left to dry. Leaf chambers and connecters were soaked in a weak Decon 90 (Decon laboratories Ltd., Sussex, UK) solution, rinsed with distilled water and left to dry. All components were completely dry before new samples were inserted in to the sample chambers.

2.6 Plant growth and anatomical measurements

2.6.1 Plant dry weight and leaf area measurements

For dry weight determination, a known fresh weight of plant tissue was dried in an oven at either 65 °C or 110 °C for over 48 hours to constant weight. Percent dry weights then allowed the calculation for reference dry weight in assays using fresh tissue. Leaf area was determined by using Delta-T Scan leaf area imager and software (Delta-T devices Ltd., Cambridge, UK).

2.6.2 Growth analyses

The following instantaneous growth analyses were carried out: leaf weight ratio (*LWR*), the amount of leaf material per total plant weight [eq. 2.2]; leaf area ratio (*LAR*), the area of leaf per total plant dry weight [eq. 2.3] and specific leaf area (*SLA*), the area of leaf per total leaf weight [eq. 2.4] (Hunt 1990) and the total leaf weight per area of leaf.

$$LWR = L_W/W \qquad \text{(dimensionless)} \qquad [2.2].$$

$$LAR = L_A/W \qquad (\text{mm}^2\ \text{mg}^{-1}) \qquad [2.3].$$

$$SLA = L_A/L_W \qquad (\text{mm}^2\ \text{mg}^{-1}) \qquad [2.4].$$

Where: W = Plant weight; L_W = Leaf weight; L_A = Leaf area

2.6.3 Anatomical and lignification analysis

Triplicate hand-cut transverse sections of ethanol-fixed leaf and root tissue were made at the proximal, middle and distal (above 0.5 cm from tip) parts of each sample. Sections were stained sequentially in 0.01 % (w/v) calcofluor M2R and 0.01 % (w/v) acridine orange for 1 minute in each stain, with an intervening wash in distilled water and visualised under a Nikon Diaphot-TMD-EF fluorescence microscope (Nikon Corporation, Tokyo 100, Japan) using a UV filter (Gates 1993). Diameters of the midrib vein and major adjacent shoot veins, root stele diameter and the number of lignified vessel members were recorded using a micrometer. Digital photographs (Nikon Coolpix950, Nikon Corporation, Tokyo 100, Japan) were taken of the sections and calibration slides. PaintShop Pro. v.6 (Jasc Software Inc., Eden Prairie, MN 55344, USA) software was used to measure the total diameter and lumen diameter of lignified vessels from the digital photographs (six lignified vessels measured per vein per cross-section in the leaves and ten lignified vessels per cross-section measured in the root) to obtain vessel wall thickness/vessel diameter ratio.

2.7 Biochemical assays

2.7.1 Sources of chemicals

Unless stated, all chemicals used were from Sigma (Sigma chemical company Ltd, Fancy road, Poole, Dorset, UK) or from BDH (BDH lab supplies, Poole, Dorset, UK). Chemicals and solvents were of analar grade unless otherwise stated.

2.8 Photosynthetic pigments in shoot tissue

Frozen leaf tissue was extracted three times, using a pestle and mortar, with 3 ml ice-cold 100 % ethanol (Fisher Scientific Ltd., UK) and about 0.3 g acid-washed sand. Extracts were pooled and centrifuged at 4600 *g* (Econospin, Sorall Instruments, Du Pont, Wilmington, Germany) for 4 minutes. The supernatant was decanted into a 25 ml volumetric flask and made up to volume with ice-cold 100 % ethanol.
The absorbance of the supernatant was measured on a spectrophotometer (Shimadzu UV150-02, Shimadzu Seisakusho Ltd., Kyoto, Japan) at 665 nm (chlorophyll-a), 649 nm (chlorophyll-b) and 470 nm (carotenoids) using 100 % ethanol as a blank at each wavelength. Pigments were quantified using the equations of Lichtenthaler (1983) [eqs. 2.5, 2.6 & 2.7]. Units were given as μg pigment ml^{-1} ethanol extract.

$$\text{Chlorophyll-a concentration} = 13.95A_{665} - 6.88A_{649} \quad [2.5].$$

$$\text{Chlorophyll-b concentration} = 24.96A_{649} - 7.32A_{665} \quad [2.6].$$

$$\text{Carotenoid concentration} = \frac{1000A_{470} - 2.05[\text{chlorophyll-a}] - 114.8[\text{chlorophyll-b}]}{245} \quad [2.7].$$

Pigment concentration was multiplied by 25 (ml) and divided by the dry weight of the sample tissue. The assay was carried out on ice and under low intensity light conditions to reduce pigment degradation.

2.9 Nitrogen concentrations of plant tissue

2.9.1 Extraction procedure for total nitrogen

Oven-dried plant tissue was digested using a modified micro-Kjeldahl digestion procedure derived from the methods of Hind (1993) and Skalar (1995). This assay converts the organic -N to ammonium (NH_4^+) –N by heating plant tissue in sulphuric acid containing potassium sulphate (K_2SO_4), to increase the reaction temperature. Selenium (Se) was added to increase oxidation of the organic matter and salicylic acid ($C_7H_6O_3$) to form 5-nitrosalicylic acid ($C_7H_5NO_5$) to quantify nitrate-N (Bremnar 1996).

Plant tissue of a known dry weight, 10 (± 0.4) mg, was added to 1 ml concentrated sulphuric acid reagent (containing 7.2 % salicylic acid w/v and 3.5 % w/v ground Kjel tablet; Kjeltabs auto, Thompson and Copper Ltd, Liverpool, UK; one Kjel tablet consisted of 1.5 g K_2SO_4 and 7.5 µg Se) and left overnight at room temperature. The mixture was subsequently heated for two hours at 100 °C in a block digester (Skalar-5620/40 digester and 5600 controller, Skalar Analytical BV, 4800 De Breda, The Netherlands). Once cooled, 3 ml of 30 % hydrogen peroxide was added to fully oxidise organic matter. The mixture was then re-heated for two hours at 330 °C. After cooling, the clear digest was poured into a 250 ml volumetric flask and made up to volume with distilled water and stored in a polypropylene bottle at 4 °C until analysed, usually within 7 days. Blank digests, containing only reagents and hydrogen peroxide, were run simultaneously. A reference material of *Platyhypnidium riparioides* (Nr. 61, individual Nr. 272; Community Bureau of Reference, European Community Council) was used for method validation and calibration, recovery was between 94-100 %.

2.9.2 Analysis procedure for total nitrogen

Determination of total reduced N concentration was carried out by automated spectrophotometry using a SAN^{plus} 4000 segmented flow analyser (Skalar Analytical, Breda, The Netherlands), connected to a SA1000 autosampler; matrix photometer (type 6250, Skalar) and an Interface unit (type SA8600, Skalar), using SAN^{plus} v. 6.2 software to run the system. The lines on the segmented flow analyser were set up for NH_4^+ according the manufacturers instructions.

Ammonia-N determination was based a modified Berthelot reaction (Skalar 1995) where Ammonia-N was chlorinated to monochloroamine, which reacts with salicylate to form 5-aminosalicylate. After oxidative coupling a green coloured complex was formed. The colour of this complex was measured at 660 nm. All reagents were prepared according to Skalar (1995). Standard curves were constructed for each set of analyses using ammonium chloride (0.02-1.00 mg N l^{-1}) for calibration after making up in diluted digested blank. Diluted non-supplemented digests were used as a blank during analysis. The SAN^{plus} v. 6.2 software converted absorbances of the complex to mg l^{-1} N of the sample.

2.10 Osmolyte concentrations in plant tissue

2.10.1 Extraction procedure

Betaine, proline and sorbitol were analysed using a sap extraction technique (Dr. John Gorham. Bangor University, UK. Personal communication and Gorham *et al.* 1984). Sap was extracted by defrosting about 200 mg of the frozen plant material, which was then placed into a 0.5 ml eppendorf PCR (polymerised chain reaction) vial with a small hole pierced in the bottom. This vial was placed inside a 1.5 ml eppendorf vial and centrifuged for 10 minutes at 22000 *g* (Mikroliter Hettich, Germany). The extracted sap was stored in the eppendorf vial at –20 °C until analysis. The remaining structural and insoluble material was stored inside the PCR vial at –20 °C until further analysis.
The remaining part of the extraction technique was based upon the method of Köhl (1996). All of the extracted sap from each sample was taken from the vial with a 200 µl micropipette and applied to a 3 ml strong cation-exchange column (Dowex 50 X8-400 resin, H^+). The column was rinsed with 4 ml distilled water and twice with 7 ml of 2.5 *N* hydrochloric acid (HCl). The three fractions were collected separately. Sorbitol (and neutral sugars) were eluted in the water fraction, proline in the first acid fraction and betaine in the first and second acid fraction. Between each batch of analyses, the Dowex resin was washed with 5 ml 2.5*N* HCl followed by distilled water until the eluant reached pH 5.5. Dowex resin was pre-equilibrated prior to use in concentrated HCl for 30 min and then washed with 5 l distilled water until the eluant reached pH 5.5. To monitor recoveries a 100 µl betaine (16.8 µmol), proline (0.14 µmol) and sorbitol (24 µmol) spike was applied to the top of the column and the spiked extracts were analysed to determine recovery levels for each assay. The sap extraction technique was

validated against a methanol:chloroform:water extraction method, where tissue of the same plant was ground with a pestle and mortar and compatible solutes extracted with the methanol:chloroform:water solvent. The sap extraction technique extracted solutes within 85-130 % of the methanol:chloroform:water extraction method.

2.10.2 Betaine analysis

The method was based upon Köhl (1996). Betaine was determined by separately adding 500 µl of each acid fraction recovered from the Dowex column to 200 µl of ice-cold tri-iodide solution (prepared using 8.85 g iodine and 10 g potassium iodide (KI) dissolved in 50 ml 1 *N* HCl by 45 minutes with stirring) within a 1.5 ml eppendorf vial and freezing at –20 °C over night. After thawing on ice, samples were centrifuged for 15 minutes at 2200 *g* (Mikroliter Hettich, Germany). The supernatant was discarded and the remaining pellet washed twice with 2.5 *N* HCl. The periodide pellet was then dissolved in 1 ml of ice-cold dichloromethane and 0.5 ml of the red-coloured mixture transferred to a UV-quartz cuvette. After the addition of 0.5 ml dichloromethane, the absorbance was measured at 365 nm in a spectrophotometer (Shimadzu UV150-02). The concentration of betaine in each acid fraction was then determined from a standard curve prepared using known amounts of betaine (0 –10 mM) with dichloromethane used as a blank.

2.10.3 Proline analysis

This method was based upon Köhl (1996); Magne & Larher (1992) and Troll & Lindsley (1955). Proline was determined by adding 2 ml of the first acid fraction from the Dowex column (and the second acid fraction, when appropriate) to 4 ml of ninhydrin reagent (prepared from 2 g of ninhydrin added to 100 ml 60 % aqueous glacial acetic acid dissolved with heating to 70 °C; this reagent is stable for 24 hours) and placed in a heating block (Skalar 5620/40) at 100 °C for one hour. The solution was cooled to room temperature and 4 ml of xylene added with vortexing (15 s). The two phases were allowed to separate and 1 ml of the pink xylene phase transferred to a UV-quartz cuvette and the absorbance measured at 515 nm in a spectrophotometer (Shimadzu UV150-02). The concentration of proline in each acid fraction was determined from a standard curve prepared from known amounts of proline (0 –50 µM) with xylene used as a blank. As the assay can detect other free amino acids, the

extraction procedure was designed to that only proline was eluted and collected as proline has a different retention time out of the column compared to other free amino acids.

2.10.4 Sorbitol analysis

This method was based upon King & Mann (1959); Brown *et al.* (1983) as commercialised by Sigma Diagnostics (1995). Sorbitol was determined by adding to a 1 ml UV plastic disposable cuvette (25 °C); 200 µl of NAD (Nicotinamide adenine dinucleotide) at 1 mg ml^{-1}; 380 µl of 0.25 M 'Trizma' buffer (Tris(hydroxymethyl)aminomethane, pH 9.6) and 400 µl of sample from the water fraction (or standard). Finally, 20 µl of sorbitol dehydrogenase (SDH) (0.5 mg ml^{-1}) was added to the cuvette and the absorbance at 340 nm was read immediately and at one-minute intervals over three minutes on a spectrophotometer (Shimadzu UV150-02 or Unicam ATI UV2). Blanks consisted of the above mixture but with distilled water substituting for the sample. Any precipitates within the mixture were allowed to settle before SDH was added. Sorbitol concentration was extrapolated from the initial rate of reaction (velocity) of known concentrations taken from a sorbitol standard curve (0-10 mM).

2.11 Total non-structural carbohydrate of shoot and root tissue

2.11.1 Extraction procedures

Carbohydrates were extracted by one of two methods:

1: The first extraction method was based upon Farrar (1993). High and low molecular weight soluble carbohydrates were extracted using successive ethanolic and water mixtures. Starch was then solubilised enzymatically after removal of the soluble carbohydrates.

Fresh, coarsely sliced, shoot or root tissue (≈ 50 mg) was added to 10 ml 90 % aqueous ethanol (Fisher Scientific, UK) in a capped 14 ml polypropylene tube (Sarstedt Ltd. Leicester, UK) at 60 °C in a heated water bath or heating block (Skalar 5620/40) for 2 hours. The extract was decanted into a 25 ml volumetric flask and the remaining tissue re-extracted in a further 10 ml ethanol. Two extractions were sufficient to extract all of the soluble carbohydrates as none were detected in a third successive extraction. The

extracts were combined and made up to volume with ethanol. The remaining tissue was further extracted with two successive 5 ml water fractions at 30 °C for 2 hours each. The two water fractions were combined.

Starch was extracted by adding the remaining tissue to a 6 ml amyloglucosidase buffer solution within a capped glass vial at 55 °C in a heated water bath for 24 hours. This solution was made by adding 0.493 mg amyloglucosidase (10 units ml^{-1}; from *Aspergillus niger* Sigma) to a sodium-acetate buffer (490 ml 0.2 M sodium acetate anhydrous to 510 ml 0.2 M acetic acid at pH 4.5) Dawson *et al.* (1989). Controls were the above mixture alone, to correct for carbohydrates present in the enzyme preparation.

2: The water fraction from the Dowex ion-exchange column (2.10.1) contained soluble carbohydrates and was assayed for carbohydrate as described below. In the case of plant material, the remaining tissue in the eppendorf PCR vial (2.10.1) was lightly ground and assayed for starch as above.

2.11.2 Phenol-sulphuric acid determination of carbohydrates

The reducing sugar content of fractions derived from extraction with ethanol, water and amyloglucosidase treatment were all determined using the phenol-sulphuric acid assay within a fume hood (Dubois *et al.* 1956; Farrar 1993). Into a dry glass boiling tube, 50 µl 80 % aqueous phenol (w/w) was added to 1 ml sample (diluted according to carbohydrate content). Cautiously, 5 ml sulphuric acid was quickly added to the mixture and left to cool. The absorbance was read at 485 nm in quartz cuvettes using water as a blank for the soluble carbohydrates and the enzyme preparation for the starch digests. The concentration of glucose equivalents was determined from a standard curve of glucose (0 –100 µg ml^{-1}). Total non-structural carbohydrates (TNC) are expressed as the combined total of soluble and insoluble carbohydrate containing fractions for each extraction method.

2.12 Soluble protein concentration in plant tissue

Fresh shoot or root tissue (100 mg) was ground in liquid nitrogen and extracted on ice in 1.5 ml cold 0.1 M 'Trizma' buffer (Tris(hydroxymethyl)aminomethane phosphate buffer) containing 10 mM ascorbic acid; 10 mM sodium metabisulphite and 2 mM

dithiothreitol (DTT) adjusted to pH 7.5. The extract was decanted into a 15 ml polypropylene tube containing 40 mg polyvinylpolypyrrolidone (PVPP), vortexted and centrifuged at 3000 *g* (Econospin, Sorall Instruments, Du Pont, Wilmington, Germany) for 10 min. Within a plastic disposible cuvette; 1 ml of one-fifth-strength Bio-Rad™ dye binding reagent (Bio-Rad, Hemel-Hemstead, UK) was added to 20 µl supernatant and the absorbance read after one hour in a spectrophotometer at 595 nm against a reagent blank. The concentration of soluble protein was determined from a standard curve prepared from γ-globulin (0 – 1.0 mg ml^{-1}).

2.13 Total soluble phenolic of plant tissue

2.13.1 Extraction procedure

This extraction method was based upon the FAO/IAEA working document (2000). Fresh shoot or root tissue (≈ 200 mg) was ground to a fine powder with liquid N_2 in a pestle and mortar and extracted on ice with 5 ml cold acetone:H_2O (7:3 v/v). The extract was decanted into a polypropylene carbohydrate tube and centrifuged at 4600 *g* (Econospin, Sorall Instruments, Du Pont, Wilmington, Germany) for 5 min. The supernatant was decanted into a cold test tube and a further 5 ml acetone:H_2O (7:3 v/v) added to the pellet, with vigorous vortexing for 1 min. After recentrifugation (5 min), the supernatants were pooled and stored at –20 °C until analysed, usually within seven days. A further 4 ml of solvent was added to the insoluble pellet and stored as above.

2.13.2 Total phenolic determination

This two-step method was based upon Marigo (1973), Waterman & Mole (1994) and the FAO/IAEA working document (2000).

A 25 µl aliquot of sample was diluted to 3 ml with distilled water and added to a test tube containing 1 ml saturated sodium carbonate solution and 0.25 ml Folin-Ciocalteu reagent (Sigma). The mixture was vortexed and left at room temperature for one hour. The absorbance of the mixture was read at 760 nm by a spectrophotometer (Shimadzu UV150-02) and the concentration of tannic acid equivalents was determined from a standard curve of tannic acid (0 –50 µg 3 ml^{-1}) with water serving as the blank. Simultaneously, a 0.5 ml aliquot of the same sample was added to 40 mg PVPP and 1.5 ml distilled water (2 g PVPP binds 8 mg phenolics; Marigo (1973). The pH was

adjusted to pH 3.5 using 2.3 mM HCl, vortexed, and kept on ice for 15 min with occasional shaking. Samples were then centrifuged for 10 min at 4600 *g* (Econospin, Sorall Instruments, Du Pont, Wilmington, Germany). Simple phenolics, flavonoids and tannins will be bound to the PVPP (Marigo 1973). The supernatant was then assayed as above but initially using 50 µl sample instead of 25 µl. The phenolic concentration of was determined by subtracting the tannic acid equivalence content of the PVPP treated sample from the tannic acid equivalence content of the non-PVPP treated sample.

2.14 Separation, identification and quantification of phenolic compounds

2.14.1 Extraction and enzyme hydrolysis of phenolic glycosides

The extraction and analysis of phenolics was based upon Mabry *et al.* (1970) and Edwards and Kessmann (1992). Shoot or root tissue (≈ 200 mg) was homogenised on ice in 5 ml cold acetone (-20 °C) using a pestle and mortar. The solvent was removed by vacuum filtration (Whatman 570 7 cm paper filters, Whatman International Ltd, Kent, UK) and the remaining residue re-extracted twice in 5 ml cold acetone:methanol (1:1 v/v) and re-filtered. The filtrate was combined and reduced to dryness and resuspended in 2 ml methanol. A 50 µl spike of quercetin (280 nmol) was added to three samples at the start of the extraction process to monitor recovery.

To obtain phenolics in their aglycone form, the glycosidic conjugates were hydrolysed using a general purpose cellulase. A 0.5 ml aliquot of the re-suspended extract was transferred to a 1.5 ml eppendorf tube and reduced to dryness under vacuum centrifugation. The residue was then re-dissolved in 0.5 ml 0.15 M citrate-phosphate buffer pH 5.0 containing 1mg ml^{-1} cellulase extract (from *Trichoderma viride*, Boehringer Mannheim, Germany), which contains β-glucosidase activity towards diverse phenolic glycosides. β-glucosidase is widely used for this purpose as about half of the naturally occurring flavonoid glycosides are β-D-glucosides (Mabry *et al.* 1970). Control samples consisted of the addition of the citrate-phosphate without cellulase to a replicate sample. Samples were incubated for 18 hours at 30 °C. Once cooled, compounds were extracted from the aqueous phase by adding three lots of 0.5 ml water-saturated ethyl acetate. Samples were vortexed and centrifuged at 2200 *g* (Mikroliter Hettich, Germany) for 1 min and the organic phase was removed to a new 1.5 ml eppendorf tube. The combined ethyl acetate phase was dried under vacuum

centrifugation and the residue re-suspended in 0.5 ml methanol. Samples were stored at –20 °C until further analysis, usually within seven days.

2.14.2 Quantification and isolation of phenolic compounds by HPLC

Separation and quantification was carried out by high performance liquid chromatography (HPLC) using a Beckman SystemGold 125P HPLC and UV-detector using SystemGold v. 8.10 software to run the system. All solvents were HPLC-grade and filtered through a 0.22 μm nylon filter (Millipore, UK) prior to use. The solvents used were 1 % formic acid (solvent A) and acetonitrile (solvent B); with a gradient of % B: initial, 20 %; 0.3 min, 20 %; 45.3 min, 60 %; 45.4 min, 100 %; 60 min, 100 %; 60.1 min, 20 %; 70 min, 20 % (total run time 70 min). Flow rate was 0.8 ml min^{-1}, injection 20 μl sample or standard. The column was a Phenosphere – 5 um packing; ODS2 column size 250 x 4.6 mm (Phenomenex, Cheshire, UK) and the UV wavelength detection was set at 287 nm. Commercial standards (Sigma) of *p*-coumaric acid, caffeic acid and ferulic acid were used to obtain calibration curves (peak area vs. concentration) in the range of 0.2 to 6.0 nmol.

For preparative purposes, 200 or 500 μl of extract was injected into the column and peaks of interest collected manually in 1.5 ml eppendorf tubes. The eluant containing the isolated compound of interest was reduced to dryness by vacuum centrifugation and resuspended in 0.5 ml methanol for analysis by HPLC co-chromatography, UV-spectral analyses (2.14.4) or mass-spectrometry (2.14.5).

2.14.3 Thin-Layer Chromatography (TLC) of phenolic compounds

Aluminium-backed TLC sheets (20 x 20 cm) coated with silica gel (60 μm) were used to separate UV absorbing and fluorescing compounds. A chloroform:methanol (9:1 v/v) solvent system was used to separate the phenolic aglycones (Mabry *et al.* 1970). TLC sheets were viewed under UV light at 254, 302 and 350 nm (Model UVM-57 302 nm, UVP Inc., San Gabriel, USA; Universal-UV lamp, 254 and 350 nm, Camlab (Glass) Ltd., Cambridge, UK). For analytical TLC, cellulase-digested extracts from the above procedure (2.14.1) were reduced to dryness and resuspended in 20 μl ethyl acetate, which was loaded onto the TLC sheet. To isolate compounds of interest, preparative TLC was used (Waterman and Mole 1994); cellulase-digested extract (1.5 ml) derived from the extraction of 1 g shoot f.wt was loaded across a TLC sheet

and developed twice in the solvent system. Compounds of interest were scraped off the plate into a 1.5 ml eppendorf tube containing 1 ml HPLC-grade methanol, vortexed (30 s), and centrifuged at 22000 *g* (Mikroliter Hettich, Germany) for 1 min. The supernatant was decanted and a further 0.5 ml methanol was added to the pellet, vortexed and centrifuged. The supernatants were combined and stored at –20 °C until further analysis by HPLC (2.14.2), UV-spectral analysis (2.14.4) and mass-spectrometry (2.13.5).

2.14.4 UV-spectra analysis

UV spectrophotometry was used to help identify the isolated phenolic compounds from collected HPLC fractions (2.14.2) or preparative TLC (2.14.3). Absorbance spectra of isolated compounds and commercial standards dissolved in methanol were determined using a Beckman DU 7500 spectrophotometer between the wavelengths of 250 nm and 500 nm and compared with published spectra.

2.14.5 Identification by LC-PDA-MS

The liquid chromatography-photodiode array-mass spectrometry (LC-PDA-MS) system consisted of a Waters Alliance 2790 LC (Waters Ltd., Hertfordshire, UK) system connected to a Waters 996 PDA and a Micromass Time-of-Flight Electro-Spray-Ionisation Mass Spectrometer (TOF-ESI-MS) (Micromass UK Ltd., Manchester, UK). The system was operated under the MassLynx software (v. 3.4, Micromass UK Ltd., Manchester UK).

Liquid chromatography was performed using 0.5 % formic acid (solvent A) and acetonitrile (hydrophobic solvent B) with a gradient of % B: initial, 5 %; 2 min, 5 %; 42 min, 100 %; 47 min, 100 %; 48 min, 5 %; 53 min, 5 % (total run time 53 min). The flow rate was 0.2 ml min^{-1} and 10 µl of sample or standard injected in methanol. The column was a Phenomenex Synergi™ 4um packing POLAR-RP 80Å, 250 x 2.0 mm (Phenomenex, UK). The eluate from the LC was connected to the PDA and UV-spectra were monitored continuously from 200 – 400 nm. Eluant from the PDA was continuously injected into the MS. The MS instrument (Micromass LCT) parameters were: polarity ES-; sample cone voltage = 20V; extraction cone voltage = 6V; desolvation temp. = 250.0 °C; source temp. = 120.0 °C; penning pressure = 5.15^{-7} mbar; nebuliser nitrogen gas flow = 4 l hr^{-1}; desolvation nitrogen gas flow = 516 l hr^{-1}; MS scan range, 100 – 800 *m/z*. Raffinose and sodium iodide was used to tune and calibrate

the MS respectively. The sample cone voltage was increased to 90 V to confirm the fragmentation pattern of caffeoyl phenylethanoid glycosides (Ryan *et al.* 1999). Commercial standards of *p*-coumaric acid, caffeic acid, ferulic acid and luteolin were used to acquire LC retention time, UV-spectra and the *m/z* ratio to enable elucidation of unknown compounds.

Isolated compounds from HPLC (2.14.2) or preparative TLC fractions (2.14.3) were directly injected into the mass spectrometer at a rate of 10 µl per minute.

2.15 Statistical analysis and data handling

A one-way analysis of variance (ANOVA) was used to test for significant differences between CO_2, N and watering treatments. An analysis of variance general linear model (ANOVA GLM) ($P < 0.05$ critical level of significance) multivariate model was used to test for significant interactions amongst CO_2, nitrogen and watering treatments, and when appropriate, followed by a Tukey's HSD *post-hoc* test. Data that did not fit the requirements of the test were natural-log transformed to increase homogeneity-of-variance. Due to pseudo-replication in the anatomical and lignin analyses, the means of the measured parameters from all cross sections per shoot or root were taken as one replicate, and a one-way ANOVA was used to test for significant differences between CO_2 treatments. All statistical analyses were carried out in SPSS v.10.00 (Chicago, Illinois, USA).

All word processing and data handling was carried out in Microsoft office 2000 (Microsoft corporation, USA) and graphs were produced using SigmaPlot 2000 for windows v. 6.10 (SPSS Inc, USA).

Chapter 3. Ecophysiological responses to increased atmospheric CO_2 under varying water and nitrogen availability

Aims

The two coastal plants *Plantago maritima* and *Armeria maritima* have contrasting metabolic responses when exposed to osmotic stress. Whilst *P. maritima* accumulates the carbon (C)-based polyhydric alcohol sorbitol (Ahmed *et al.* 1979), *A. maritima* accumulates the nitrogen (N)-based quaternary ammonium compound betaine and the imino acid proline (Stewart & Lee 1974). Considering the fact that these two species hyperaccumulate C or N-based compatible solutes, the aim of the study within this chapter was to determine how the growth, nutrient status and photosynthesis of these plants are affected by drought and whether these effects are modified by elevated atmospheric CO_2 and reduced N availability. The section below introduces the commonly observed responses in plants exposed to elevated CO_2 and how such responses are varied by altered N availability. This chapter complements chapter four, which describes changes in the carbohydrate and compatible solute concentrations in response to the above growth conditions.

3.1 Introduction

In the natural environment, conditions are often far from optimal and many factors limit the growth of plants. Water and CO_2 are common limiting factors for growth and therefore it is important to understand the interactions of water supply in plants and responses to the predicted increase in atmospheric CO_2. Many coastal plants depend on utilising compatible solute metabolism when subjected to water deficits by accumulating either N-based or C-based compatible solutes in the cytoplasm of the plant cells (Stewart *et al.* 1979). The diversion of C and N away from growth to maintain an equal water potential of the cytoplasm and the vacuole could be of an order that would compete with growth (Jefferies *et al.* 1979; Yeo 1983). Few studies have concentrated on the effects of elevated CO_2 on coastal plants that are exposed to frequent periods of drought where compatible solute metabolism is an important function for survival (Lenssen 1993; Lenssen *et al.* 1995; Gray & Mogg 2001). Therefore, there is an important need to evaluate how the growth and photosynthesis of plants under ambient and elevated CO_2 conditions are affected by the accumulation of compatible solutes.

Biomass

Native plants show a range of responses to elevated atmospheric CO_2 (Saralabai *et al.* 1997; Luo 1999), such as increased biomass (Baxter 1994); reduced photosynthetic capacity (Baxter *et al.* 1995); reduced N concentration (Wolfe 1998) and increased water conservation (Lenssen *et al.* 1995). However, many native plants grow under unfavourable conditions such as low N and/or drought, so that the partitioning of dry matter between the root and shoot under elevated CO_2 is also dependent on the availability of soil nutrients (particularly N) and moisture (Bazzaz 1990; Baxter *et al.* a & b 1994; Stitt & Krapp 1999). Generally, most plants cannot fully utilise the increased C assimilated under elevated atmospheric CO_2 and over time, plants alter the allocation of assimilates and dry matter partitioning to best utilise the increased availability of C (Farrar & Williams 1991). A low N supply from the soil and elevated atmospheric CO_2 can cause an increase in the root:shoot ratio in the majority of plants (Wilson 1988; Rogers *et al.* 1996), especially if root growth is increased as an extra sink for photosynthetic metabolites. In turn, this will enhance the ability of the roots to acquire N for further shoot growth (Farrar & Williams 1991; Wolfe *et al.* 1998). Therefore, the combined effect of elevated CO_2, low N and drought may all, and separately, affect the differential growth of the shoots and roots.

Dry matter allocation

As well as changes in biomass, leaf area can alter in plants growing under elevated CO_2, though whether the leaf area increases, decreases or stays the same can also be dependent on the availability of N. The leaf area of trees, crops and native species is increased on average by 24 % under elevated CO_2 (Pritchard *et al.* 1999). The investment of fixed C into leaf area can be measured in terms of leaf area ratio (*LAR*) (fraction of leaf area to total plant weight); leaf weight ratio (*LWR*) (fraction of plant weight that is leaf) and specific leaf area (*SLA*) (area per unit weight of leaf) (Farrar & Williams 1991). Indeed, increases in leaf area can be proportionally less than the increase in leaf or total plant weight. Previous studies have shown the ratio of leaf area to total plant weight (*LAR*) on average decreases 16 % and the ratio of leaf weight to total plant weight (*LWR*) decreases by 10 % for plants exposed to elevated, compared to those grown in ambient CO_2 (Pritchard *et al.* 1999). This suggests that plants allocate less C to the production of new leaf area at elevated CO_2 and that increases in growth are proportionally higher in the roots than in the shoots (Wolfe *et al.* 1998).

Specific leaf area (*SLA*) describes the leaf area over a unit of leaf dry biomass. Plants with a low *SLA* have thicker or denser leaves, the consequence of which can be an increase in the number of chloroplasts and a potentially increased photosynthetic capacity (Körner & Meendez-Riedl 1989; Evans & Poorter 2001). In previous studies, *SLA* has been shown to be reduced on average by 16 % in plants exposed to elevated CO_2 (Pritchard *et al.* 1999). Whilst this reduction can be due to increases in leaf thickness, it has also been shown that increases in the accumulation of non-structural carbohydrates can reduce *SLA*. This is particularly found in plants exposed to elevated CO_2 whilst grown with low N availability (Bazzaz 1990).

Photosynthesis and nitrogen concentrations

Under ambient CO_2, photosynthesis in C_3 plants is limited by CO_2 availability; therefore elevated CO_2 can potentially enhance photosynthesis. However, as production of photosynthetic assimilates exceeds demands for growth, usually due to nutrient limitations (Baxter *et al.* 1994 b; Harmens *et al.* 2000), photosynthesis may eventually 'acclimate' or 'down-regulate' to a level that can be maintained by sink growth (Arp 1991; Eamus 1991; Baxter *et al.* 1995). Acclimation is a process that reduces the amount of proteins involved in photosynthesis, which effectively increases the amount of N available for enhancing sink development. Reductions in photosynthetic capacity in plants under elevated CO_2 can also be caused in part by a reduction in photosynthetic components such as chlorophyll and carotenoids (Arp 1991). Chlorophyll can be reduced by 33 % as a long-term response to elevated CO_2 (Cook *et al.* 1998; Davey *et al.* 1999). Such overall reductions in N in plants are a common occurrence in plants exposed to elevated CO_2. Results from 378 published studies on plants exposed to elevated CO_2 have shown that there is on average a 14 % and a 9 % reduction in shoot and root N concentrations, respectively. As less N is required in the shoot more N can be allocated to the root system (Chapin 1980; Cotrufo *et al.* 1988).

The main intention of this study was to determine the different biochemical and physiological responses to drought in *Plantago maritima* and *Armeria maritima* when exposed to elevated atmospheric CO_2. The combination of N limitation and drought with elevated CO_2 is predicted to compound and increase the known difference in responses of the two species. In response to elevated CO_2, the amount of nitrogenous compatible solutes may decrease in the N-accumulating *A. maritima*, as N that would otherwise be allocated to compatible solutes could be allocated to other growth functions, especially when available N is limited. Conversely, the amount of the C-based sorbitol may increase in the C-accumulating *P. maritima* as this species can use C more effectively by allocating large amounts of C to sorbitol. If the N and C concentrations are affected within these plants, as compatible solutes have a broad significance in the N and C economy, then this will be reflected in the plant growth (*e.g.* dry weights, leaf area, dry matter allocation and water content). This response may change in droughted plants where there are further increases in C and N-based compatible solutes and there is internal competition for C and N between allocation for growth and osmolyte production in *P. maritima* and *A. maritima*.

3.2 Materials and methods

3.2.1 Experimental set-up – CEH Bangor Solardome facility

Ten week experiment

Clonal shoots of *P. maritima* and *A. maritima* were collected in June 2000 and established at the University of Durham Botanical Gardens glasshouse facility (Chapter 2.3.1; 2.3.2). After four weeks, the plants were transferred to the CEH Solardome facility (Chapter 2.2.3) where similar-sized plants of *P. maritima* and *A. maritima* were individually placed in 250 cm^3 pots filled with 600 g of dry silica sand (Chapter 2.3.1). Plants were equally divided between the four Solardomes. Within each Solardome, 24 plants were subjected to either a drought treatment (30 % field capacity (FC)) (Chapter 2.3.3) with a high N (28 mg l^{-1} N (NH_4NO_3)) or low N (2.8 mg l^{-1} N) $1/4^{th}$ strength Long-Ashton nutrient solution addition (Chapter 2.3.2), or a well-watered treatment (80 % FC) with a high or low N nutrient solution addition (96 plants per species per dome). Pots were randomised within each dome twice per week after watering. The start of the experiment was staggered over five days from the 1^{st} August 2000 to allow adequate time for harvesting all the plants in one session. Plants were harvested after five and ten weeks exposure to the treatments.

3.3 Growth responses to changes in carbon, nitrogen and water availability in *Plantago maritima* and *Armeria maritima*

The effects of varying CO_2, N and water availability on plant biomass, water content, leaf area and dry-matter partitioning in *P. maritima* and *A. maritima* are summarised in table 3.1. The table numbers 1.1.1 to 1.1.20 in the text refer to the statistical analyses presented in Appendix 1.1. The controls for elevated CO_2, low N and drought treatments are ambient CO_2, high N and well-watered conditions.

Table 3.1. Summary of statistically-significant effects, from tables 1.1.1. to 1.1.18 (Appendix 1.1), of elevated CO_2; nitrogen availability or drought on the growth parameters of *Plantago maritima* and *Armeria maritima* after five and ten weeks exposure.
↑ = increase in growth measurement compared to control; ↓ = decrease in growth measurement compared to control; n.d. = no difference between treatment and control. Subscript numbers ($_5$ or $_{10}$) relate to which week the measurement was significantly different to the control.

Total, shoot, root dry weight and root:shoot ratio (r:s)		***Plantago maritima***	***Armeria maritima***
Response to:	**Growth conditions**	**Response**	**Response**
Elevated CO_2	Well-watered; high N	n.d.	n.d.
	Well-watered; low N	↓ $total_5$ $root_5$	↑ $shoot_{10}$ ↓ $r:s_5$
	Drought; high N	n.d.	n.d.
	Drought; low N	n.d.	n.d.
Low N	Ambient CO_2; well-watered	↓ $total_5$ $shoot_{10}$ ↑ $r:s_{5,10}$	↓ $total_{10}$ $shoot_{5,10}$ $root_{10}$ ↑ $r:s_{5,10}$
	Ambient CO_2; Drought	↓ $total_5$ $shoot_{5,10}$ ↑ $r:s_5$	↓ $total_{10}$ $shoot_{5,10}$ ↑ $r:s_{10}$
	Elevated CO_2; well-watered	↓ $total_{5,10}$ $shoot_{5,10}$ $root_{5,10}$ ↑ $r:s_{10}$	↓ $total_{10}$ $shoot_{10}$ ↑ $r:s_{10}$
	Elevated CO_2; Drought	↓ $total_{5,10}$ $shoot_{10}$ $root_{5,10}$	↓ $total_{5,10}$ $shoot_{5,10}$ $root_{10}$
Drought	Ambient CO_2; High N	n.d.	↓ $shoot_5$ $root_{10}$ ↓ $r:s_{10}$
	Ambient CO_2; Low N	↓ $r:s_{10}$	↓ $r:s_5$
	Elevated CO_2; High N	n.d.	n.d.
	Elevated CO_2; Low N	↑ $shoot_{10}$	↓ $total_{10}$ $shoot_{10}$ $root_{10}$

Total, shoot and root water content (g H_2O g^{-1} dwt)		***Plantago maritima***	***Armeria maritima***
Response to:	**Growth conditions**	**Response**	**Response**
Elevated CO_2	Well-watered; high N	↓ $shoot_5$	↑ $root_5$
	Well-watered; low N	↑ $total_{5,10}$ $shoot_{5,10}$ $root_{10}$	↓ $total_{5,10}$ $shoot_{5,10}$
	Drought; high N	↓ $shoot_5$	↓ $total_{10}$ $root_{10}$
	Drought; low N	↓ $shoot_5$	↑ $total_{10}$ $root_{10}$
Low N	Ambient CO_2; well-watered	↓ $total_{5,10}$ $shoot_{5,10}$	↓ $total_{5,10}$ $shoot_{5,10}$ $root_{10}$
	Ambient CO_2; Drought	↓ $total_{5,10}$ $shoot_{5,10}$	↓ $total_{5,10}$ $shoot_{5,10}$ $root_{10}$
	Elevated CO_2; well-watered	↓ $total_{10}$ $shoot_{10}$	↓ $total_{5,10}$ $shoot_{5,10}$ $root_{5,10}$
	Elevated CO_2; Drought	↓ $total_{5,10}$ $shoot_{5,10}$	↓ $shoot_{5,10}$
Drought	Ambient CO_2; High N	n.d.	↓ $shoot_{5,10}$
	Ambient CO_2; Low N	↑ $shoot_{5,10}$	↓ $shoot_{5,10}$ ↑ $root_{10}$
	Elevated CO_2; High N	↓ $total_{10}$ $root_{10}$	↓ $total_{5,10}$ $shoot_{5,10}$ $root_{5,10}$
	Elevated CO_2; Low N	↓ $total_5$ $shoot_{5,10}$ $root_5$	↑ $total_{10}$ $shoot_{5,10}$ $root_{10}$

Leaf area; *LAR*; *SLA*; *LWR*		***Plantago maritima***	***Armeria maritima***
Response to:	**Growth conditions**	**Response**	**Response**
Elevated CO_2	Well-watered; high N	↓ $leaf\ area_5$	↓ SLA_{10}
	Well-watered; low N	↑ SLA_{10}	↓ $SLA_{5,10}$ ↑ LWR_5
	Drought; high N	n.d.	↓ $leaf\ area_5$ LAR_5 SLA_5
	Drought; low N	n.d.	↓ $leaf\ area_5$ LAR_5 SLA_5
Low N	Ambient CO_2; well-watered	↓ $leaf\ area_{5,10}$ $LAR_{5,10}$ SLA_{10} $LWR_{5,10}$	↓ $leaf\ area_{5,10}$ $LAR_{5,10}$ SLA_{10} $LWR_{5,10}$
	Ambient CO_2; Drought	↓ $leaf\ area_{10}$ LAR_{10} SLA_{10} LWR_5	↓ $leaf\ area_{5,10}$ $LAR_{5,10}$ $SLA_{5,10}$ LWR_{10}
	Elevated CO_2; well-watered	↓ $leaf\ area_{5,10}$ LAR_{10} SLA_{10} LWR_{10}	↓ $leaf\ area_{5,10}$ $LAR_{5,10}$ $SLA_{5,10}$ $LWR_{5,10}$
	Elevated CO_2; Drought	↓ $leaf\ area_{5,10}$	↓ $leaf\ area_{5,10}$ LAR_5 SLA_5
Drought	Ambient CO_2; High N	↓ SLA_5	↓ SLA_{10} ↑ LWR_{10}
	Ambient CO_2; Low N	↓ SLA_{10}	↑ $leaf\ area_5$ ↓ SLA_{10} ↑ LWR_5
	Elevated CO_2; High N	↓ $leaf\ area_{10}$ LAR_{10}	n.d.
	Elevated CO_2; Low N	↓ SLA_5	↑ LAR_{10} SLA_{10}

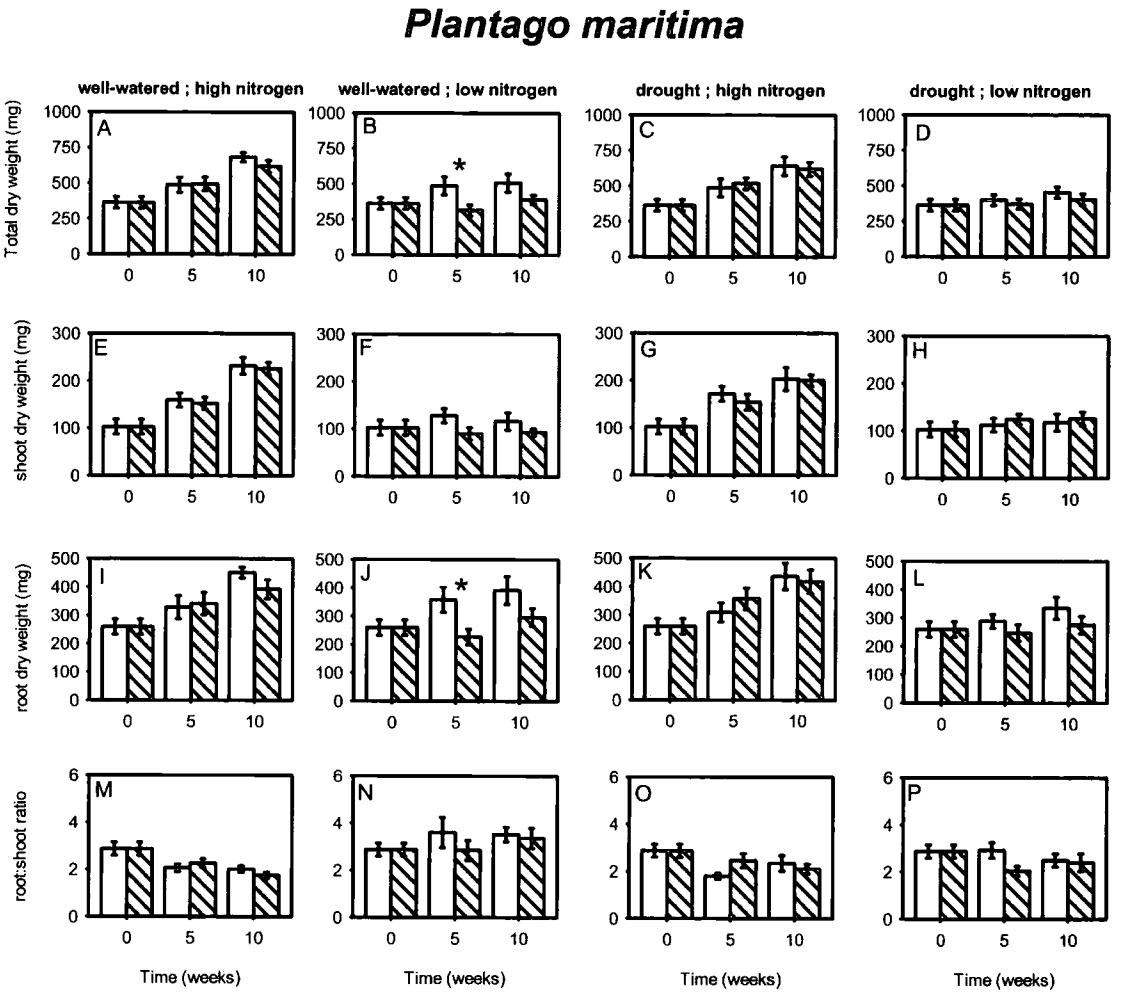


Figure 3.1. The effect of 360 (open bars) and 600 (hatched bars) μmol CO_2 mol^{-1} in combination with 28 mg l^{-1} N (high N) or 2.8 mg l^{-1} N (low N) on total (A-D), shoot (E-H) and root dry weight (I-L) and root:shoot ratio (M-P) of *Plantago maritima*. Plants were grown over ten weeks under well-watered (80% field capacity) or drought (30% field capacity) conditions. Data represent mean ± one SE. $n = 12$. Significant effects of elevated CO_2 are indicated by * ($P \leq 0.05$).

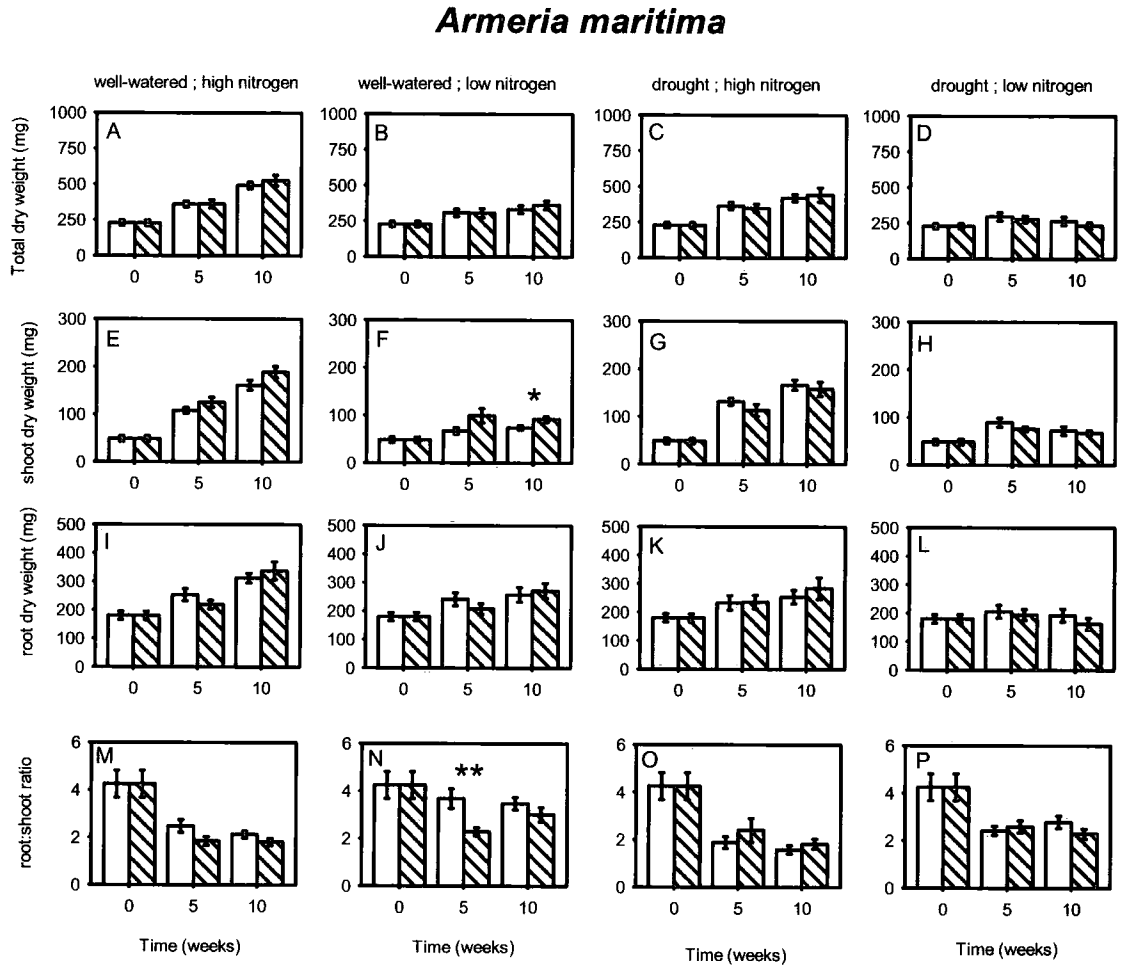


Figure 3.2. The effect of 360 (open bars) and 600 (hatched bars) μmol CO_2 mol^{-1} in combination with 28 mg l^{-1} N (high N) or 2.8 mg l^{-1} N (low N) on total (A-D), shoot (E-H) and root dry weight (I-L) and root:shoot ratio (M-P) of *Armeria maritima*. Plants were grown over ten weeks under well-watered (80% field capacity) or drought (30% field capacity) conditions. Data represent mean ± one SE. $n = 12$. Significant effects of elevated CO_2 are indicated by * ($P \leq 0.05$) and ** ($P \leq 0.01$).

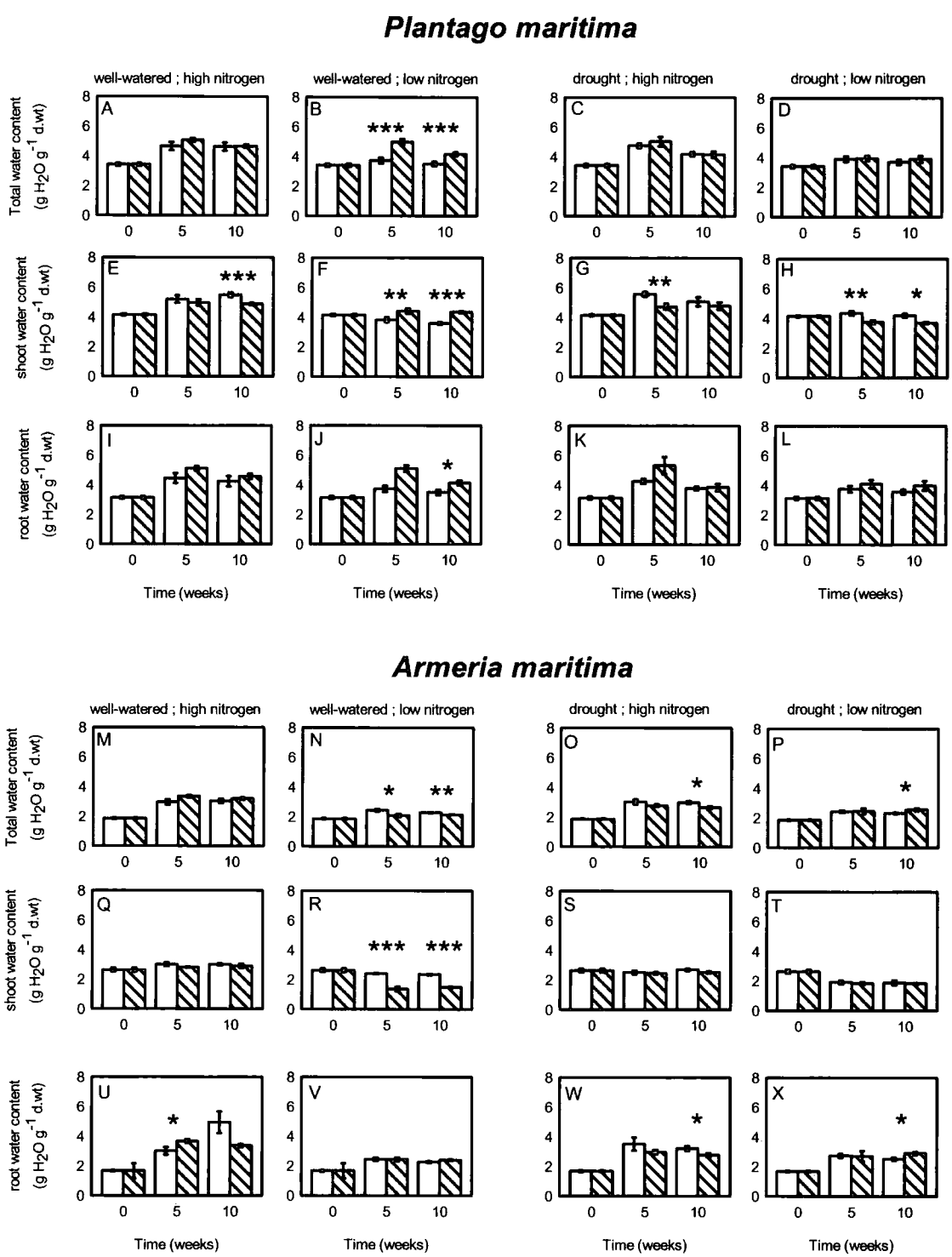


Figure 3.3. The effect of 360 (open bars) and 600 (hatched bars) μmol CO_2 mol^{-1} in combination with 28 mg l^{-1} N (high N) or 2.8 mg l^{-1} N (low N) on total (A-D; M-P), shoot (E-H; Q-T) and root water concentration (I-L; U-X) of *Plantago maritima* and *Armeria maritima*. Plants were grown over ten weeks under well-watered (80% field capacity) or drought (30% field capacity) conditions. Data represent mean ± one SE. $n = 12$. Significant effects of elevated CO_2 are indicated by * ($P \leq 0.05$); ** ($P \leq 0.01$) and *** ($P \leq 0.001$).

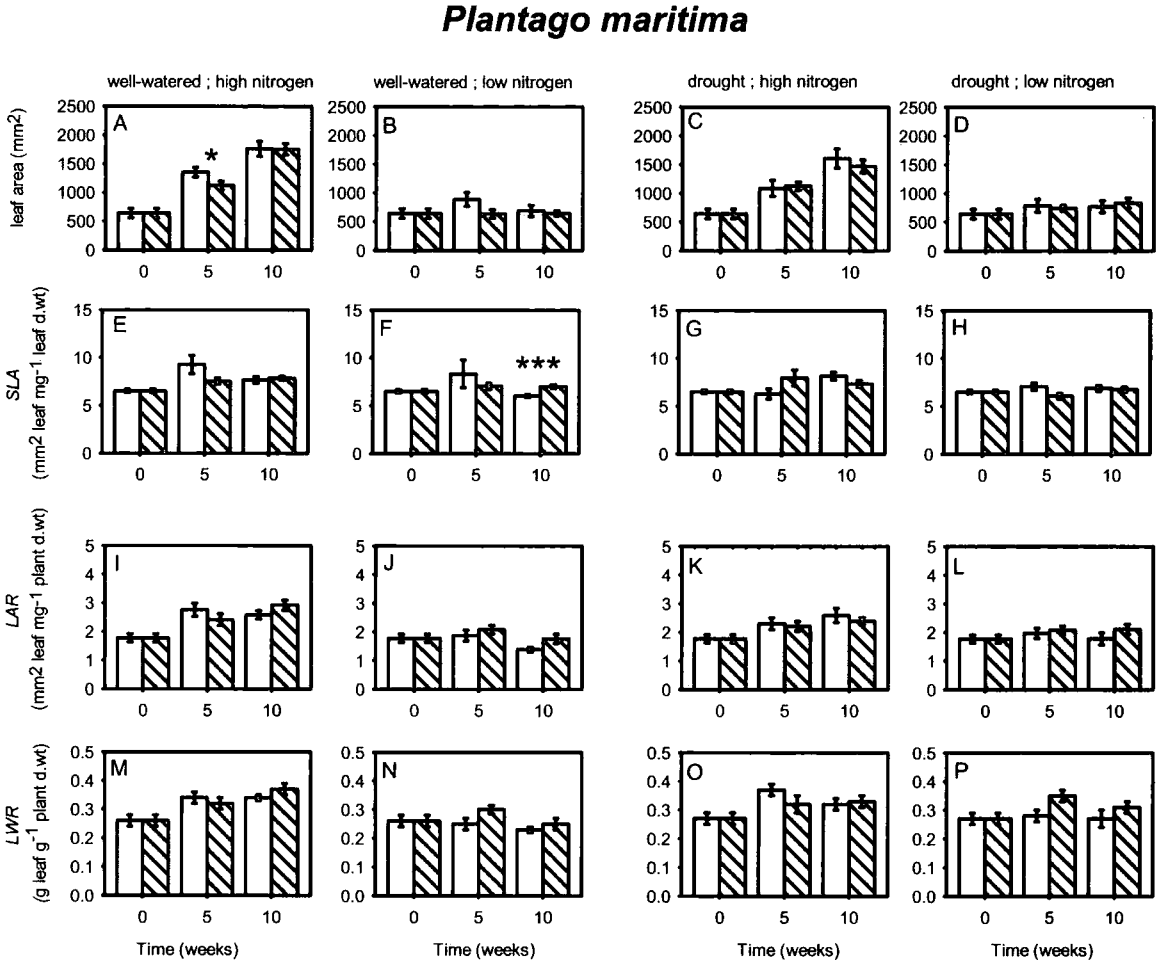


Figure 3.4. The effect of 360 (open bars) and 600 (hatched bars) μmol CO_2 mol^{-1} in combination with 28 mg l^{-1} N (high N) or 2.8 mg l^{-1} N (low N) on leaf area (A-D), specific leaf area (*SLA*) (E-H); leaf area ratio (*LAR*) (I-L) and leaf weight ratio (*LWR*) (M-P) of *Plantago maritima*. Plants were grown over ten weeks under well-watered (80% field capacity) or drought (30% field capacity) conditions. Data represent mean ± one SE. $n = 12$. Significant effects of elevated CO_2 are indicated by * ($P \leq 0.05$) and *** ($P \leq 0.001$).

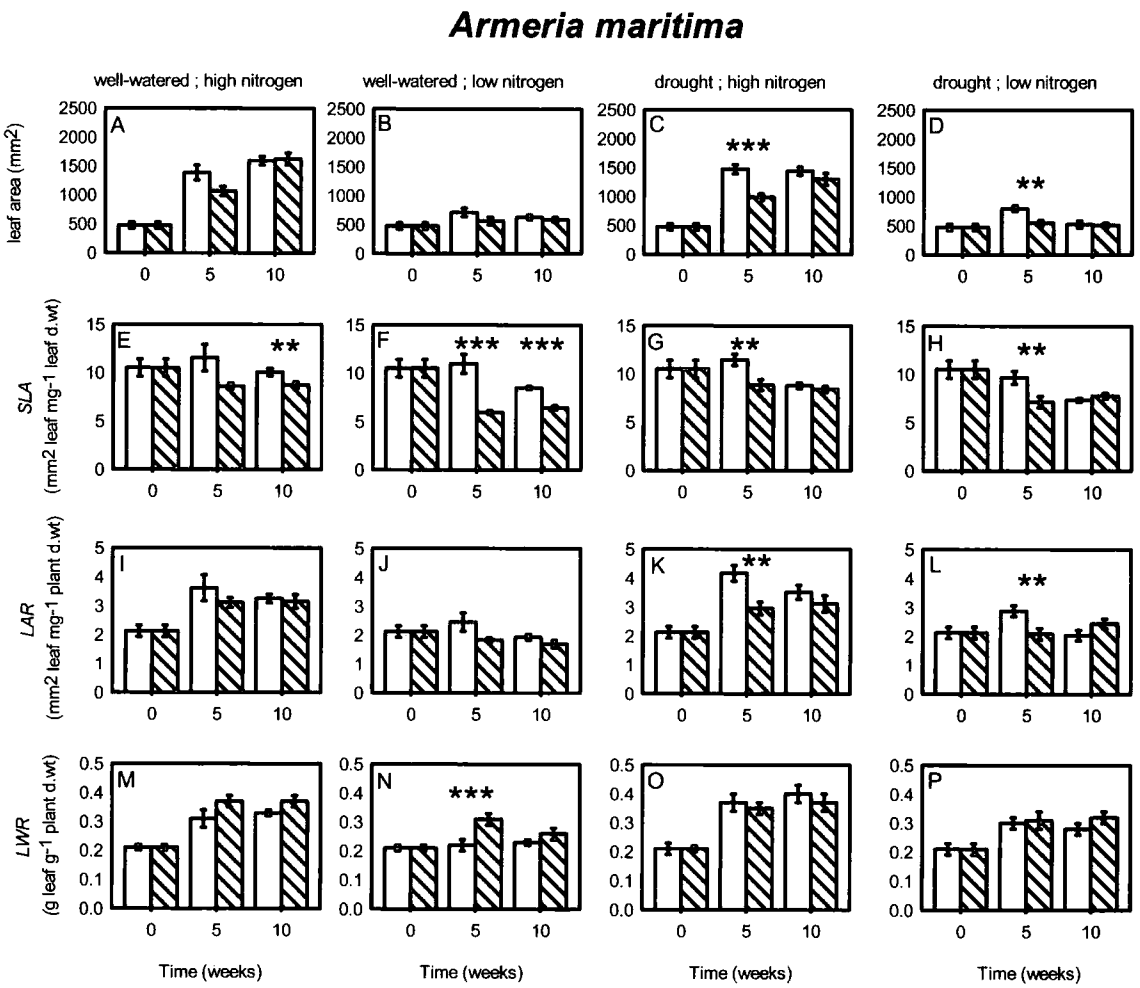


Figure 3.5. The effect of 360 (open bars) and 600 (hatched bars) μmol CO_2 mol^{-1} in combination with 28 mg l^{-1} N (high N) or 2.8 mg l^{-1} N (low N) on leaf area (A-D), specific leaf area (*SLA*) (E-H); leaf area ratio (*LAR*) (I-L) and leaf weight ratio (*LWR*) (M-P) of *Armeria maritima*. Plants were grown over ten weeks under well-watered (80% field capacity) or drought (30% field capacity) conditions.
Data represent mean ± one SE. $n = 12$. Significant effects of elevated CO_2 are indicated by * ($P \leq 0.05$); ** ($P \leq 0.01$) and *** ($P \leq 0.001$).

3.3.1 Effect of elevated CO_2 on growth

Plantago maritima

i. Well-watered plants

Compared with ambient CO_2, elevated CO_2 had little effect on biomass, leaf area or dry matter partitioning in *P. maritima*. Elevated CO_2 had significant effects only when combined with low N availability, causing reductions in plant biomass (Figs. 3.1 B, J; Table 1.1.1) and a sustained increase in the plant water content (Fig. 3.3 B, F, J; Table 1.1.7).

ii. Droughted plants

There were no significant effects of elevated CO_2 upon the biomass, leaf area and dry matter partitioning in droughted plants. However, there were transient decreases in shoot water content in plants grown at elevated CO_2 (Fig 3.3 E, G, H: Table 1.1.7).

Armeria maritima

i. Well-watered plants

As in *P. maritima*, elevated CO_2 did not greatly affect the biomass of *A. maritima*, when compared to plants grown at ambient CO_2. It was only in plants with a low N supply that responded to an increase in CO_2 as the shoot biomass increased and the root:shoot ratio decreased (Figs. 3.2 F, N; Table 1.1.4). The *SLA* and *LWR* also responded differently to elevated CO_2 availability than *P. maritima* (Fig. 3.5). In particular, the *SLA* was reduced (Figs. 3.5 E, F; Table 1.1.16) and *LWR* was increased in response to elevated CO_2 (Fig. 3.5 N; Table 1.1.16). In plants combined with low N supply, there was a sustained reduction in the plant water content (Fig. 3.3 N, R; Table 1.1.10).

ii. Droughted plants

When droughted, the leaf area, *LAR* and *SLA* were lowered by elevated CO_2, but the effects were only significant after five weeks (Figs. 3.5 C, D, G, H, K, L; Table 1.1.16). At high N availability, elevated CO_2 decreased the total and root water content (Figs. 3.3 O, W; Table 1.1.10) though at low N supply the total and root water contents were increased (Figs. 3.3 P, X; Table 1.1.10).

3.3.2. Effect of low N availability on growth

Plantago maritima

i. Well-watered plants

Low N availability caused a reductions in the total and shoot biomasses, compared to plants at high N availability (Figs. 3.1 A & B, E & F; Table 1.1.2). The root biomass was also decreased in plants grown at elevated CO_2 (Figs. 3.1 I & J; Table 1.1.2). As the reduced biomass was not as pronounced in the root compared to the shoot this caused an increased root:shoot ratio (Figs. 3.1 M & N; Table 1.1.2). The low N supply also reduced leaf area (Figs. 3.4 A & B; Table 1.1.14), *LAR*, *SLA* and *LWR* (Figs. 3.4 E & F, I & J, M & N; Table 1.1.14). The total and shoot water concentrations were also decreased (Figs. 3.3 A & B, E & F; Table 1.1.8).

ii. Droughted plants

Low N availability reduced the total and shoot biomasses, compared to plants at high N availability (Figs. 3.1 C & D, G & H; Table 1.1.2). The root biomass was also decreased in plants grown at elevated CO_2 (Figs. 3.1 K & L; Table 1.1.2). The low N supply reduced leaf area (Figs. 3.4 C & D; Table 1.1.14) and *LAR*, *SLA* and *LWR* were decreased though only in plants grown at ambient CO_2 (Figs. 3.4 G & H, K & L, O & P; Table 1.1.14). The total and shoot water concentrations were also reduced by low N availability (Figs. 3.3 A & B, E & F; Table 1.1.8).

Armeria maritima

i. Well-watered plants

Compared to plants grown at a high N availability, low N availability decreased the total and shoot biomass (Figs. 3.2 A & B, E & F; Table 1.1.5). Low N also decreased the root biomass, though only in plants grown under ambient CO_2 (Figs. 3.2 I & J; Table 1.1.5). These changes caused a respective increase in the root:shoot ratio (Figs. 3.2 M & N; Table 1.1.5). There was a sustained decrease in the leaf area (Figs. A & B; Table 1.1.17) and *LAR*, *SLA* and *LWR* in response to low N supply (Figs. 3.5 E & F, I & J, M & N; Table 1.1.17). The total and shoot water concentration was also decreased by low N availability (Figs. 3.3 M & N, Q & R; Table 1.1.11). However, unlike *P. maritima*, the root water content was also decreased (Figs. 3.3 U & V; Table 1.1.11).

ii. Droughted plants

Compared to plants grown at a high N availability, low N availability decreased the total and shoot biomass (Figs. 3.2 C & D, G & H; Table 1.1.5). Low N also decreased the root biomass, though only in plants grown under elevated CO_2 (Figs. 3.2 K & L; Table 1.1.5). There was a sustained decrease in the leaf area (Figs. C & D; Table 1.1.17), *SLA* and *LAR* (Figs. 3.5 G & H, K & L; Table 1.1.17). The total, shoot and root water contents were also decreased, though mainly in plants grown at ambient CO_2 (Figs. 3.3 O & P, S & T, W & X; Table 1.1.11).

3.3.3. Effect of drought on growth

Plantago maritima

i. High N supply

Drought did not greatly affect the plant biomass. There were only transient decreases in leaf area (Figs. 3.1 A & C; Table 1.1.3) and *LAR* and *SLA* (Figs. 3.1 E & G; I & K; Table 1.1.3). The plant water content was only reduced in plants grown at elevated CO_2 (Figs. 3.3 A & C, I & K; Table 1.1.9).

ii. Low N supply

The shoot biomass was increased in response to drought though only in plants that were grown at elevated CO_2 (Figs. 3.1 F & H; Table 1.1.3) and the root:shoot ratio was decreased in plants at ambient CO_2 (Figs. 3.1 N & P; Table 1.1.3). The *SLA* was decreased (Figs. 3.4 F & H; Table 1.1.15). The shoot water content was increased (Figs. 3.3 F & H; Table 1.1.9), however, the plant water content was decreased in plants grown at elevated CO_2 (Figs. 3.3 B & D, F & H, J & L; Table 1.1.9).

Armeria maritima

i. High N supply

The shoot and root biomass (Figs. 3.2 E & G, I & K; Table 1.1.6) were reduced in droughted plants at ambient CO_2, compared to plants that were well-watered. This led to a decrease of the root:shoot ratio (Figs. 3.2 N & P, M & O; Table 1.1.6). The *SLA* was reduced (Figs. 3.5 E & G; Table 1.1.18) and *LWR* was increased (Figs. 3.5 M & O; Table 1.1.18) by drought in plants grown at ambient CO_2. Drought reduced the total, shoot and root water content (Figs. 3.3 M & O, Q & S, U & W; Table 1.1.12) particularly those exposed to elevated CO_2.

ii. Low N supply

The plant biomass was significantly reduced in droughted plants that were exposed to elevated CO_2 (Figs 3.2 B & D; F & H; J & L; Table 1.1.6). The leaf area and *LWR* was increased and the *SLA* was decreased only in response to drought in plants with grown at ambient CO_2 (Figs. 3.5 B & D, F & H, J & L; Table 1.1.18). This effect did not occur in plants that were grown at elevated CO_2, as drought increased, rather than decreased, *LAR* and *SLA* (Figs. 3.5 F & H, J & L; Table 1.1.18).
The shoot water content was decreased, and the root water content was increased in response to drought when grown at ambient CO_2 (Figs. 3.3 R & T, U & X; Table 1.1.12). However, the whole plant water content was increased when grown at elevated CO_2 (Figs. 3.3 N & P, R & T, V & X; Table 1.1.12).

3.4. Plant nitrogen and photosynthetic changes in *Plantago maritima* and *Armeria maritima* in response to carbon, nitrogen and water availability

The effects of varying CO_2, N and water availability on N content, the concentrations of photosynthetic pigments and photosynthetic capacity in *P. maritima* and *A. maritima* are summarised in table 3.2. The table numbers 1.2.1 to 1.2.14 and 1.3.1 to 1.3.8 in the text refer to the statistical analyses presented in Appendix 1.2 and 1.3. The controls for elevated CO_2, low N and drought treatments are ambient CO_2, high N and well-watered conditions.

Table 3.2. Summary of statistically-significant effects, from tables 1.2.1. to 1.3.6 (Appendix 1.2 & 1.3), of elevated CO_2; nitrogen availability or drought on the nitrogen content and photosynthetic pigments and capacity of *Plantago maritima* and *Armeria maritima* after five and ten weeks exposure.
↑ = increase in nitrogen and photosynthetic measurements compared to control; ↓ = decrease in nitrogen and photosynthetic measurements compared to control; n.d. = no difference between treatment and control. Subscript numbers ($_5$ or $_{10}$) relate to which week the measurement was significantly different to the control.

Total, shoot and root N concentrations		***Plantago maritima***	***Armeria maritima***
Response to:	**Growth conditions**	**Response**	**Response**
Elevated CO_2	Well-watered; high N	n.d.	n.d.
	Well-watered; low N	↑ $root_{10}$	n.d.
	Drought; high N	↓ $total_5$ $root_5$	↓ $shoot_{10}$ ↑ $root_{10}$
	Drought; low N	n.d.	n.d.
Low N	Ambient CO_2; well-watered	↓ $total_5$ $shoot_5$ $root_5$	↓ $total_5$ $shoot_{5,10}$ $root_5$
	Ambient CO_2; Drought	↓ $total_5$ $shoot_{10}$ $root_5$	↓ $total_{5,10}$ $shoot_5$ $root_{10}$
	Elevated CO_2; well-watered	↓ $total_5$ $shoot_5$ $root_5$ ↑ $root_{10}$	↓ $total_{5,10}$ $shoot_5$
	Elevated CO_2; Drought	↓ $shoot_5$	↓ $total_{5,10}$ $shoot_{5,10}$
Drought	Ambient CO_2; High N	n.d.	↑ $total_{10}$ $root_{10}$
	Ambient CO_2; Low N	↓ $shoot_{10}$	n.d.
	Elevated CO_2; High N	n.d.	↑ $shoot_{10}$
	Elevated CO_2; Low N	n.d.	n.d.

Carotenoid (carot); Chlorophyll (chloro); Photosynthetic capacity (photo)		***Plantago maritima***	***Armeria maritima***
Response to:	**Growth conditions**	**Response**	**Response**
Elevated CO_2	Well-watered; high N	↓ $carot_5$ $chloro_5$ $photo_5$	↓ $carot_{10}$ $chloro_5$ $photo_{5,10}$
	Well-watered; low N	n.d.	↓ $chloro_{5,10}$
	Drought; high N	↓ $photo_5$	n.d.
	Drought; low N	↓ $carot_5$	↑ $carot_{10}$ ↓ $photo_{10}$
Low N	Ambient CO_2; well-watered	↓ $carot_5$ $chloro_5$	↓ $chloro_5$ $photo_5$
	Ambient CO_2; Drought	↑ $carot_5$ ↓ $photo_{10}$	↓ $carot_5$ $chloro_5$ $photo_5$
	Elevated CO_2; well-watered	↓ $carot_5$	↓ $carot_{5,10}$ $chloro_{5,10}$
	Elevated CO_2; Drought	n.d.	↓ $photo_{10}$
Drought	Ambient CO_2; High N	n.d.	↓ $chloro_5$ $photo_5$
	Ambient CO_2; Low N	↑ $carot_5$	↓ $photo_5$
	Elevated CO_2; High N	n.d.	↑ $chloro_5$
	Elevated CO_2; Low N	n.d.	↑ $chloro_5$

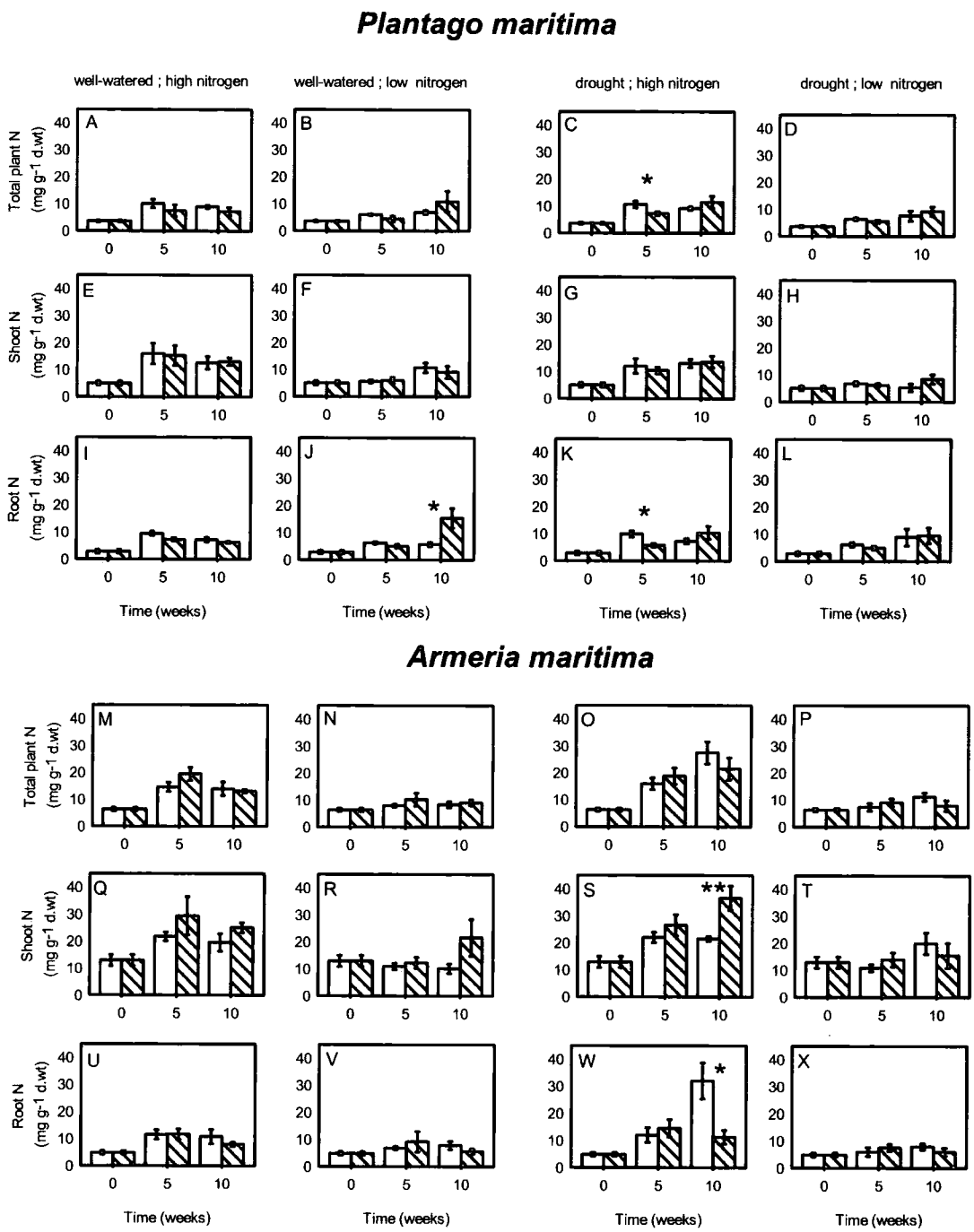


Figure 3.6. The effect of 360 (open bars) and 600 (hatched bars) μmol CO_2 mol^{-1} in combination with 28 mg l^{-1} N (high N) or 2.8 mg l^{-1} N (low N) on total plant N (A-D; M-P), shoot N (E-H; Q-T) and root N (I-L; U-X) in *Plantago maritima* and *Armeria maritima*. Plants were grown over ten weeks under well-watered (80% field capacity) or drought (30% field capacity) conditions. Data represent mean ± one SE. $n = 12$. Significant effects of elevated CO_2 are indicated by * ($P \leq 0.05$); ** ($P \leq 0.01$) and *** ($P \leq 0.001$).

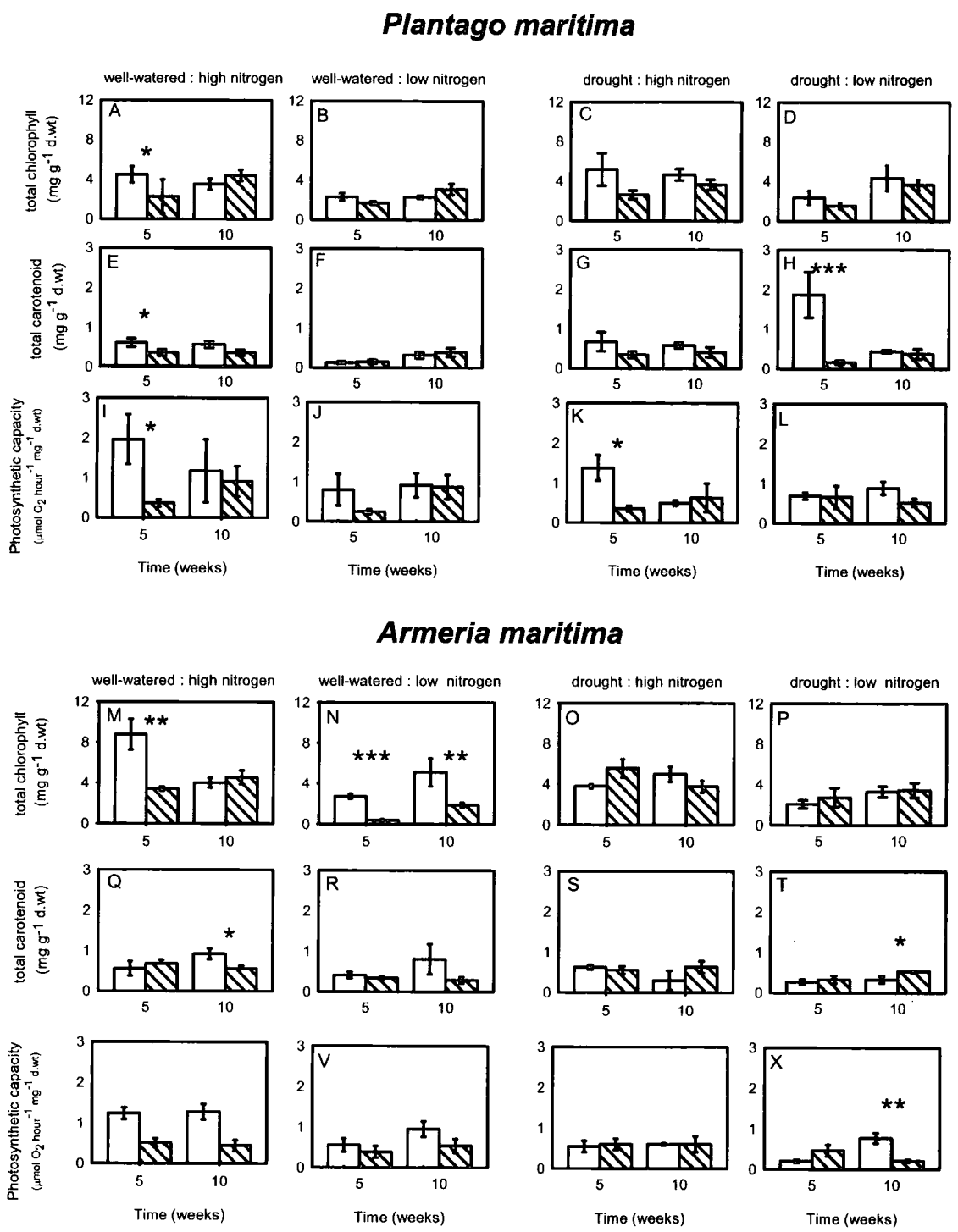


Figure 3.7. The effect of 360 (open bars) and 600 (hatched bars) µmol CO_2 mol^{-1} in combination with 28 mg l^{-1} N (high N) or 2.8 mg l^{-1} N (low N) on carotenoid (A-D; M-P), chlorophyll (E-H; Q-T) and photosynthetic capacity (I-L; U-X) in *Plantago maritima* and *Armeria maritima*. Plants were grown over ten weeks under well-watered (80% field capacity) or drought (30% field capacity) conditions.
Data represent mean ± one SE. $n = 12$. Significant effects of elevated CO_2 are indicated by * ($P \leq 0.05$); ** ($P \leq 0.01$) and *** ($P \leq 0.001$).

3.4.1. Effect of elevated CO_2 on plant N and photosynthesis

Plantago maritima

i. Well-watered plants

There were no significant changes in the shoot N concentration in *P. maritima* grown at elevated CO_2, compared to plants grown at ambient CO_2. However, the root N concentrations were increased, though only in plants grown at low N availability (Figs. 3.6 J; Table 1.2.1). Elevated CO_2 reduced the carotenoid and chlorophyll concentrations and photosynthetic capacity in plants that were grown with high N availability (Figs. 3.7 A, E, I. K; Table 1.3.1).

ii. Droughted plants

The total and root N concentrations were decreased in response to elevated CO_2, though only in plants with high N availability (Figs. 3.6 C, K; Table 1.2.1). The carotenoid concentrations and photosynthetic capacity were decreased (Figs. 3.7 T & X; Table 1.3.1).

Armeria maritima

i. well-watered plants

Elevated CO_2 did not affect the N concentration in well-watered plants. Total chlorophyll concentrations were decreased by elevated CO_2 (Figs. 3.7 M, N; Table 1.3.4). The photosynthetic capacity and carotenoid concentrations were decreased in plants grown at elevated CO_2 though only in plants grown at high N supply (Fig. 3.7 Q, U; Table 1.3.4)

ii. droughted plants

Nitrogen concentrations were lower in the shoots and higher in the roots, though only in with high N availability (Figs. 3.6 S, W; Table 1.2.4). The carotenoid concentrations were higher, and the photosynthetic capacity was decreased in plants grown under elevated CO_2 at low N availability (Fig. 3.7 T, X; Table 1.3.4).

3.4.2. Effect of low N availability on plant N and photosynthesis

Plantago maritima

i. well-watered plants

Low N availability generally resulted in decreased total, shoot and root N concentrations (Figs. 3.6 A & B, E & F, I & J; Table 1.2.2). Low N availability decreased the carotenoid and chlorophyll concentrations, when compared to plants grown at high N availability (Figs. 3.7 A & B; E & F; Table 1.3.2).

ii. droughted plants

In plants exposed to drought, only shoot N showed a significant reduction in plants grown at ambient CO_2 (Figs. 3.6 G & H; I & J; Table 1.2.2). Only at ambient CO_2 did low N availability increase carotenoid concentrations and reduce photosynthetic capacity (Figs. 3.7 G & H, K & L; Table 1.3.2).

Armeria maritima

i. well-watered plants

Reduced N availability decreased the total and shoot N concentrations, compared to plants with high N availability (Figs. 3.6 M & N, Q & R; Table 1.2.5). The root N concentrations were only decreased in plants exposed to ambient CO_2 (Figs. 3.6 U & V; Table 1.2.5). Generally, carotenoid and chlorophyll concentrations were lowered in response to low N availability (Figs. 3.7 M & N, Q & R; Table 1.3.5). Photosynthetic capacity was also decreased in response to low N though only in plants exposed to ambient CO_2 (Figs. 3.7 U & V; Table 1.3.5)

ii. droughted plants

Reduced N availability decreased the total and shoot N concentrations, compared to plants with high N availability (Figs. 3.6 O & P, S & T; Table 1.2.5). The root N concentrations were only decreased in plants exposed to ambient CO_2 (Figs. 3.6 W & X; Table 1.2.5). Carotenoid and chlorophyll concentrations were lowered in response to low N availability, though only in plants exposed to ambient CO_2 (Figs. 3.7 M & N, Q & R; Table 1.3.5). Photosynthetic capacity was also decreased in response to low N (Figs. 3.7; W & X; Table 1.3.5).

3.4.3. Effect of drought on plant N and photosynthesis

Plantago maritima

Drought had little effect on the N concentrations in *P. maritima*. Only a lowering of shoot N occurred in plants grown at ambient CO_2 with a low N supply (Figs. 3.6 F & H; Table 1.2.3). Also, there was only an increase in the carotenoid concentrations in response to drought, in plants grown at ambient CO_2 with a low N supply (Figs. 3.7 F & H; Table 1.3.3).

Armeria maritima

Drought caused an increase in the total and root N concentrations in *A. maritima* grown at ambient CO_2 with high N supply (Fig. 3.6 M & O; U & W; Table 1.2.6) and in the shoot N concentrations in plants grown at elevated CO_2 with high N supply (Figs. 3.6 Q & S; Table 1.2.6). The chlorophyll concentrations and photosynthetic capacity were decreased in response to drought when grown at ambient CO_2 (Figs. 3.7 M & O, U & W; V & X; Table 1.3.6). However, in plants grown at elevated CO_2, there were only increases in chlorophyll concentrations (Fig. 3.7 M & O, N & P; Table 1.3.6).

3.5. Conclusions and Discussion

The main objective of this study was to determine the different biochemical and physiological responses to drought in *P. maritima* and *A. maritima* when exposed to elevated atmospheric CO_2. In the present investigation, *P. maritima* and *A. maritima* have shown to have opposing responses to elevated CO_2, as *A. maritima* was more responsive to changes in C and N availability than *P. maritima*, especially in terms of decreasing the amount of fixed C into new leaf matter. However, low N availability, compared to plants grown at a high N supply, had more effect on biomass and dry matter partitioning than elevated CO_2. It was also hypothesised that the growth and photosynthetic responses may change in droughted plants where there might be further increases in C and N-based compatible solutes and internal competition for C and N between allocation for growth and osmolyte production in *P. maritima* and *A. maritima*. However, drought did not have any detrimental affect on the biomass in plants with an altered C or N supply. The reported physiological responses to changes and interactions of drought, N availability and atmospheric CO_2 concentration are discussed in the following sections.

3.5.1. Plant responses to drought

Biomass and dry matter partitioning

Drought usually significantly affects the growth of plants, however, *P. maritima* and *A. maritima* are considered to be drought-tolerant (Goldsmith 1967). This may explain why these plants were not greatly affected by such treatment, and there was little effect on the growth responses to drought when combined with low N or elevated CO_2 treatments. It is interesting that the root biomass was reduced in droughted plants in both species as increased rooting would have allowed an increase in the uptake of water in drought situations (Wullschleger *et al.* 2002). These results are consistent with a study by Buckland *et al.* (1991) and Köhl (1996) who found that drought caused a 10 – 20 % decrease in biomass in the salt-marsh ecotype of *A. maritima* at ambient CO_2. There was also a tendency for the lowering of root growth to be less than the shoot growth, so causing a lower the root:shoot ratio (Köhl (1996). In the present study, there were also increases in the allocation of dry matter to the foliage of droughted *A. maritima* plants as *LWR* and *LAR* were increased. This implies that although there was a greater allocation of resources to the leaf as a whole, there was a possible increase in the thickness of the leaves or an increase in plant dry matter. Any increase in leaf

area, *LWR* or *LAR* may cause an increase in transpiration and is therefore partly responsible for the lower water concentrations in droughted plants growing under ambient and elevated CO_2 (Wolfe *et al.* 1998; Poorter & Perez-Soba 2001). Although it is not possible to explain the advantage of increased shoot matter in drought situations, it may be that the plants have a tighter control over other water reducing functions such as by reducing stomatal conductance and hence evaporative water loss (Popilsilova & Catsky 1999).

The main response to drought in the present study was a reduction in water content, particularly in the shoots. The results in the present study are in agreement with responses observed in *A. maritima* by Köhl (1996) where drought (30 % FC) also had more of an effect on the leaves with reductions in the water content from 5 – 25 % of the control. Root water contents in the study by Köhl (1996) were on average between 8-10 g H_2O g^{-1} dry weight and shoot water content was on average between 4 and 5 g H_2O g^{-1} dry weight. Interestingly, the water content was not affected by drought in *P. maritima* grown under ambient CO_2 with a high N supply (Fig. 3.3). However, if the N supply was reduced, the shoot water content was increased. The opposite occurred in droughted plants grown at elevated CO_2 where the plant water content was reduced at higher availabilities of N. The water concentration of droughted plants can increase in droughted plants as cell size is increased to accommodate increases in osmotic compounds (Glenn & O`Leary 1984; Walsh 2000). This may explain why there were increases in the water content of some droughted plants, though this does not explain why it was only the case in plants growing with a low N supply.

Plant N; photosynthetic pigment concentrations and photosynthetic capacity

Drought had little effect on the N concentration of *P. maritima*, compared to well-watered plants. However, in *A. maritima,* drought significantly increased the total and root N concentrations grown at ambient CO_2 with a high N supply (Fig. 3.6). There was also an increase in the shoot N concentrations in *A. maritima* grown at elevated CO_2 with a high N supply. Interestingly, these increases did not occur when the N availability was low. These increases in N concentrations may be due to an increased accumulation of N-based compatible solutes in *A. maritima*. This hypothesis will be further explored in chapter 4.

There was also a species-specific response in the photosynthetic response to drought. In *P. maritima*, drought did not seriously affect the photosynthetic pigment concentrations or photosynthetic capacity, though they were seriously altered in *A. maritima* with

contrasting responses when grown at ambient or elevated CO_2 (Fig. 3.7). The chlorophyll concentrations and photosynthetic capacity were decreased in response to drought when grown at ambient CO_2 but, in droughted plants grown at elevated CO_2, there were only increases in chlorophyll concentrations. This decrease of chlorophyll, rather than carotenoids in *A. maritima*, could be due to the fact that chlorophyll degrades faster than carotenoids in droughted plants (Yordanov *et al.* 2000). Such chlorophyll degradation was also shown in a study by Schwanz & Polle (2001) who compared drought and non-drought tolerant plants (Oak, *Quercus robur*; and Pine, *Pinus pinaster* respectively). In their study, the drought tolerant species had a less of a carotenoid response to drought when compared to the non-tolerant species. However, under elevated CO_2, the non-drought tolerant species had a lower loss of pigments causing some ameliorative effects when under drought conditions. Therefore, the damaging effects of drought can be alleviated under elevated CO_2 as the amount of chlorophyll and carotenoid degradation is reduced.

3.5.2. Plant responses to low nitrogen availability

Biomass and dry matter partitioning

Low N availability, compared to plants grown at a high N supply, had more effect on biomass and dry matter partitioning than elevated CO_2. The response to low N availability in *A. maritima* was similar to *P. maritima* as the total, shoot and root biomasses were all decreased (Fig. 3.2). The main difference between the species in response to low N supply was that in *A. maritima* the response was more prominent after ten weeks whereas the low N supply affected the biomass of *P. maritima* from week five. However, the opposite occurred for leaf area and dry matter partitioning as the leaf area, *LAR*, *SLA* and *LWR* were all reduced from week five in response to low N supply, as opposed to changes occurring mainly after ten weeks in *P. maritima* (Fig. 3.5). This implies that although the biomass does not respond to low N availability as quick in *A. maritima*, this species has higher morphological plasticity, so it can alter its dry matter partitioning sooner than *P. maritima*. The lowering of *LAR* in plants with a low N supply is related to decreases in *SLA*, *LWR* and a shift in allocating resources from the shoot to the root for biomass production. These responses are all seen in *P. maritima* and *A. maritima* in the present study and could be due to decreases in cell expansion rates and/or the total numbers of cells in the leaves (Chapin 1980; Van Arendonk *et al.* 1997). Reductions in *SLA* can be due to decreases in leaf thickness or increases in the percent dry matter. De Pinheiro Henriques & Marcelis (2000)

suggested that increases in percent dry matter in plants grown at a low N supply could be due to a lower concentration of nitrates being stored in the vacuole for osmoregulation. This would result in less water uptake by the cells and cause increased dry matter accumulation. However, as *P. maritima* and *A. maritima* can use compounds other than nitrates for osmoregulation it is possible that reductions in *SLA* in these studies are due to an increase in leaf thickness. The water concentration in response to a low N supply in *A. maritima* was also similar to that of *P. maritima* (Fig. 3.3). However, in *A. maritima* the root water content was decreased as well as the total and shoot water content. These results are in agreement with the salt marsh varieties of *P. maritima* and *A. maritima*, where *A. maritima* had a more varied response to altered N availability than *P. maritima* at ambient CO_2, (Jefferies 1977).

Plant N; photosynthetic pigment concentrations and photosynthetic capacity

Low N supply decreased N concentrations in *A. maritima* in a similar fashion to *P. maritima*, though the lowering of N in *A. maritima* also occurred after ten weeks (Fig. 3.6). These plant N concentrations are in agreement with N concentrations measured in the salt marsh varieties of the study species (Stewart *et al.* 1973). *Armeria maritima* had 19-26 mg N g^{-1} dry weight and *P. maritima* ranged from 9 to 18 mg N g^{-1} dry weight depending where on the salt marsh the samples were taken. The differences in N concentrations were attributed to increases in nitrate reductase activity, with *A. maritima* possessing a higher nitrate reductase activity than *P. maritima*, which might also account for the altered N content between the species in the present study. Changes in N availability can decrease photosynthetic rates (Peri *et al.* 2002) as N supply is lowered and N demand is increased, then growth is usually reduced and N from older leaves an be exported for new growth (Chapin 1980). The changes in photosynthetic pigments were quite different in *A. maritima* when compared to *P. maritima*, as the photosynthetic pigments were also reduced in response to a low N supply (Fig. 3.7). This reduced the photosynthetic capacity of plants grown at ambient CO_2. As mentioned in the previous section (3.5.1), it could be that the N, which is bound to such pigments, is reallocated for other processes, such as production of nitrogenous compatible solutes, when N is in short supply. This would particularly be the case when the plants are droughted. However, this lowering of pigment concentrations did not occur in droughted plants at elevated CO_2.

3.5.3. Plant responses to elevated atmospheric CO_2

Biomass and dry matter partitioning

For both species, it is interesting that the only conditions in which plant-biomass was affected by elevated CO_2, compared to plants grown under ambient CO_2, was in well-watered plants that had a low N supply. A review by Stitt & Krapp (1999) concluded that there is usually an increase in biomass with elevated CO_2, but only when there is an adequate N supply. This appeared to hold true for *P. maritima* though only in the sense that there was a reduction in biomass when plants were grown at a combined treatment of elevated CO_2 and low N supply, compared to plants at ambient CO_2. However, in *A. maritima* the shoot biomass increased in response to elevated CO_2 combined with a low N supply. This could be due to the fact that *A. maritima* possess higher activities of nitrate reductase than *P. maritima* (Stewart *et al.* 1973). This may allow *A. maritima* to better utilise the high CO_2 supply at low N conditions. Also, root:shoot ratios of *P. maritima* and *A. maritima* were not greatly affected and this is in agreement with studies on other coastal plants exposed to elevated CO_2 (Lenssen *et al*, 1995). The non-responsiveness of *Plantago maritima* to elevated CO_2, in terms of biomass and dry matter partitioning, is unlike other *Plantago* species such as *Plantago lanceolata* and *Plantago major* that show an increase in biomass in response to elevated CO_2 (den Hertog *et al.*1993; Klus *et al.* 2001).

The greatest difference in the inter-specific response to elevated CO_2 was in the level of change in the dry-matter partitioning. Leaf area, *SLA* and *LAR* were all significantly decreased in *A. maritima*, but not so much in *P. maritima*, under elevated CO_2. Also, the *LWR* was increased by elevated CO_2 in well-watered *A. maritima* that had a low N supply. These results imply that there was more investment into leaf production as a whole. This is in agreement with studies on two other coastal plants (*Aster tripolium* and *Puccinellia maritima*) where a decrease in *SLA* was also found when plants were exposed to high salt and elevated CO_2 concentrations (Lenssen *et al.* 1995). Lowered *SLA* could be due to either increases in leaf thickness or an increase in the dry matter accumulation per unit leaf area, such as increased non-structural carbohydrates within the tissue (den Hertog *et al.* 1998). Recalculation of *SLA* on a structural dry weight basis (i.e. deducting the total non-structural carbohydrate (TNC) from the sample dry weight) can remove any CO_2 effect on *SLA*. However, as TNC concentrations did not change in *A. maritima* (Chapter 4), the reduced *SLA* could be due to increased leaf thickness instead, with more cell layers or increased cell size (Baxter *et al.* 1995;

Roumet *et al.* 1999). Unfortunately it was not possible to measure cell size at the time of the experiment. As elevated CO_2 caused an increased *LWR* in well-watered plants grown at low N availability along with a concurrent reduction in *SLA*, it suggests that the morphology of the leaves altered along with an increase in the investment of dry matter to the leaves (den Hertog *et al.* 1998). It was hypothesised that changes in *SLA* and *LAR* would not occur in *P. maritima* because excess C is allocated to the non-structural sugar alcohol, sorbitol so that an increase of other non-structural carbohydrates does not occur. This is discussed further in chapter 4. Reductions in *LAR* at elevated CO_2 may be due to an adaptive acclimation mechanism as the plant adjusts the balance between C assimilation and utilization. It is interesting that the reduced *LAR* at elevated CO_2 only occurred in droughted plants of *A. maritima*. The reduced leaf area as a ratio of the whole plant weight implies that the plant can reduce its evaporative water loss by reducing leaf area whilst maintaining photosynthetic capacity at elevated CO_2 (Quick *et al.* 1991; Wolfe *et al.* 1998). The change in plant biomass and dry matter partitioning in *A. maritima* and *P. maritima* in response to elevated CO_2 was not observed when the plants were droughted. Although the leaf area, *SLA* and *LAR* were still lowered by elevated CO_2 when combined with drought in *A. maritima*, compared to well-watered plants at ambient CO_2, the effects were significant after five weeks and had disappeared by ten weeks. These results are in partial agreement with a study by Arp *et al.* (1998); who found that water supply did not show a significant interaction with elevated CO_2 in six perennial heathland species. In their study, N supply had the greatest effect on biomass at elevated CO_2, as in the present investigation. However, the results from *A. maritima* were not in agreement with that study as watering regime and CO_2 supply did have a significant treatment interaction.

The elevated CO_2 effect on the water content was also species-specific (Fig. 3.3). In *P. maritima*, the water content was increased when compared to well-watered plants at ambient CO_2 whilst in *A. maritima* the water content was decreased in response to elevated CO_2. However, a combined elevated CO_2 and drought treatment caused the opposite response to the CO_2 effect in well-watered plants. This shows that drought can alter water conservation in *P. maritima* and *A. maritima* in response to elevated CO_2. The changes in water content at a combined treatment of elevated CO_2 and drought could be due to altered leaf transpiration as a decrease in transpiration rates is commonly observed in plants exposed to elevated CO_2 (Wullschleger *et al.* 2002). This hypothesis is tested in chapter 5.

Plant N; photosynthetic pigment concentrations and photosynthetic capacity

In agreement with the hypothesis that there would be a species-specific response to elevated CO_2, due to the different processes of compatible solute accumulation, the response to elevated CO_2 was slightly different in *A. maritima* when compared to *P. maritima*. Overall, the N concentration in the shoots of *A. maritima* was 40 – 50 % higher than the shoot N concentrations in *P. maritima* (Fig. 3.6). Whilst elevated CO_2 did not affect shoot N concentrations in *P. maritima*, there were some increases in the root N concentrations in well-watered plants. This may be due to the reallocation of N from the shoot to root growth in order to increase the rate and amount of N uptake from the soil. The increase in N supply could be used to increase shoot biomass, so allowing the plant to further benefit from the increased C supply (Stitt & Krapp 1999). In *A. maritima*, there was an increase in root N concentration though only in droughted plants with a high N supply. Again, this could be due to reallocation of N from the shoot as the shoot N concentration was reduced. A common response to elevated CO_2 is a reduction in chlorophyll concentration (eg. 10 % reduction in chlorophyll in young birch *Betula pendula*), with the decrease in chlorophyll being correlated to decreases in leaf N concentrations (Nakano *et al.* 1997; den Hertog *et al.* 1998; Rey & Jarvis 1998). The chlorophyll concentrations can be reduced so that the N used in the chlorophyll-binding protein can be allocated to other N-demanding functions when the N-supply is low (Rey & Jarvis 1998; Evans & Poorter 2001). However, in *A. maritima*, the reallocated N was probably not taken from chlorophyll- or carotenoid-binding proteins, as there was not a reduction in these pigments (den Hertog *et al.* 1998). In addition, as photosynthesis is usually related to plant N concentrations, if plant N is reduced, this can be correlated to a decrease in the photosynthetic capacity (Baxter *et al.* 1995; Cook 1998; Davey *et al.* 1999). Interestingly, in *P. maritima*, there was still a decrease in the photosynthetic capacity in response to elevated CO_2 even when the concentrations of photosynthetic pigments were not lowered. This implies that other photosynthetic processes may be responsible for the reduction in photosynthetic capacity. A likely candidate for this would be changes in the activity or concentration of the enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco; E.C. 4.1.1.39; Stitt 1991). As Rubisco is not saturated at the current atmospheric CO_2 concentration, any increase in the CO_2 substrate will increase photosynthetic rates in the short-term. However, increased rates of photosynthesis are usually not sustainable as other nutrients become limiting. This can lead to a reduction in the concentration of

Rubisco, and hence photosynthetic capacity (Bowes 1991; Stitt 1991; Stitt & Krapp 1999). The change in Rubisco activity in response to long-term exposure to elevated CO_2 is further explored in chapter 6.

3.5.4. General conclusions

1. An increased CO_2 supply to *P. maritima* did not alter plant biomass, dry matter partitioning or N concentrations. However, water concentration was lowered in the shoot and increased in the root and there was a general decrease in photosynthetic pigments and capacity.
2. An increased CO_2 supply to *A. maritima* did not alter plant biomass. However, *SLA* and *LAR* were reduced. There were variable responses in the water concentrations, but the trend was that they were reduced by elevated CO_2. There were modest increases in the N concentrations in the shoots and a decrease in the photosynthetic pigments and capacity, though mainly in well-watered plants.
3. Low N availability in *P. maritima* reduced plant biomass and increased root:shoot ratio, leaf area, *SLA*, *LAR* and *LWR*. Shoot water concentrations were reduced but root concentrations were not. There were modest reductions in the N concentrations, photosynthetic pigments and photosynthetic capacity, though mainly in well-watered plants.
4. Low N availability in *A. maritima* reduced plant biomass and increased root:shoot ratio, leaf area, *SLA*, *LAR* and *LWR*. The changes in dry matter partitioning occurred at an earlier time than those in *P. maritima*. The shoot and root water contents and plant N concentrations were reduced. The photosynthetic pigments and capacity were reduced, though mainly in plants grown at ambient CO_2.
5. Drought did not alter plant biomass, N concentrations, photosynthetic pigments or photosynthetic capacity in *P. maritima*. However, the *SLA* was reduced along with reductions in the water concentrations, though mainly in plants grown at elevated CO_2.
6. Drought reduced plant biomass and root:shoot ratio in *A. maritima*. The *SLA* was reduced and *LWR* was increased, though mainly in plants exposed to ambient CO_2. The water concentrations were reduced though this response varied with N supply. The N concentrations were increased, though only in plants with a high N supply, and the chlorophyll concentrations and photosynthetic capacity were reduced when grown at ambient CO_2. In plants grown at elevated CO_2 there were increases in the chlorophyll concentrations, though this did not affect the photosynthetic capacity.

Chapter 4. Compatible solute production and partitioning of *Plantago maritima* and *Armeria maritima* in response to increased atmospheric CO_2 under varying water and nitrogen availability

Aim

The aim of the study within this chapter was to determine how the accumulation of compatible solutes and non-structural carbohydrates in *Plantago maritima* and *Armeria maritima* were affected by drought and whether these effects were further influenced by elevated atmospheric CO_2 and soil nitrogen (N) availability. This chapter complements chapter three, which describes changes in the growth, nutrient status and photosynthesis in response to the above growth conditions.

4.1 Introduction

Photosynthesis is usually stimulated when plants are exposed to elevated CO_2 causing increased non-structural carbohydrate concentrations, particularly starch (Baxter *et al.* 1995; Farrar 1999; Stitt & Krapp 1999). In addition, shoot N concentrations can decrease as N is reallocated from proteins involved in photosynthesis to other functions such as growth (Poorter *et al.* 1997; Stitt & Krapp 1999). Such changes in the allocation of carbon (C) and N may affect the regulation of either N- or C-based compounds involved in plant compatible solute metabolism, which is important for plant survival in adverse environments (Popp & Smirnoff 1995). However, it is unclear whether or not C can be incorporated into such C-based solutes as an extra C sink under elevated CO_2 and if N can be similarly reallocated when grown under elevated CO_2, particularly with low N availability.

Any partitioning of C and N into compatible solutes makes substantial demands on the plant C and N economy and so may cause a direct effect on the concentrations of soluble and insoluble C (reduced sugars and starch) and N (Storey *et al.* 1977; Stewart & Larher 1980). For example, these soluble nitrogenous compatible solutes may be concentrated at up to 250 mM within the cell and can account for over 20 % of total plant N concentration (Singh *et al.* 1973; Storey *et al.* 1977; Stewart & Rhodes 1978; Jefferies *et al.* 1979). Also, sorbitol can account for as much as 50 % of C allocated to the total non-structural carbohydrate pool within plant tissue (Jefferies *et al.* 1979).

In view of the different metabolite changes induced in different plant species in response to stress, there is an important need to evaluate how the accumulation of compatible solutes in plants is affected by ambient and elevated CO_2 concentrations.

This study was designed to manipulate the C and N requirement of *A. maritima* and *P. maritima* to investigate the relationship between C allocation in non-structural carbohydrates and the use of both C and N compatible solutes. The main hypothesis of this study was that with differing availability of N supplies, the biochemical responses to drought in terms of compatible solute metabolism in *P. maritima* and *A. maritima* would result in contrasting biochemical responses to elevated atmospheric CO_2. In response to elevated CO_2, the proportion of nitrogenous compatible solutes might be expected to decrease in the N-accumulating *A. maritima*, as under limiting conditions available N would be required for growth functions. Conversely, with increased CO_2 availability and low N, the amount of the C-based sorbitol might be expected to increase in *P. maritima* during drought as this species allocate large amounts of C to sorbitol as well as to other non-structural carbohydrates.

4.2 Methods

The experimental design was as stated in section 3.2.1. The extraction and analyses for betaine, proline, sorbitol and non-structural carbohydrates are described in detail in sections 2.10 and 2.11 and the statistical analysis procedures detailed in section 2.15. The total shoot and root N concentrations quoted within this chapter (section 4.6) are taken from the previous chapter (section 3.4.).

4.3 Results: Effect of resource availability on compatible solutes, soluble carbohydrates and starch

The effects of varying CO_2, N and water availability on compatible solutes, soluble carbohydrates and starch in *P. maritima* and *A. maritima* are summarised in Table 4.3. Tables 2.1.1 to 2.2.6 in the text refer to the statistical tables in appendix 2.

Table 4.1. Concentrations of the compatible solutes sorbitol and proline, soluble carbohydrates and starch in the shoot and root of *Plantago maritima* after five and ten week's growth. Plants were exposed to either ambient (360 μmol mol^{-1}) or elevated (600 μmol mol^{-1}) atmospheric CO_2. Data represent metabolite concentrations under treatments, well-watered and high N availability, well-watered with low N availability, drought with high N availability and drought with low N availability. Data represent mean ± one SE. n = 5-6. Significant differences between CO_2 treatments are indicated by * ($P \leq 0.05$); ** ($P \leq 0.01$).

Compound			Five weeks		Ten weeks	
			Atmospheric CO_2 concentration (μmol CO_2 mol^{-1})			
	Treatment		360	600	360	600
Sorbitol (mg g^{-1} d.wt)	Watered + N	Shoot	32.30 ±7.27	40.09 ±14.52	54.45 ±12.28	52.11 ±6.31
		Root	3.32 ±1.19	10.30 ±3.90	25.70 ±7.03	25.32 ±7.33
	Watered – N	Shoot	31.44 ±11.50	41.72 ±12.62	47.32 ±12.16	45.37 ±8.01
		Root	25.84 ±7.11	19.94 ±2.26	21.21 ±1.54	17.14 ±6.48
	Drought + N	Shoot	57.33 ±5.42	28.45 ±9.33	53.80 ±11.73	42.95 ±12.16
		Root	22.18 ±8.71	22.25 ±8.11	15.02 ±3.72	11.41 ±3.77
	Drought - N	Shoot	55.98 ±7.46	46.71 ±4.27	68.74 ±5.34	29.36 ±8.19 **
		Root	27.89 ±2.78	43.76 ±6.57	13.22 ±0.86	27.86 ±5.97
Proline (mg g^{-1} d.wt)	Watered + N	Shoot	0.03 ±0.01	0.01 ±0.01	0.03 ±0.02	0.00 ±0.00
		Root	0.03 ±0.01	0.04 ±0.02	0.00 ±0.00	0.00 ±0.00
	Watered – N	Shoot	0.06 ±0.01	0.04 ±0.01	0.05 ±0.01	0.02 ±0.01
		Root	0.06 ±0.02	0.10 ±0.01	0.00 ±0.00	0.00 ±0.00
	Drought + N	Shoot	0.01 ±0.01	0.05 ±0.02	0.04 ±0.02	0.02 ±0.01
		Root	0.00 ±0.00	0.08 ±0.05	0.03 ±0.01	0.01 ±0.01
	Drought - N	Shoot	0.03 ±0.02	0.02 ±0.01	0.04 ±0.01	0.03 ±0.01
		Root	0.03 ±0.01	0.13 ±0.03 *	0.02 ±0.01	0.01 ±0.00
Soluble carbohydrate (mg glucose equivalents g^{-1} d.wt)	Watered + N	Shoot	27.62 ±3.11	27.85 ±2.35	32.83 ±4.71	28.69 ±1.53
		Root	53.26 ±12.30	38.67 ±9.37	72.83 ±10.80	68.93 ±8.02
	Watered – N	Shoot	40.85 ±1.67	38.82 ±1.64	30.49 ±4.74	39.83 ±6.57
		Root	62.25 ±11.42	99.24 ±7.60 *	78.83 ±11.38	111.63 ±8.60
	Drought + N	Shoot	37.09 ±5.31	30.56 ±3.07	26.70 ±5.19	26.47 ±5.27
		Root	44.81 ±8.96	51.67 ±13.05	49.57 ±7.54	54.23 ±16.81
	Drought - N	Shoot	40.70 ±3.66	36.50 ±4.60	38.45 ±5.96	28.56 ±3.02
		Root	60.01 ±10.03	79.23 ±21.84	81.35 ±14.63	62.67 ±8.22
Starch (mg g^{-1} d.wt)	Watered + N	Shoot	14.05 ±3.70	36.02 ±13.46	33.02 ±7.87	29.24 ±6.45
		Root	24.30 ±9.48	0.40 ±0.02 *	16.30 ±6.33	29.46 ±4.04
	Watered – N	Shoot	28.79 ±12.09	68.36 ±14.56	30.25 ±3.67	60.42 ±3.71 **
		Root	3.11 ±1.33	0.40 ±0.02	16.54 ±1.40	31.90 ±5.04 *
	Drought + N	Shoot	34.85 ±3.05	4.29 ±0.49 **	21.12 ±4.01	61.33 ±17.44
		Root	0.33 ±0.02	12.16 ±6.23	26.30 ±4.09	20.76 ±6.05
	Drought - N	Shoot	20.01 ±8.41	66.21 ±27.80	48.23 ±10.00	33.20 ±11.97
		Root	0.35 ±0.01	11.37 ±3.05 *	21.18 ±2.77	18.70 ±4.49

Table 4.2. Concentrations of the compatible solutes betaine and proline, soluble carbohydrates and starch in the shoot and root of *Armeria maritima* after five and ten week's growth. Plants were exposed to either ambient (360 μmol mol^{-1}) or elevated (600 μmol mol^{-1}) atmospheric CO_2. Data represent metabolite concentrations under treatments, well-watered and high N availability, well-watered with low N availability, drought with high N availability and drought with low N availability. Data represent mean ± one SE. n = 5-6. Significant differences between CO_2 treatments are indicated by * ($P \leq 0.05$); ** ($P \leq 0.01$); *** ($P \leq 0.001$).

Compound			Five weeks		Ten weeks	
			Atmospheric CO_2 concentration (μmol CO_2 mol^{-1})			
	Treatment		360	600	360	600
Betaine (mg g^{-1} d.wt)	Watered + N	Shoot	7.97 ±0.43	9.70 ±0.51 *	13.86 ±2.18	6.20 ±1.74 *
		Root	4.44 ±0.72	3.46 ±0.37	4.61 ±3.61	1.87 ±0.75
	Watered – N	Shoot	7.55 ±1.35	9.81 ±2.97	4.50 ±0.79	7.77 ±2.89
		Root	1.44 ±0.41	3.45 ±0.56 *	0.01 ±0.00	5.89 ±1.21 **
	Drought + N	Shoot	12.69 ±1.18	6.76 ±1.28 *	6.96 ±0.56	9.09 ±2.34
		Root	6.99 ±0.25	4.03 ±0.93 *	3.41 ±0.80	1.27 ±0.63
	Drought - N	Shoot	7.75 ±1.12	4.22 ±1.30	6.55 ±0.72	4.20 ±1.22
		Root	4.56 ±0.51	5.00 ±1.38	4.98 ±1.76	5.79 ±2.27
Proline (mg g^{-1} d.wt)	Watered + N	Shoot	0.10 ±0.02	0.19 ±0.05 *	0.31 ±0.10	0.32 ±0.10
		Root	0.04 ±0.01	0.05 ±0.00	0.25 ±0.05	0.49 ±0.06 *
	Watered – N	Shoot	0.05 ±0.01	0.06 ±0.00	0.40 ±0.12	0.14 ±0.04
		Root	0.02 ±0.01	0.03 ±0.01	0.28 ±0.03	0.41 ±0.08
	Drought + N	Shoot	0.15 ±0.03	0.23 ±0.08	0.42 ±0.10	0.64 ±0.22
		Root	0.24 ±0.02	0.08 ±0.02 **	0.30 ±0.09	0.40 ±0.11
	Drought - N	Shoot	0.07 ±0.01	0.06 ±0.01	0.39 ±0.06	0.52 ±0.14
		Root	0.12 ±0.01	0.11 ±0.03	0.39 ±0.10	0.34 ±0.09
Soluble carbohydrate (mg glucose equivalents g^{-1} d.wt)	Watered + N	Shoot	12.66 ±1.73	18.86 ±1.08 *	28.73 ±5.51	30.10 ±5.09
		Root	28.79 ±2.70	26.14 ±1.45	81.79 ±13.70	56.43 ±10.69
	Watered – N	Shoot	11.54 ±2.27	11.00 ±1.69	26.31 ±2.54	22.85 ±5.94
		Root	21.37 ±4.29	30.31 ±4.46	70.84 ±17.20	64.97 ±9.88
	Drought + N	Shoot	14.86 ±1.88	12.33 ±1.62	24.11 ±4.29	50.45 ±11.79
		Root	28.62 ±6.05	26.23 ±4.10	64.15 ±10.13	56.93 ±11.57
	Drought - N	Shoot	9.68 ±0.20	12.00 ±2.65	21.64 ±3.96	14.65 ±2.09
		Root	28.05 ±0.97	27.75 ±7.38	63.27 ±10.55	53.98 ±5.97
Starch (mg g^{-1} d.wt)	Watered + N	Shoot	36.26 ±2.74	8.38 ±2.46 ***	18.68 ±5.57	24.24 ±6.50
		Root	55.86 ±9.90	9.50 ±2.48 **	30.08 ±5.49	36.87 ±9.63
	Watered – N	Shoot	58.86 ±8.23	47.22 ±25.56	50.73 ±6.25	30.80 ±4.72 *
		Root	60.10 ±7.90	11.83 ±2.96 ***	48.37 ±9.71	28.19 ±8.59
	Drought + N	Shoot	3.31 ±1.88	5.33 ±2.35	19.26 ±1.83	22.72 ±6.40
		Root	9.63 ±3.86	13.99 ±0.90	13.51 ±4.25	17.52 ±11.20
	Drought - N	Shoot	6.84 ±1.06	6.36 ±2.04	27.84 ±7.79	15.59 ±4.57
		Root	18.05 ±3.06	25.54 ±6.63	9.50 ±2.13	26.52 ±6.22

Table 4.3. Summary of statistically-significant effects, from appendix tables 2.1.1 to 2.2.6 of elevated CO_2, nitrogen availability or drought on the compatible solute and carbohydrate status and percent N and C allocated to compatible solutes of *Plantago maritima* and *Armeria maritima* after five and ten weeks exposure.

↑ = increase in growth measurement compared to control; ↓ = decrease in growth measurement compared to control; n.d. = no difference between treatment and control. Subscript numbers ($_5$ or $_{10}$) relate to which week the measurement was significantly different to the control.

Shoot and root compatible solute concentrations; sorbitol, proline, betaine and % N or C allocation to these compounds		*Plantago maritima*	*Armeria maritima*
Response to:	Growth conditions	Response	Response
Elevated CO_2	Well-watered; high N	n.d.	↑ shoot betaine proline$_5$ & ↓ shoot betaine$_{10}$ ↑ root proline$_{10}$
	Well-watered; low N	↑ root % N_5	↑ root betaine$_{5,10}$ % N_{10}
	Drought; high N	↓ root % N_{10}	↓ shoot & root betaine$_5$ root proline$_5$ % shoot N_5
	Drought; low N	↓ Shoot sorbitol$_{10}$ ↑ root proline$_5$	↓ % shoot N_5
Low N	Ambient CO_2; well-watered	↑ Root sorbitol$_5$, % N_5 % C_5 ↓ Shoot % N_{10}	↓ shoot betaine$_{10}$ root betaine$_5$ & ↓ shoot proline$_5$
	Ambient CO_2; Drought	↓ Root % N_{10}	↓ shoot betaine$_5$ root betaine$_5$ & ↓ shoot & root proline$_5$
	Elevated CO_2; well-watered	↑ Root proline$_5$ % N_5	↑ root betaine$_{10}$ % N_{10} ↓ shoot & root proline$_5$
	Elevated CO_2; Drought	n.d.	n.d.
Drought	Ambient CO_2; High N	↑ Shoot sorbitol$_5$ ↑ root proline$_{10}$ ↑ Root % C_5 % N_{10}	↑ shoot & root betaine$_5$ shoot % N_5 & ↓ shoot betaine$_{10}$ ↑ root proline$_5$
	Ambient CO_2; Low N	↓ Root sorbitol, ↑ root proline$_{10}$	↑ root betaine$_5$↑ root proline$_5$
	Elevated CO_2; High N	n.d.	n.d.
	Elevated CO_2; Low N	↑ Root sorbitol$_5$ % $C_{5,10}$	↑ shoot proline$_{10}$

Shoot and root soluble carbohydrate (CHO) and starch concentrations		*Plantago maritima*	*Armeria maritima*
Response to:	Growth conditions	Response	Response
Elevated CO_2	Well-watered; high N	↓ root starch$_5$	↑ shoot CHO_5 ↓ shoot & root starch$_5$
	Well-watered; low N	↑ root CHO_5 shoot & root starch$_{10}$	↓ shoot starch$_{10}$ root starch$_5$
	Drought; high N	↓ shoot starch$_5$	n.d.
	Drought; low N	↑ root starch$_5$	n.d.
Low N	Ambient CO_2; well-watered	↑ shoot CHO_5	↑ shoot starch$_{10}$
	Ambient CO_2; Drought	↑ shoot starch$_{10}$	n.d.
	Elevated CO_2; well-watered	↑ shoot CHO_5 root $CHO_{5,10}$ shoot starch$_{10}$	↓ shoot CHO_5
	Elevated CO_2; Drought	n.d.	↓ shoot CHO_{10}
Drought	Ambient CO_2; High N	↑ shoot starch$_5$ ↓ root starch$_5$	↓ shoot & root starch$_5$
	Ambient CO_2; Low N	n.d.	↓ shoot & root starch$_5$ root starch$_{10}$
	Elevated CO_2; High N	n.d.	↓ shoot CHO_5
	Elevated CO_2; Low N	↓ root CHO_{10} ↑ root starch$_5$	n.d.

4.3.1 *Effect of elevated CO_2 on compatible solutes, soluble carbohydrates and starch*

Plantago maritima

i. Well-watered plants

There were no significant effects of elevated CO_2 upon shoot and root concentrations of sorbitol and proline and in shoot soluble-carbohydrate concentrations, compared to plants grown at ambient CO_2 (Table 4.1). However, there were significant increases in root soluble-carbohydrate ($P \leq 0.05$ table 2.1.4) and both shoot and root starch concentrations ($P \leq 0.01$ table 2.1; $P \leq 0.05$ table 2.1.4), when such plants were grown at low N availability. Plants grown at elevated CO_2 had decreased root starch concentrations, compared to plants grown at ambient CO_2 ($P \leq 0.05$ tables 4.1 & 2.1.4).

ii. Droughted plants

A combination of elevated CO_2 and drought treatment gave rise to a decrease in sorbitol and starch concentration in shoots, and a transitory increase in root proline and starch concentration after five weeks. These decreases in sorbitol and increases in proline concentrations were statistically significant in plants subjected to low N availability ($P \leq 0.01$ table 2.1.1; $P \leq 0.05$ table 2.1.4 and $P \leq 0.05$ table 2.1.4) and high N availability for starch ($P \leq 0.01$ table 2.1.1). Betaine was not detected in *P. maritima.*

Armeria maritima

i. Well-watered plants

Shoot betaine, proline and soluble-carbohydrate concentrations were higher in plants grown at elevated CO_2 with high N availability ($P \leq 0.05$ table 2.1.7), when compared to plants grown at ambient CO_2. However, after ten weeks, shoot betaine concentrations were decreased ($P \leq 0.05$, tables 2.1.7). In plants grown at elevated CO_2 combined with low N availability, root betaine concentrations were increased ($P \leq 0.05$ & $P \leq 0.01$; table 2.1.10). Starch concentrations were lowered by elevated CO_2 in plants with high N availability (tables 2.1.7 & 2.1.10).

ii. Droughted plants

In plants that were exposed to combined elevated CO_2 and drought, betaine and root proline concentrations were significantly lowered at high N supply ($P \leq 0.05$ & $P \leq 0.01$; tables 2.1.7 & 2.1.10). Sorbitol was not detected in the tissue of *A. maritima.*

4.3.2 *Effect of low N availability on compatible solutes, soluble carbohydrates and starch*

Plantago maritima

i. Well-watered plants

Shoot concentrations of sorbitol or proline or root starch concentrations were not affected by low N availability alone. Low N availability led to an initial increase in root sorbitol, proline and soluble carbohydrate concentrations when grown at ambient and elevated CO_2, respectively ($P \leq 0.05$ table 2.1.5) (table 4.1). Low N availability generally increased the shoot soluble-carbohydrate and starch concentrations when grown at ambient and elevated CO_2 ($P \leq 0.01$ and $P \leq 0.05$ table 2.1.2).

ii. Droughted plants

Low N availability, compared to high N availability, significantly increased the shoot starch concentrations in plants grown at ambient CO_2 ($P \leq 0.05$ table 2.1.2).

Armeria maritima

i. Well-watered plants

Low N availability, compared to plants grown at high N availability, lowered shoot proline and betaine concentrations ($P \leq 0.05$ & $P \leq 0.01$ table 2.1.8), lowered root betaine concentrations ($P \leq 0.05$ table 2.1.11) and increased shoot starch concentrations ($P \leq 0.01$ table 2.1.8) in plants at ambient CO_2. However, in plants that were exposed to elevated CO_2, low N availability increased the root betaine concentrations ($P \leq 0.05$ table 2.1.11) and lowered the proline and shoot soluble-carbohydrate concentrations ($P \leq 0.05$ tables 2.1.8 and 2.1.11).

ii. Droughted plants

Shoot and root betaine and proline concentrations were decreased by low N availability coupled with drought in plants exposed to ambient CO_2 after week five ($P \leq 0.05$ table 2.1.8 and $P \leq 0.01$ table 2.1.11).

4.3.3 *Effect of drought on compatible solutes, soluble carbohydrates and starch*

Plantago maritima

i. High nitrogen availability

Drought caused a significant increase in the shoot sorbitol concentrations ($P \leq 0.05$; table 2.1.3) in plants exposed to ambient CO_2 with high N availability, compared to well-watered plants (table 4.1). Root proline concentrations were increased by drought in plants exposed to ambient CO_2 irrespective of N supply ($P \leq 0.05$; table 2.1.6). Drought caused an increase and decrease in the shoot and root starch concentrations respectively ($P \leq 0.05$ table 2.1.3 and table 2.1.6) in plants grown at ambient CO_2.

ii. Low nitrogen availability

Root sorbitol concentrations were decreased in plants exposed to ambient CO_2 and increased in plants exposed to elevated CO_2 ($P \leq 0.01$; table 2.1.6). Root starch and soluble-carbohydrate concentrations increased in droughted plants grown at elevated CO_2 ($P \leq 0.05$ and $P \leq 0.01$, table 2.1.6).

Armeria maritima

i. High N availability

Drought alone caused an increase in the concentrations of shoot and root betaine ($P \leq 0.01$ table 2.1.9 & $P \leq 0.05$ table 2.1.12) and root proline ($P \leq 0.000$ table 2.1.12) in plants grown at ambient CO_2, when compared to well-watered plants (table 4.3). However, at ten weeks, there was a decrease in shoot betaine concentrations in plants grown at ambient CO_2 ($P \leq 0.05$ table 2.1.9). Drought decreased the shoot soluble-carbohydrate concentrations in plants grown at elevated CO_2 ($P \leq 0.05$ table 2.1.9).

ii. Low N availability

Root betaine and proline concentrations were generally increased by drought when grown at ambient CO_2, compared to well-watered plants ($P \leq 0.01$ & $P \leq 0.000$ table 2.1.12). There were decreases in the shoot and root starch concentrations for all droughted plants grown at ambient CO_2, irrespective of N supply ($P \leq 0.000$ table 2.1.9 & $P \leq 0.01$ table 2.1.12). At week ten, reductions in starch concentrations only persisted in roots of droughted plants grown at low N availability at ambient CO_2 ($P \leq 0.05$ table 2.1.12).

4.4 Results: Allocation of nitrogen and carbon to compatible solutes

The effects of varying CO_2, N and water availability on the allocation of N and C to compatible solutes in *P. maritima* and *A. maritima* are summarised in table 4.3.

4.4.1 *Allocation of N to nitrogen-based compatible solutes*

Armeria maritima

In response to elevated CO_2, shoots of droughted *A. maritima* had significantly decreased the percent N allocated to compatible solutes, after five weeks (Fig. 4.1 A; $P \leq 0.05$ Table 2.2.1). Although not statistically significant, there was also an increase in the N allocated to compatible solutes in the roots of well-watered plants that had low N availability (Fig. 4.1 A). However, after ten weeks, this increase of N allocation to compatible solutes in the roots in well-watered plants, in response to elevated CO_2, was significantly different (Fig. 4.1 B; $P \leq 0.01$ Table 2.2.1).

In plants grown at low N availability alone, there was a general trend to increase the percentage N allocated to compatible solutes, compared to plants grown at high N availability. This increase was statistically significant in the roots of well-watered plants that were exposed to elevated CO_2 for ten weeks (Fig. 4.1 B; $P < 0.05$ table 2.2.2).

There was a general trend to increase the percentage N allocated to compatible solutes in droughted plants, particularly those grown at ambient CO_2, compared to plants that were well-watered. This increase was statistically significant in the shoots of droughted plants that were exposed to ambient CO_2 with high N availability after five weeks (Fig. 4.1 B; $P < 0.05$ table 2.2.3).

Plantago maritima

In *P. maritima*, there was considerably less N allocated to compatible solute production, than the percentage N allocation measured in *A. maritima*, with only proline being detected (Figs. 4.1 C & D). Although the percent of N allocated to proline was negligible, in response to elevated CO_2, there was a transitory increase in the N allocated to proline in the roots of well-watered plants with low N availability after five

weeks (Fig. 4.1 C; $P < 0.01$ table 2.2.1) and in the roots of droughted plants with high N availability after ten weeks (Fig. 4.1 D; $P < 0.05$ table 2.2.1), compared to plants at ambient CO_2.

Low N availability, compared to plants grown at high N availability, significantly increased the percentage N allocated to proline in the shoots of well-watered *P. maritima* exposed to ambient CO_2 after ten weeks and in the roots of well-watered plants exposed to both ambient and elevated CO_2 after week five (Fig. 4.1 C; $P < 0.05$ & $P < 0.01$ respectively, table 2.2.2). However, after week ten, there was a significant decrease in the percent N allocation in droughted plants grown at ambient CO_2, compared to plants grown at high N availability (Fig. 4.1 D; $P < 0.001$ table 2.2.2).

There was no effect of drought on the percent N allocation to proline after week five, compared to plants that were well-watered (Fig. 4.1 C). However, after week ten there was a significant reduction in the percent N allocation of the roots of plants that were grown at ambient CO_2 with high N availability, compared to similar plants that were well-watered (Fig. 4.1 D; $P \leq 0.000$ table 2.2.3).

4.4.2 *Allocation of C to carbon-based compatible solutes*

In *P. maritima*, there were no statistically-significant increases in the C allocated to sorbitol within the total non-structural carbohydrate pool (non-structural carbohydrate pool is the sum total of sorbitol, soluble sugars and starch) in plants exposed to elevated CO_2, compared to plants at ambient CO_2. (Fig. 4.2 A; table 2.2.4). However, the general trend was a reduction in the C allocated to sorbitol in plants that were droughted under elevated CO_2, especially in the shoots, compared to plants at ambient CO_2.

Generally, low N availability alone did not affect the percent C allocated to sorbitol in the shoots of plants, irrespective of CO_2 supply, compared to plants grown at high N availability. There was a significant increase in the root C allocated to sorbitol in well-watered plants grown at ambient CO_2 at week five (Fig. 4.2 B; $P < 0.05$ table 2.2.5).

In the shoots of droughted plants there were slight increases in the percent C allocated in plants that were grown at ambient CO_2 after week five (Fig. 4.2 A), compared to well-watered plants. However, after week ten, there was a slight reduction of the C

allocated to sorbitol in shoots of droughted plants grown at elevated CO_2 with high N availability (Fig. 4.2 B). Despite these trends, the alterations in C allocation to sorbitol in plant shoots were not significant. In the roots, there were significant increases in the percent C allocated to sorbitol. These increases were statistically significant in plants grown at ambient CO_2 with high N supply after week five (Fig. 4.2 A; $P < 0.05$ table 2.2.6) and in plants grown at elevated CO_2 with low N availability at both weeks five and ten (Figs. 4.2 A & B; $P < 0.05$ table 2.2.6), when compared to well-watered plants.

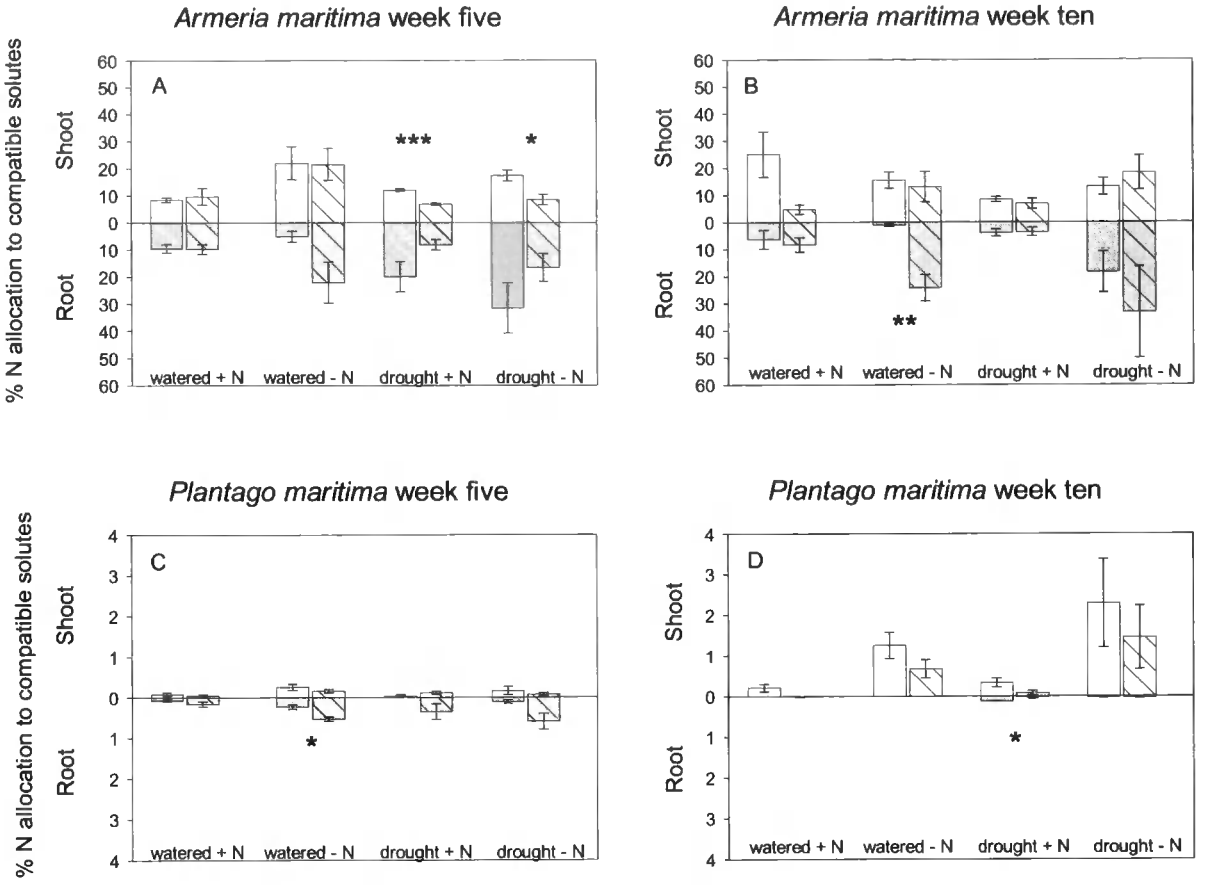


Figure 4.1. Betaine and proline content of the shoot (unshaded) and root (shaded) as a percentage of the total shoot and root nitrogen in *Armeria maritima* (A & B) and *Plantago maritima* (C & D), after five and ten week's exposure to treatment. Plants were exposed to either ambient (360 μmol mol^{-1}) (open bars) or elevated (600 μmol mol^{-1}) (striped bars) atmospheric CO_2. Data represents the percentage N allocation to these compatible solutes under the treatments; well-watered and high N availability; well-watered with low N availability; drought with high N availability and drought with low N availability. Data represent mean ± one SE. n = 5-6. Significant differences between CO_2 treatments are indicated by * ($P \leq 0.050$); ** ($P \leq 0.010$); *** ($P \leq 0.001$). Note differences in scale between the two species.

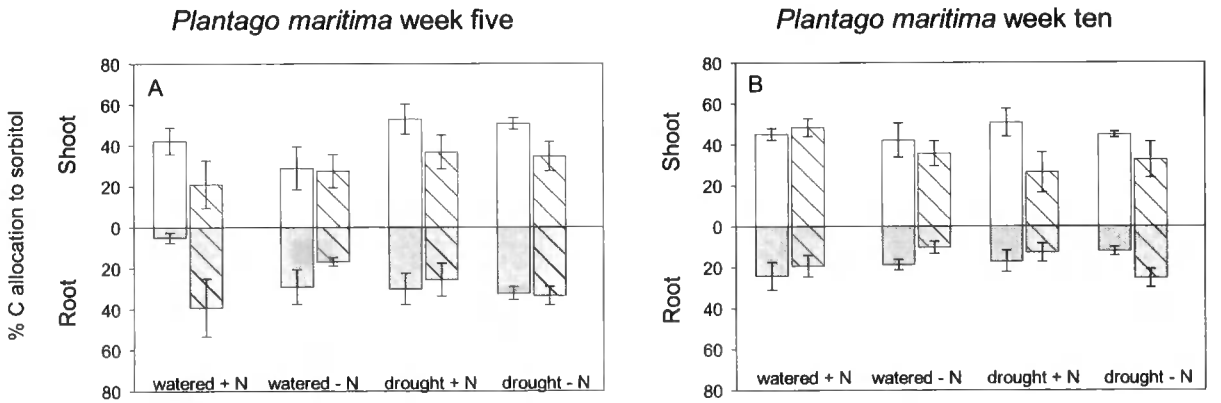


Figure 4.2. The percentage allocation of carbon to sorbitol within the total non-structural carbohydrate pool in the shoot (unshaded) and root (shaded) of *Plantago maritima* (A & B), after five and ten week's exposure to treatment. Plants were exposed to either ambient (360 μmol mol^{-1}) (open bars) or elevated (600 μmol mol^{-1}) (striped bars) atmospheric CO_2. Data represents the percentage C allocation to sorbitol under the treatments; well-watered and high N availability; well-watered with low N availability; drought with high N availability and drought with low N availability. Data represent mean ± one SE. n = 5-6.

4.5 Conclusion and discussion

4.5.1 Plant responses to drought

The metabolic response to drought is in agreement with Stewart *et al.* (1979) who first described that *A. maritima* accumulated high concentrations of betaine and proline whilst *P. maritima* accumulated sorbitol in a salt-marsh habitat. Although these species accumulate such compounds in high concentrations, as described in Stewart *et al* (1979), the intensity of increase in concentration in response to drought is unlike that measured by Ahmed *et al.* (1979) where a 100 fold increase in sorbitol was measured in the roots of *P. maritima* exposed to high external concentrations of NaCl.
As expected in *P. maritima*, sorbitol concentrations increase in response to drought, which is in agreement with other studies (Wang & Stutte 1992; Popp & Smirnoff 1995). However, sorbitol concentrations only increased in plants grown at ambient CO_2 with a high N supply, and at low N supply root sorbitol concentrations were lower than well-watered plants after ten weeks (Table 4.1). An exception was an increase in the root sorbitol concentrations in plants grown at elevated CO_2 with low N availability. These results are also consistent with other studies (Ahmed *et al.* 1979) in that the concentration of sorbitol in the shoots is greater than that of the roots (Table 4.1). Sorbitol synthesis is mainly constrained to source leaves, meaning that most of the sorbitol measured in the roots would have been transported there, which explains the higher concentrations in the shoots rather than the roots. In a drought situation, the water stress would be at its highest in the roots, where the difference in internal and external water potential would be the greatest (Sheehy-Skeffington & Jeffrey 1988). Overall, the increase in solutes in the shoots would cause a negative gradient of water from the roots to the shoots thereby increasing water extraction from the soil. The fact that there was more C allocated to sorbitol in terms of the total non-structural carbohydrate pool does suggest that sorbitol was being used to play a role in osmoregulation and there was a shift in end-point synthesis for carbon (Popp & Smirnoff 1995). However, there was a reduction in the C allocated to sorbitol in the shoots of plants grown at elevated CO_2 with high N supply after ten weeks which could be due to a shift from sorbitol to sucrose synthesis (see below). Although there was more C being allocated to sorbitol in droughted plants, especially the roots, the overall growth of *P. maritima*, when droughted, was not seriously affected as previously determined by Yeo (1983) (Chapter 3; Fig. 3.1).

The control mechanisms of C partitioning into sorbitol over other carbohydrates are largely unknown. A study by Escobar-Gutierrez *et al.* (1998) showed, that in peach (*Prunus persica*) leaves, sorbitol synthesis accounted for more than 50 % of the newly fixed CO_2. In a study by Walsh (2000), up to 80 % of the non-structural carbohydrate pool was sorbitol in *P. maritima*. This is consistent with the current study, which determined that up to 50 % of the C in the total non-structural carbohydrate pool is incorporated into sorbitol (Fig. 4.2), suggesting that sorbitol is a major end-product of photosynthesis in this species. Escobar-Gutiérrez *et al.* (1998) found, by ^{14}C labelling, that sorbitol was preferentially synthesised over sucrose when photosynthetic rates were low, especially when subjected to drought. This accounts for the increase in the ratio of sorbitol to sucrose when the net photosynthetic assimilation rate is decreased with drought (Table 4.1; Fig. 4.2). However, if net photosynthetic rates are increased in response to elevated CO_2, it is possible that sucrose would be preferentially synthesised over sorbitol, even under drought conditions (Escobar-Gutiérrez & Gaudillère 1997). Although the photosynthetic capacity was reduced by elevated CO_2, the net photosynthetic rates could still have been increased (Chapter 3, Fig. 3.7). The fact that sorbitol was not accumulated in response to drought in some circumstances, especially after ten weeks, could give weight to the argument that sorbitol is not only used as a compatible solute but also as a C storage and translocation compound for use when growth conditions improves (Bohnert *et al.* 1995; Popp & Smirnoff 1995; Walsh 2000). The proline concentration in *P. maritima* was negligible which is in line with previous studies on this species (Ahmed *et al.* 1979; Jefferies *et al.* 1979).

In *A. maritima*, drought increased the betaine and proline concentrations but like *P. maritima*, only at ambient CO_2 (Table 4.2). This is in agreement with other studies of osmotic stress in *A. maritima* and generally for proline and betaine production (Singh *et al.* 1973; Stewart & Larher 1980; Irigoyen *et al.* 1992; Köhl 1996). The exceptions were an increase in the shoot proline in plants grown at elevated CO_2 with low N supply and a decrease in the shoot betaine concentrations in plants grown at ambient CO_2 with a high N supply after ten weeks, compared to well-watered plants. It is also in agreement with the study by Storey *et al.* (1977) who showed that the concentration of soluble nitrogenous compatible compounds could account for up to 20 % of the plant N concentration as the percent of N allocated to compatible solutes increased in droughted plants, compared to well-watered plants, particularly in the roots (Fig. 4.1).

Other studies have found seasonal increases in these compounds with betaine being present all year round whilst proline accumulated more so in winter months which may support the hypothesis that these compounds are also being used as a N store (Köhl 1997). The fact remains that the percent N allocated to betaine and proline increased, especially in the shoots of plants grown at ambient CO_2 after five weeks, and this suggests that these compounds are being used in osmoregulation. However, this does not explain why there was not a significant increase when droughted plants were exposed to elevated CO_2, compared with well-watered plants grown at elevated CO_2 (Table 4.2).

4.5.2 Plant responses to low nitrogen availability

There are usually reductions in nitrogenous compounds in plants grown at low N supply so it was hypothesised that there would be decreases in betaine and proline concentrations. This was true in the case of *A. maritima* when plants were grown at ambient CO_2 (Table 4.2). However, although the concentration of these compounds was reduced, the percent N allocated to these compounds was increased, particularly in the roots that were grown at elevated CO_2 (Fig. 4.1). So, although the total N concentration of the plant was lowered by low N availability, the plant still allocated N to the betaine and proline biosynthesis. The increase of proline, and hence N allocation to proline, has been explained by increases in the Δ^1-pyrroline-5-carboxylate synthetase (the initial enzyme to convert L-Glutamic acid to proline) as gene transcripts for this enzyme have been shown to increase under N starvation, hypersalinity and water stress (Delauney & Verma 1993; Yoshiba *et al.* 1997). The same has been shown for betaine in that increases occur in mRNA transcripts for betaine aldehyde dehydrogenase (BADH; to convert betaine aldehyde to glycine betaine) in salt-stressed plants (Hanson *et al.* 1995).

It has also been proposed that *A. maritima* uses these compounds as a N store, for use for extra N when flowering or growth during spring (Jefferies *et al.* 1979; Jefferies 1980; Batanouny & Ebeid 1981). It is likely that proline would be used as the storage compounds, rather than betaine, as it has a higher metabolic turn over rate and it can be easily converted into other amino acids (Jefferies 1980; Wyn Jones 1980). Therefore it is interesting that this species still allocated N to these compounds, even when N availability was so obviously low, meaning that the N requirement of this species is inherently higher than that of *P. maritima* (Stewart & Rhodes 1978).

Concentrations of C compounds can also increase in plants grown at low N availability (Bryant *et al.* 1983), compared to plants grown at high N availability. However, in *A. maritima*, the non-structural carbohydrate compounds were only increased in the shoots of well-watered plants grown at ambient CO_2 and the soluble-carbohydrate concentrations were reduced in plants grown at elevated CO_2 (Table 4.2). This implies that the C can still be incorporated into growth, or it could be that photosynthesis is reduced, as shown by a reduction in photosynthetic capacity (see Chapter 3; fig. 3.7). In addition, sorbitol was hypothesised to increase in plants grown at a low N supply in *P. maritima*, compared to plants that had a high N supply. However, this only occurred in well-watered plants and not in droughted plants, though there were increases in the starch and soluble-carbohydrate concentrations at low N supply (Table 4.1).

4.5.3 Plant responses to elevated atmospheric CO_2

Most of the literature on carbon partitioning is based on plants that have sucrose or starch as the major final products of photosynthesis (Stitt & Krapp 1999). The hypothesis that there would be an increase in the sorbitol concentrations of *P. maritima* in response to elevated CO_2 did not hold true. The sorbitol concentrations, and the percent C allocated to sorbitol, were actually reduced by elevated CO_2, though this only occurred in droughted plants (Table 4.1; Fig. 4.2). Also, the soluble-carbohydrate concentrations were not affected by elevated CO_2. However, the starch concentrations were altered in plants coupled with a high N supply although they were only significantly increased when coupled with a low N supply (Table 4.1). This is in agreement with other studies that show starch is the main carbohydrate that is increased by elevated CO_2 (den Hertog *et al.* 1996; Poorter *et al.* 1997). It is interesting that *Plantago maritima* only significantly reduced its sorbitol concentration in droughted plants when grown at elevated CO_2. This could be due to changes in the carbon metabolism of the plant (Pan *et al.* 1998) or changes in the water-use-efficiency (*WUE*) of the plants when they are droughted (Bowes 1993). For example, if increased stomatal closure with elevated CO_2 and drought occurs, then it is possible that the plant conserves more water causing a lesser demand for compatible solute production (Wullschleger *et al.* 2002). However, the water contents of the plants were reduced by elevated CO_2, (see chapter 3, fig. 3.3) which implies that the plants are losing water when exposed to drought and elevated CO_2. A study by Pan *et al.* (1998) showed that in apple leaves exposed to elevated CO_2, the concentration of sorbitol was actually

increased. This was explained by increased C supply to sorbitol as photosynthetic rates were also increased, rather than a reduction in the degradation of sorbitol. However, another study on apple leaves grown at elevated CO_2 showed that sorbitol concentrations only increased in concentration in source and not in sink leaves (Wang *et al.* 1999). Also, there was a tendency for the apple leaves to accumulate starch over other carbohydrates in plants exposed to elevated CO_2, which also occurred in the current study (Table 4.1). As *P. maritima* had a lower photosynthetic capacity when grown under elevated CO_2 (chapter 3; Fig. 3.7) then the C supply for sorbitol synthesis would have been reduced causing the lowering of sorbitol concentrations. This means that the plant favours C storage into starch rather than into osmoregulatory processes and compounds (Pan *et al.* 1998). However, it is evident from the biomass data (Chapter 3, Fig. 3.1) that this shift in C assimilation from sorbitol to other non-structural carbohydrates does not affect the growth of the plant.

The results from the current study show that the allocation of N to compatible solutes is not only altered by increases in water stress but it can be altered by the supply of N and interestingly, C (Table 4.2 and Fig. 4.1). The hypothesis that there would be a decrease in the betaine and proline concentrations in *A. maritima* in plants exposed to elevated CO_2 held true for plants that were droughted, but not for plants that were well-watered (Table 4.2). This is compliant with models such as the carbon-nutrient balance (Bryant *et al.* 1983) that predicts N-based compounds would decrease as N is allocated to other plant functions, mainly growth (Cotrufo *et al.* 1998; Gleadow *et al.* 1998). However, such models also predict increases in C-based compounds but this only occurred in the soluble-carbohydrate concentration in well-watered plants with high N supply after five weeks (Table 4.11) (Peñuelas & Estiarte 1998). It is possible that N, which would otherwise be used for betaine and proline synthesis, was used for other demanding functions, such as maintaining growth, especially if the N supply from the soil was limiting growth (Stitt & Krapp 1999). Therefore, like *P. maritima*, the plant might be reallocating resources, in this case N, from osmoregulatory functions to other plant processes when grown under drought and elevated CO_2 conditions. It could be that other functions like increased *WUE* alter the osmoregulatory processes within the plant. However, like in *P. maritima*, a decrease in the compatible solute concentration was not matched by changes in the plant water content (chapter 3; Fig. 3.3). The fact that there were also increases in the betaine and proline concentrations in well-watered plants, and that the percent N allocated to compatible solutes when N supply was lower in plants

exposed to elevated CO_2, could be due to increased soluble N availability, compared to plants grown at ambient CO_2. If N is reallocated from photosynthetic functions, as shown by a reduction in chlorophyll within this study (Chapter 3; Fig. 3.7) then more soluble N could be present within the plant. It was shown in *Eucalyptus cladocalyx*, that although the total N concentration decreased in response to elevated CO_2, the percent N allocated to cyanogenic glycosides was increased (Gleadow *et al.* 1998). Also, the increase in proline has been associated with protein hydrolysis induced by water stress (Irigoyen *et al.* 1992) and it is possible that the proteolytic release of the precursors glutamate or arginine could increase proline concentrations (Stewart & Larher 1980). Therefore, if Rubisco and chlorophyll-binding proteins are hydrolysed in plants exposed to elevated CO_2 (Fig. 3.7), then the increase in proline could be an artefact of that rather than an increase for osmoregulatory purposes. However, this does not explain why there was a decrease in betaine after ten weeks in well-watered plants. It is possible that this transient increase in betaine is due to it being a temporary N store, or N transport system within the plant, whilst the plant is acclimating to new growth conditions. This shows that the blanket hypothesis that all nitrogenous compounds are reduced in plants grown at elevated CO_2 should be approached with caution and that it is important to study individual compounds rather than assuming that all N compounds are decreased (Stitt & Krapp 1999). It is also usually common to observe increases in starch and soluble-carbohydrates in plants exposed to elevated CO_2 (Stitt 1991). However, this did not occur in *A. maritima*, as the trend was a decrease in these compounds though only in well-watered plants (Table 4.2). It was hypothesised in chapter 3 that the increased specific leaf area (*SLA*) (Fig. 3.5) could be due to increased concentrations of non-structural carbohydrates, however, as this did not occur the decrease in *SLA* was probably due to changes in leaf thickness or cell size (den Hertog *et al.* 1998).

In summary, these results show that concentrations of sorbitol in *P. maritima* and betaine and proline in *A. maritima* do increase when these plants are subjected to reduced water availability. However, this response is dependent on how much N and C is available to the plant. The small and mainly invariable level of changes in concentration of these compounds may indicate that a true drought condition was not achieved in this experiment, as other studies have reported much larger increases in concentrations of compatible solutes (Ahmed *et al.* 1979). In *P. maritima*, the sorbitol concentrations were reduced in droughted plants that are grown under elevated CO_2 and likewise for betaine and proline in *A. maritima*. The concentrations of betaine and proline in *A. maritima* were shown to increase in well-watered plants when grown at elevated CO_2. The cause of such a response to elevated CO_2 was suggested to be either a change in the photosynthetic and stomatal functioning and/or shifts in carbon partitioning to sucrose and starch and N allocation to other growth functions.

Chapter 5: Water potential and photosynthetic status of *Plantago maritima* and *Armeria maritima* under increasing soil water deficit

Aim

The aim of the study reported within this chapter was to investigate the effect of increasing soil water deficit on biomass, water content, water potential and photosynthesis of *Plantago maritima* and *Armeria maritima*. The study concentrated on the effects on these parameters of drought at ambient CO_2 and went on to study how the responses are altered by elevated atmospheric CO_2.

5.1 Introduction

Drought resistance involves a range of cellular and metabolic adaptations, which affect plant water relations. As shown in chapters three and four, *P. maritima* and *A. maritima* possess different physiological and metabolic strategies when responding to reduced water availability. This chapter further explores the photosynthetic responses involved in drought and elevated atmospheric CO_2 conditions.

Changes in photosynthesis occur in response to drought (Yordinov *et al.* 2000). Usually, the first response to drought is stomatal closure (reduced stomatal conductance g_s) causing decreases in transpiration (E) as stomatal resistance increases. If net photosynthetic rates (A) are maintained during drought then the water-use-efficiency (*WUE*) (A/g_s or A/E) will increase due to a higher reduction of g_s or E than of A (Wall 2001). The initial increase in *WUE* may not be sustained after prolonged exposure to drought, when compared to well-watered plants, as photosynthesis is constrained by stomatal closure (Flexas *et al.* 2001). As g_s is reduced, there is usually a decrease in the internal intercellular CO_2 concentration (C_i). The ratio of A to the internal intercellular CO_2 concentration (C_i) (apparent carboxylation efficiency) (A/C_i) facilitates an estimate of the ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) status within the plant. An increased C_i is a product of impaired photosynthetic processes, which ultimately lead to a lower A/C_i ratio. A decreased A/C_i ratio over time will reflect photosynthetic adjustment that will take place, not as an immediate response to drought, but as a result of prolonged stomatal closure and a decrease in the concentration or activity of Rubisco (Flexas *et al.* 2001).

Decreased stomatal conductance is also a common plant response to elevated atmospheric CO_2, associated with increased *WUE* (Bazzaz 1990; Wolfe *et al.* 1998). This response to elevated CO_2 will cause some degree of water conservation within the plant as stomatal apertures are reduced and may benefit the plant during short-term drought occurrences. Increases in *WUE* have been shown in wild species such as *Dactylis glomerata*, *Filipendula vulgaris*, *Festuca rupicola* and *Salvia nemorosa* under elevated CO_2 and this appears to help plants in xeric conditions (Tuba *et al.* 1998). The latter authors concluded that the 'greatest impact of elevated CO_2 will be on plant water relations and drought survival rather than on photosynthetic productivity under drought conditions'. Therefore, it is possible that elevated CO_2 alleviates water stress by increasing *WUE* (Eamus 1991; Lombardini *et al.* 1997; Saralabai *et al.* 1997).

It has been suggested that reductions in transpiration (E) by reduced stomatal conductance (g_s) may affect the plant water potential (Ψ), particularly during water stress (Wullschleger *et al.* 2002). Changes in plant water potentials are indicative of how the osmotic and turgor pressures of the plants are responding to external water deficit (Tyree & Jarvis 1982; Wyn Jones & Gorham 1983). Water potential and its components are measured in megapascals (MPa) where zero MPa would be pure water. The description of plant water potential (Ψ) is usually comprised of four components (Wyn Jones & Gorham 1983) [eq. 1]; 1, the osmotic potential (Ψ_π), which relates to the solute status of the cell and is lowered (MPa becomes more negative) by the addition of solutes to the water; 2, the pressure potential (Ψ_p), which relates to the turgor of the cell and the water potential is usually positive unless the plant is wilted; 3, the gravity potential (Ψ_g) relating to the effect of gravity on the free energy of water and which depends on height and 4, the matric potential (Ψ_m) which is a measure of the effect of a soil matrix on a substance's ability to absorb or release water. Therefore, increases in the solute concentration and reductions in cell turgor pressure (wilting) within the plant cause more negative water potentials. The relationship between these potentials is given below [eq. 5.1]:

$$\Psi = \Psi_\pi + \Psi_p + \Psi_g + \Psi_m \qquad [5.1]$$

The gravitational component is usually ignored, as its contribution is negligible when vertical distances are less than five metres (Passioura 1982). The plant can lose water by stomatal transpiration and by water moving out of the cell if the external soil water

potential is more negative than the cell water potential. When transpiration exceeds water absorption, cell turgor falls (Ψ_p) as cell volume decreases. Therefore, plants can maintain high water potentials by lowering transpiration rates (g_s). Also, accumulating solutes, such as compatible solutes, can lower (ie. render more negative) the osmotic potential component (Ψ_π) of water potential so that the gradient between the water potential outside and the water potential inside the cell is reduced (Lombardini *et al.* 1997). The concentrations of compatible solutes have been shown to increase in *P. maritima* (sorbitol) and *A. maritima* (betaine and proline) as water availability decreases (Ahmed 1979; Stewart *et al.* 1979; Wyn Jones & Gorham 1983; chapter four of this study).

If elevated CO_2 reduces g_s and E, this could lead to increased (less negative) plant water potential. Wall (2001), tested whether elevated CO_2, which caused decreased transpiration, would increase shoot water potentials (less negative). He found that, in wheat grown under elevated CO_2, there were less negative leaf water potentials, even under drought conditions. It has also been shown that the water potentials of soybean plants (*Glycine max*) and tomato (*Lycopersicon esculentum*) exposed to elevated CO_2 were 0.5 MPa and 0.22 MPa higher (less negative) respectively, than of plants from ambient CO_2 (Paez *et al.* 1984; Wullschleger *et al.* 2002). A review of papers during the 1990`s revealed that in general, elevated CO_2 leads to higher water potentials in plants with a lower stomatal conductance (Wullschleger *et al.* 2002). However, this response can be species-specific. Tognetti *et al.* (2000 b) reported that *Erica arborea* had less negative (higher) osmotic potentials in plants grown at elevated CO_2 but another species, *Juniperus communis*, had lower (more negative) osmotic potentials at elevated CO_2. This higher osmotic potential may affect the plants' capacity for drought tolerance. However, if the osmotic potential is lowered at elevated CO_2, this might have ameliorating effects on drought, especially if leaf conductance is also reduced (Allen *et al.* 1998). Therefore, by adjusting plant water potential in plants growing at ambient or elevated CO_2, plants may be better able to extract soil water. The increased *WUE* will also lower the water lost via transpiration in plants grown at elevated CO_2. The increased time in which plant cells can remain turgid may provide the plant enough time to survive the drought period before water is freely available again.

The main hypothesis of the first section of this chapter was that there is a relationship between reduced water availability to *P. maritima* and *A. maritima* and a lowering of the water potential (becomes more negative) of the shoot tissue. The lowering of the water potential should be mainly due to the increases in the compatible solute concentrations as shown in chapter four. It was also hypothesised that any initial photosynthetic responses to drought, such as stomatal closure (reduced g_s), will cause an increase in the *WUE* and that prolonged exposure to drought will cause a reduction of *WUE* and in the A/C_i ratio over time as the photosynthetic apparatus acclimates to the drought environment. Such changes in g_s and E may ultimately affect the water content and growth of the plant.

Reductions in g_s and E, caused by increased atmospheric CO_2 availability, may reduce the need for compatible solute production in *P. maritima* and *A. maritima*. Significantly, reductions in the concentrations of compatible solutes in these plants exposed to elevated CO_2 were determined in chapter four. Therefore, the aim of the study within the second section of this chapter was to test the hypothesis that reduced g_s and E, and increased *WUE*, in *P. maritima* and *A. maritima* under increased CO_2 availability, which in turn alters the water concentration of the plant, are partly responsible for the alterations in the compatible solute concentrations in plants exposed to drought and elevated CO_2. A reduction in osmolyte production, along with reduced g_s and E, will lead to increased water potential (less negative) when compared to droughted plants grown under ambient CO_2 concentrations. Such changes in g_s and E may ultimately affect the water content and growth of the plant.

5.1.1 Experimental set-up– University of Durham

Ten-week drought study

Tillers of *P. maritima* and *A. maritima* (144 from each species) were taken from plants collected as above and established outside at the CEH-Bangor Solardome field station for eight months, and potted into 9 cm diameter (200 cm^3) pots filled with 370 g of dry silica sand (Chapter 2.3.1). The plants were placed into the growth room facility at the University of Durham (Chapter 2.2.1) and set out in a randomised block design. After two weeks, the plants were randomly assigned one of the following six treatments (24 plants per treatment per species): 100, 80, 60, 40, 20 % field capacity of sand, and sand watered to saturation point. Plants were watered with $1/4^{th}$ strength Long Ashton solution modified to contain 28 mg l^{-1} N (NH_4NO_3) every seven days and pots were randomised twice per week. The experiment ran for 10 weeks (70 days) (4^{th} March to 12^{th} May 2003) and consisted of six harvests.

Plants were harvested in groups of 24 individuals per species (four replicates per water treatment) and harvests were split over 2 days. At each harvest, photosynthetic gas-exchange was determined from intact leaves for plants growing at 100 % FC and 20 % FC (Chapter 2.4.1) before leaves from all treatments were carefully excised, weighed and placed within a psychrometer chamber to determine leaf water potential (Chapter 2.5). Leaves and roots were then detached and weighed and either dried (65 °C, 48 h) for dry weight determination (2.6.1) or frozen in liquid N_2 prior to being stored at –20 °C.

5.1.2 Experimental set-up – CEH Bangor growth cabinet facility

Four-week drought and elevated CO_2 study

Tillers of *Plantago maritima* and *Armeria maritima* (80 of each species) were taken from plants established outside at the CEH-Bangor Solardome field station and potted into 9 cm diameter (200 cm^3) pots filled with 370 g of dry silica sand (Chapter 2.3.1). Plants of each species were randomly assigned and placed inside one of two growth cabinets as detailed in section 2.2.2. Briefly, growth cabinets, had day/night temperature regimes of 20°C/15°C and 14 hours light (550 µmol m^{-2} sec^{-1}): 10 hours dark photoperiod. Relative humidity was set at 60% (0.93 kPa – atmospheric pressure).

Once plants were established, cabinets were set to contain either an atmosphere of ambient CO_2 (360 μmol CO_2 mol^{-1}) or of elevated CO_2 (600 μmol CO_2 mol^{-1}) concentrations. Plants were subjected to one of two watering regimes (a well-watered, 100 % field capacity and a drought, 20 % field capacity) (section 2.3.3). After every seven days of CO_2 treatment plants were watered with $1/4^{th}$-strength Long –Aston nutrient solution modified to contain 28 mg N dm^{-3} as NH_3NO_4. The experiment ran for four weeks (28^{th} June to 27^{th} July 2002) and consisted of five harvests.

Plants were harvested in groups of four per species, per treatment, every seven days. Harvests were split over 2 days, with the plants grown at ambient CO_2 harvested on the first day and the plants grown at elevated CO_2 harvested on the second day. At each harvest, photosynthetic gas-exchange was determined from intact leaves using a CIRAS-1 IRGA (section 2.4.1) before leaves and root were carefully excised, weighed and placed within a psychrometer chamber to determine leaf and root water potential (Chapter 2.5). Tissue was either dried (65 °C, 48 h) for dry weight determination or frozen in liquid N_2 prior to being temporarily stored at -40 °C until transported in dry ice to the University of Durham, where samples were stored at –20 °C.

5.2 Results: ten-week drought study

5.2.1 Photosynthetic parameters of *Plantago maritima* and *Armeria maritima* exposed to decreased water availability

The photosynthetic rates are not shown for day 0 or day 7 as the leaf material was too small to fit into the IRGA chamber and photosynthetic rates for *A. maritima* on day 21 are not presented due to faulty equipment precluding measurements. The net photosynthetic rates were slightly higher in droughted, than well-watered plants, for both species (Fig. 5.1 A & B), with a significant increase on day 28 in *P. maritima* ($F = 16.582_{1,4}$ $P = 0.015$). The stomatal conductance and transpiration rates did not vary significantly between watering treatments in *P. maritima*, but there were small increases in the stomatal conductance and transpiration rates in droughted *A. maritima*, when compared to well-watered plants. A significant increase in the stomatal conductance occurred on day 28 in *A. maritima* ($F = 128.0_{1,4}$ $P = 0.000$). This response was consistent although not significant over time.

Despite the minimal responses to drought, *P. maritima* had slightly higher *WUE*'s when expressed as a function of transpiration (E) and stomatal conductance (g_s) (Fig. 5.2 A & C). In *A. maritima*, there was not an increase in *WUE* in response to drought when expressed as a function of transpiration (Fig. 5.2 B). However, when expressed as a function of stomatal conductance, there was a tendency for lower *WUE*, caused by an increase of stomatal conduction over photosynthetic rate (Fig. 5.2 D). However, none of the differences were statistically significant. The A/C_i ratios were higher in plants with reduced water availability with a significant increase shown by *P. maritima* after 28 days ($F = 28.661_{1,4}$ $P = 0.006$). The A/C_i ratio did not decrease over time in droughted plants.

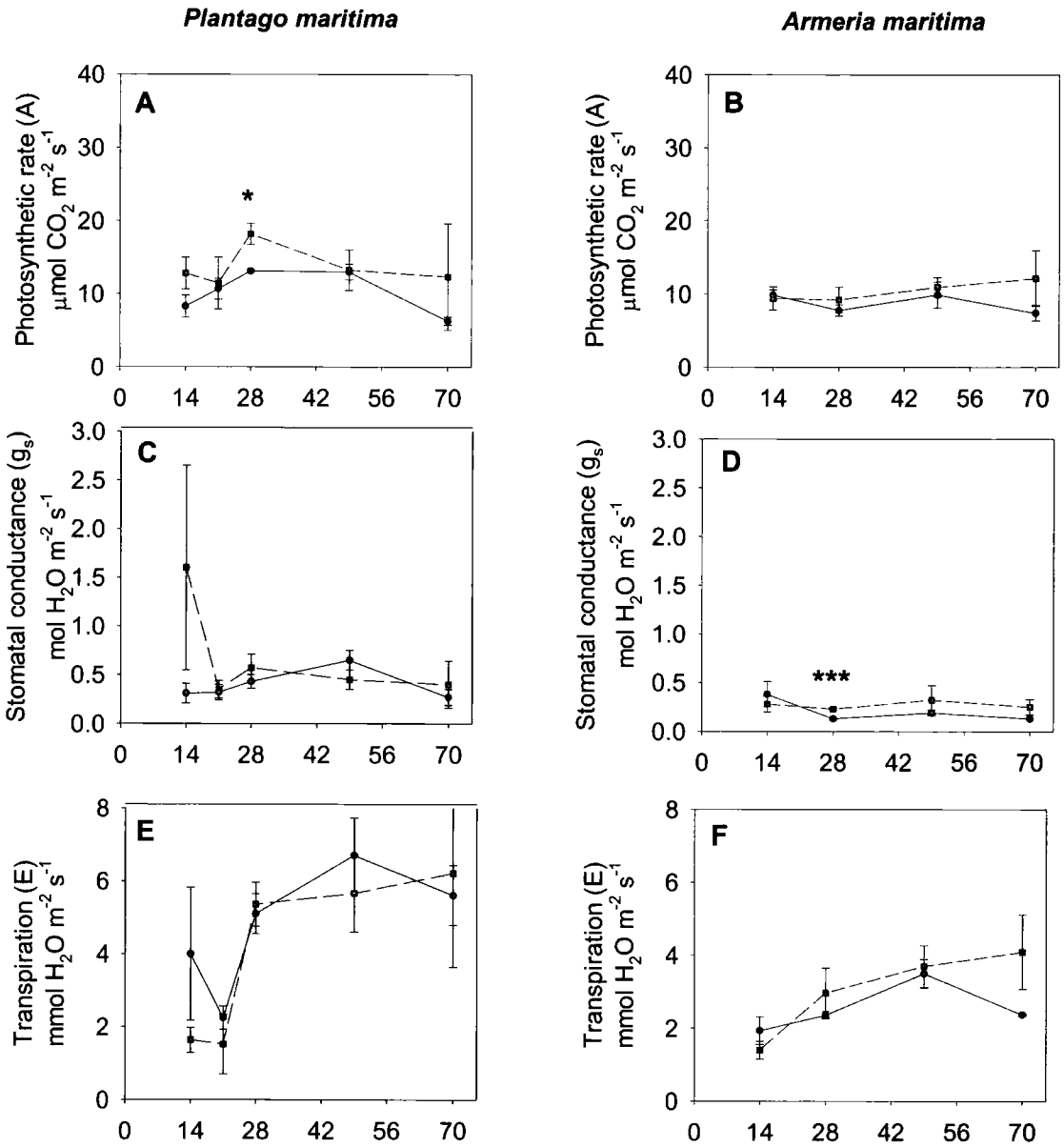


Figure 5.1. Net photosynthesis (A), stomatal conductance (g_s) and transpiration (E) of *Plantago maritima* (A, C, E, G) and *Armeria maritima* (B, D, F, H) grown under well-watered (100 % FC) (solid line) or drought (20 % FC) (dashed line) conditions over a period of 70 days. Data represent mean ± SE. n = 3. Significant effect of drought are indicated by * = $P \leq 0.05$; *** = $P \leq 0.001$.

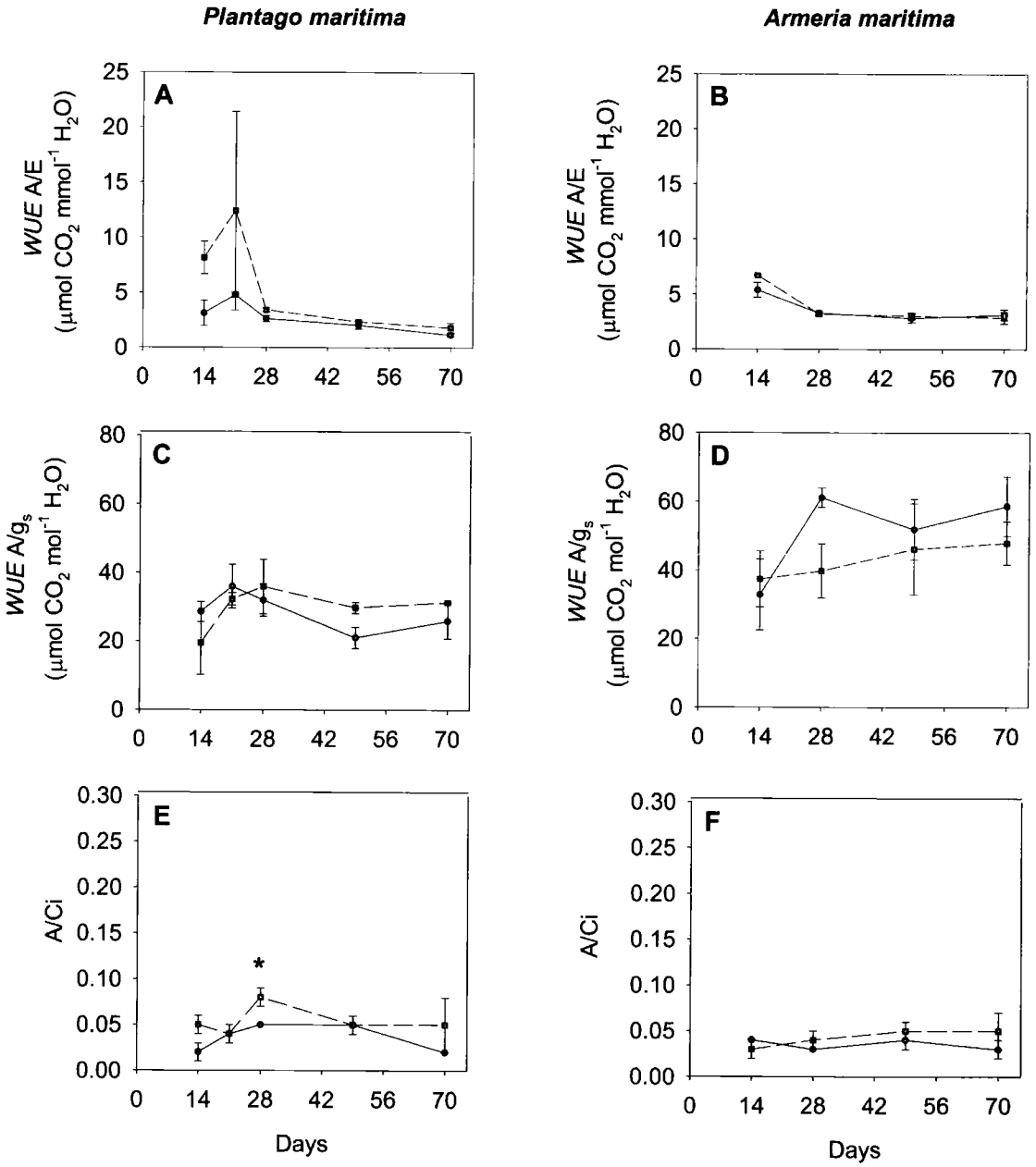


Figure 5.2. Water-use efficiency based on stomatal conductance (*WUE* A/E) (A, B); and based on transpiration (*WUE* A/g_s) (C, D) and A/C_i (E, F) of *Plantago maritima* and *Armeria maritima* grown under well-watered (100 % FC) (solid line) or drought (20 % FC) (dashed line) conditions over a period of 70 days. Data represent mean ± SE. n = 3. Significant effects of drought are indicated by * = $P \leq 0.01$.

5.2.2 Leaf water potential

Both species had slightly higher (less negative) water potentials as a result of drought, compared to well-watered plants over time, especially after 28 days growth (Figs. 5.3 A & B). These increases were significant in *P. maritima* after days 28 and 49 ($F = 1.652_{1,4}$ $P = 0.005$ and $F = 8.270_{1,4}$ $P = 0.045$). In contrast, there were no significant differences between droughted and well-watered plants of *A. maritima*.

5.2.3 Response of water potential to changes in percent soil-water content

By subjecting the plants to varied levels of soil water (Field capacity, FC) it was possible to measure how the leaf water potential was altered in both species. When all the water potential measurements over the ten-week period were correlated to the percent water content of the sand in the plant pots, *P. maritima* showed a significant positive correlation as the soil water content increased ($r^2 = 0.083$; adjusted $R^2 = 0.074$; $F = 9.106_{1,101}$ $P = 0.003$) (Fig. 5.4 A), whilst *A. maritima* showed no change in its water potential under different soil water availabilities ($r^2 = 0.007$; adjusted $R^2 = 0.000$; $F = 0.664_{1,101}$; $P = 0.417$) (Fig. 5.4 B).

When the water potential was broken down to a per-harvest basis, *P. maritima* consistently (apart from day 21) had lower water potential (less negative) with increasing water deficit (Figs. 5.5 A – F). Plants of *A. maritima* started off showing the typical plant response of decreased (more negative) water potential with a reduction in sand water content over time in for the first 14 days (Figs. 5.5 A & B). However, after the first 14 days, the water potential was slightly increased with a reduction in the water availability. Significant correlations occurred on day 28 for both species (Fig. 5.5 D; Table 5.1).

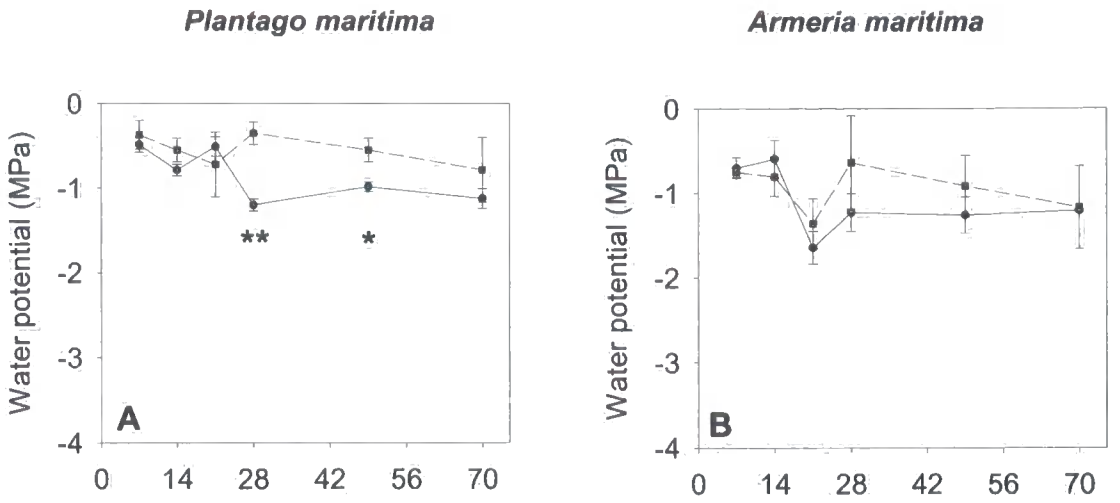


Figure 5.3. Shoot water potential of *Plantago maritima* (A) and *Armeria maritima* (B) grown under well-watered (100 % FC) (solid line) or drought (20 % FC) (dashed line) over a period of 70 days. Data represent mean ± SE. n = 3. Significant effects of drought are indicated by * = $P \leq 0.05$; ** = $P \leq 0.01$.

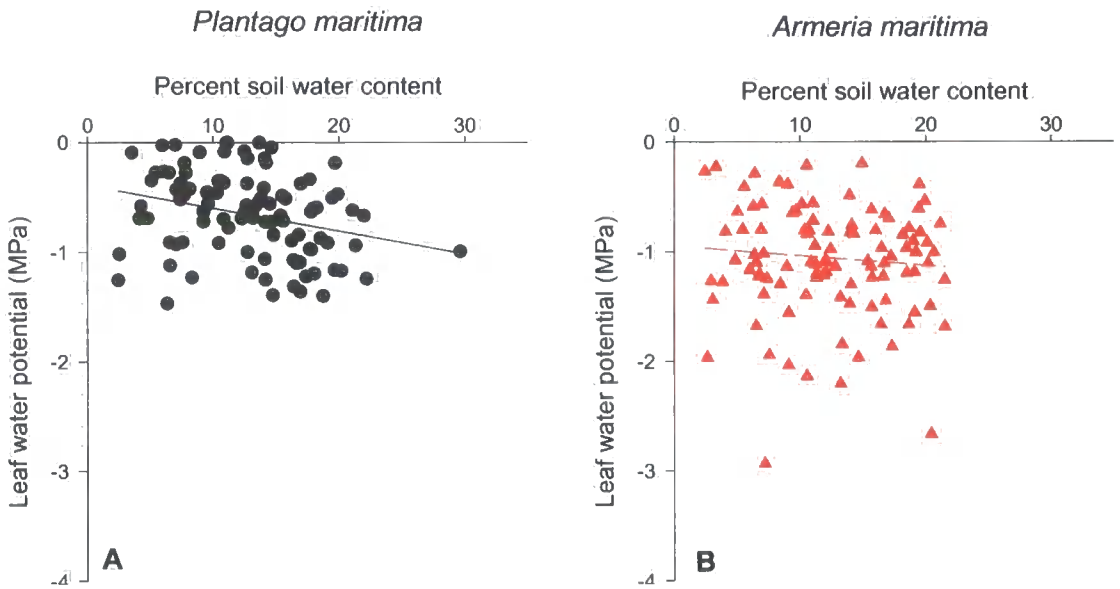


Figure 5.4. Correlations between percent soil-water content and leaf water potential in *Plantago maritima* (A) and *Armeria maritima* (B). Individual data points are derived from all harvests. Line represents linear regression.
Plantago maritima, (y = -0.390 + -0.020x) $r^2 = 0.083$; adjusted $R^2 = 0.074$; $F = 9.106_{1,101}$ $P = 0.003$ and *Armeria maritima* (y = -0.990 + -0.008x) $r^2 = 0.007$; adjusted $R^2 = 0.000$; $F = 0.664_{1,101}$ $P = 0.417$. n = 102.

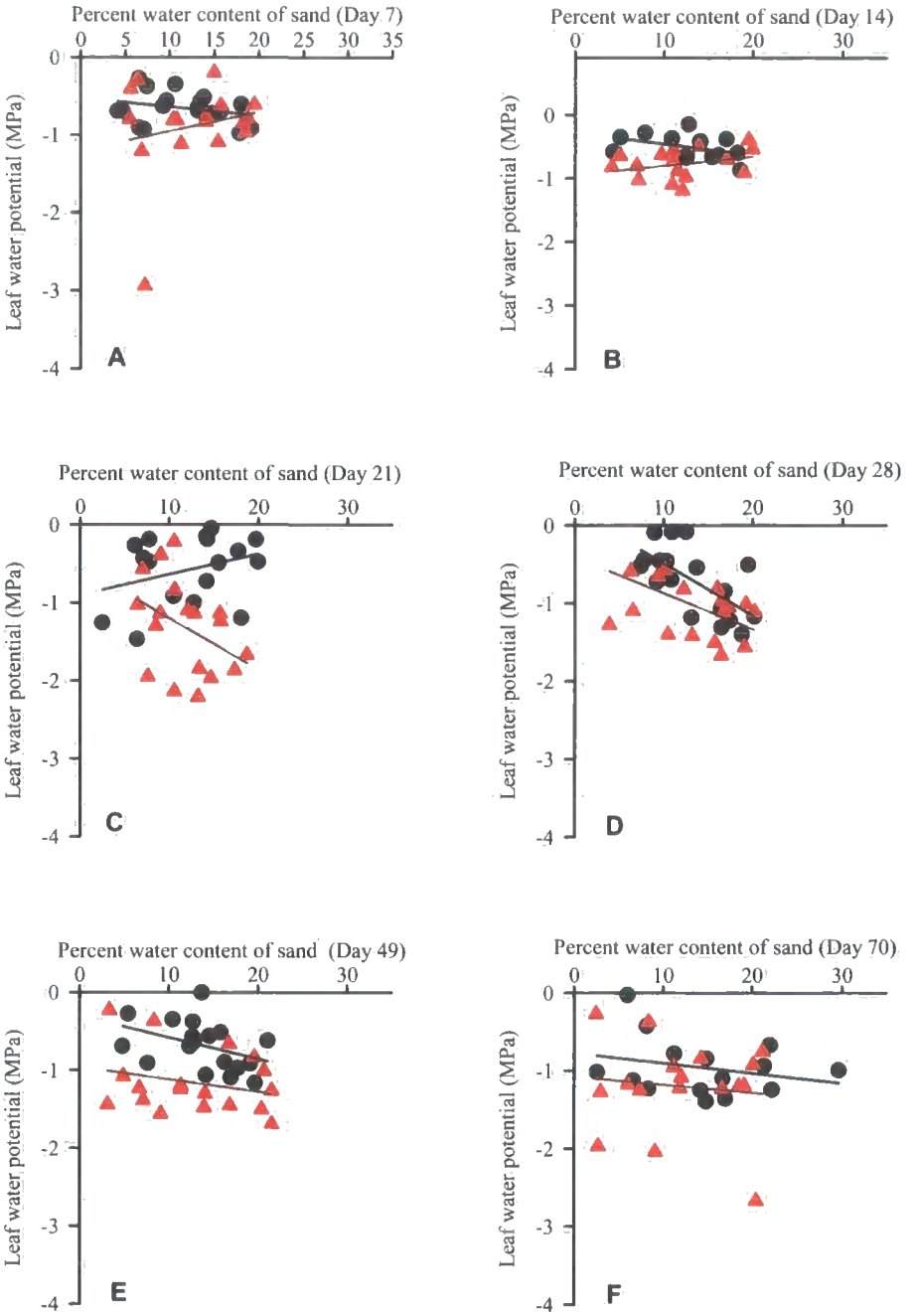


Figure 5.5. Correlations between soil-water content and leaf water potential in *Plantago maritima* (circles) and *Armeria maritima* (triangles) over a period of 70 days. Lines represent linear regressions.

Table 5.1. Results from the linear regression equation and ANOVA to test for significant correlations between soil-water content and leaf water potential in *Plantago maritima* and *Armeria maritima* over 70 days. Significance level is set at $P < 0.05$.

				ANOVA		
	Days	Regression equation	R^2	*Adjusted* r^2	F	P
Plantago maritima	7	y = -0.523 + -0.011x	0.072	0.014	$1.236_{1,16}$	0.283
	14	y = -0.276 + -0.018x	0.174	0.099	$2.320_{1,11}$	0.156
	21	y = -0897 + 0.026x	0.100	0.040	$1.663_{1,15}$	0.217
	28	**y = 0.180 + -0.067x**	**0.424**	**0.388**	**$11.773_{1,16}$**	**0.003**
	49	y = -0.301 + -0.028x	0.163	0.110	$3.105_{1,16}$	0.097
	70	y = -0.772 + -0.013x	0.067	0.000	$0.931_{1,13}$	0.352
	Days					
Armeria maritima	7	y = -1.198 + 0.025x	0.042	0.000	$0.653_{1,15}$	0.432
	14	y = -0.940 + 0.01x	0.092	0.027	$1.414_{1,14}$	0.254
	21	y = -0.541 + -0.066x	0.159	0.107	$3.037_{1,16}$	0.101
	28	**y = -0.411 + -0.046x**	**0.244**	**0.197**	**$5.173_{1,16}$**	**0.037**
	49	y = -0.959 + -0.016x	0.064	0.005	$1.093_{1,16}$	0.311
	70	y = -1.077 + -0.010x	0.013	0.000	$0.197_{1,15}$	0.664

5.2.4 Plant biomass and water concentrations

A reduction in the water availability reduced the shoot and root biomass in *P. maritima* with a significant reduction in the shoot biomass on day 49 ($F = 23.73_{1,4}$ $P = 0.008$). In contrast, the biomass did not alter over time in *A. maritima* (Figs. 5.6 A - D).
The water concentrations of the shoot and root in *P. maritima* were lowered by reduced water availability with significant reductions in the shoot on day 14 ($F = 11.396_{1,4}$ $P = 0.028$) and in the root on days 21 ($F = 160.865_{1,3}$ $P = 0.001$); 49 ($F = 33.452_{1,4}$ $P = 0.004$) and 70 ($F = 35.919_{1,4}$ $P = 0.004$) (Figs. 5.6 E & G). However, although the water concentration of the shoot and root in *A. maritima* were lowered by reduced water availability there were no statistically significant reductions (Figs. 5.6 F & H).

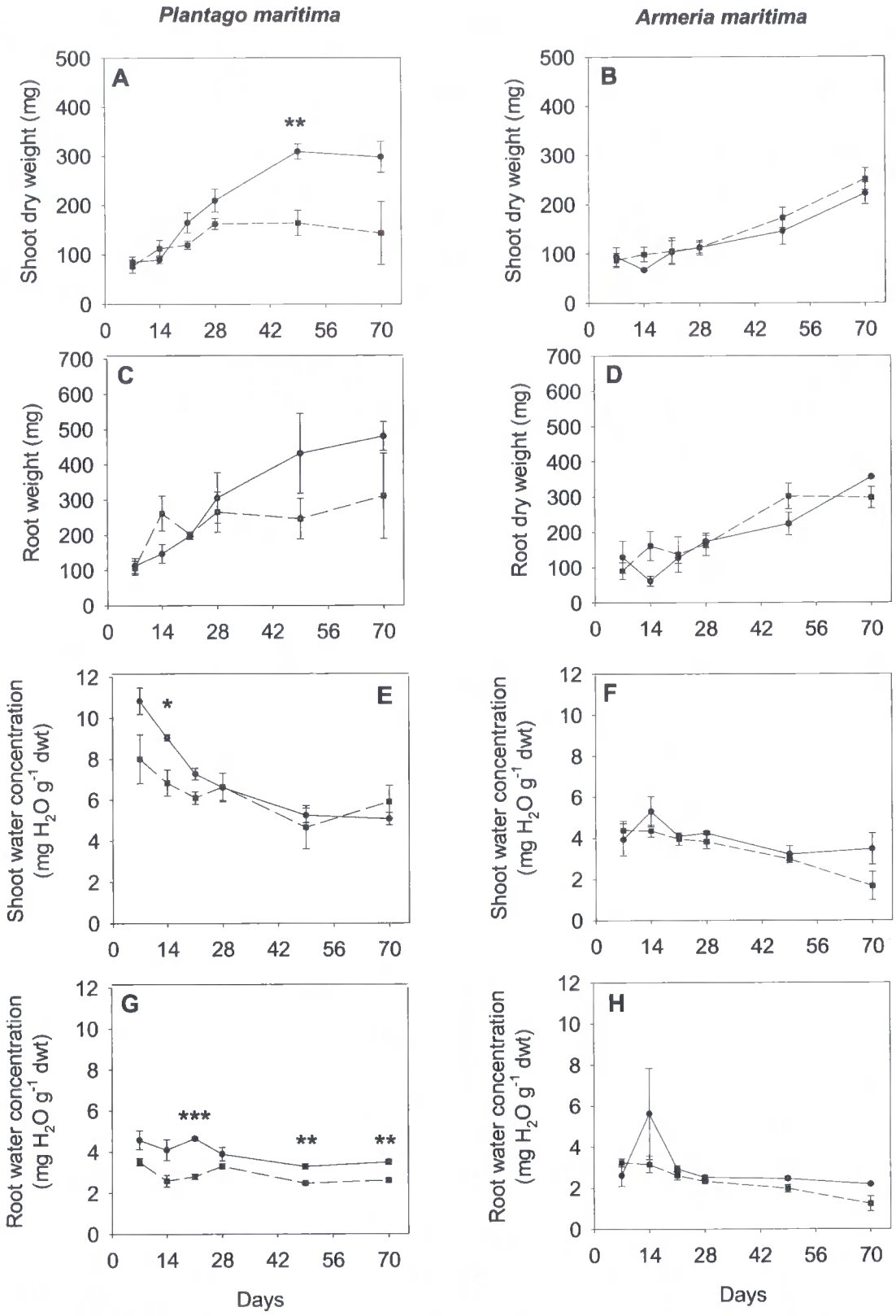


Figure 5.6. Shoot and root biomass and water concentration of *Plantago maritima* (A, C, E, G) and *Armeria maritima* (B, D, F, H) grown under well-watered (100 % FC) (solid line) or drought (20 % FC) (dashed line) conditions over a period of 70 days. Data represent mean ± SE. n = 3. Significant effects of drought are indicated by * = $P \leq 0.05$; ** = $P \leq 0.01$; *** = $P \leq 0.001$.

5.2.5 Conclusions and discussion: ten-week drought study

Plant water potential

The main hypothesis of the first section of this chapter was that there is a relationship between reduced water availability to *P. maritima* and *A. maritima* and a lowering of the water potential (becomes more negative) of the shoot tissue as compatible solute concentrations increase in droughted plants (Chapter 4). However, both species had higher (less negative) water potentials as a result of drought, compared to the corresponding well-watered plants over time, especially after 28 days (Figs. 5.3 A & B). The majority of plants show a more negative water potential in response to drought (Wyn Jones & Gorham 1983), therefore it is interesting that *P. maritima* showed a negative correlation to increased soil water content (Fig. 5.4 A; Fig. 5.5), whilst *A. maritima* had no change in its water potential under different soil water availabilities (Fig. 5.4 B; Fig. 5.5). These results are in agreement with the study of Jefferies *et al.* (1979), where the salt-marsh variety of *P. maritima*, showed less negative leaf water potentials in plants with a higher sorbitol concentration when grown under saline conditions (ie, a leaf water potential of –1.2 MPa with a sorbitol concentration of 30 mM compared to a leaf water potential of –2.8 MPa with a sorbitol concentration of 12 mM). However, Köhl (1997 b) found that the root water potential in *A. maritima* was more negative when grown in high external salt concentrations.
The maintenance of shoot water potential could be brought about by a number of other factors such as stomatal closure (Schulze & Hall 1982; Beadle *et al.* 1993). This would increase the cell turgor component of water potential (Wyn Jones & Gorham 1983). The turgor maintenance is important in drought situations, as it is this that will ultimately extend the period of carbon acquisition (Osmond 1980). Also, cell membrane and wall properties may alter in *A. maritima* causing the cells to become more desiccation tolerant (Ferris & Taylor 1994). However, there were no decreases in the stomatal opening or transpiration in *P. maritima*, which refutes the hypothesis that stomatal closure was partly responsible for the increased leaf water potential (Figs. 5.1 E & F). Also, *A. maritima* had higher rates of water loss by transpiration due to higher stomatal conductance. Technically, higher rates of transpiration in droughted plants should lower the leaf water potential, though in this species the water potential was not altered when compared to the well-watered plants. Compatible solute accumulation in plant cells results in a decrease of the cell osmotic potential, which helps in maintaining

water absorption and cell turgor pressure (Wyn Jones & Gorham 1983; Chapter 4), which will help in maintaining stomatal opening and photosynthesis. This may explain the higher rates of transpiration, stomatal conductance and water potential. It has been found in crop plants that if the stomata close at a lower water content the plants may recover from drought quicker if the stomata will also open at a lower water content when they are provided with water (Serraj & Sinclair 2002).

Plant photosynthesis, transpiration and water-use-efficiency

The driving force of transpirational water loss is represented by the gradient of water potential between the leaf interior and the atmosphere (Burghardt & Riederer 2003). Therefore, the reduction of stomatal opening is a quick way of regulating against water stress and drought tolerant species can still photosynthesise even if stomata are closed (hence a higher *WUE*) (Clark *et al.* 1999). It was hypothesised that any initial photosynthetic responses to drought, such as stomatal closure (reduced g_s), will cause an increase in the *WUE*. This hypothesis held true for *P. maritima* and *A. maritima* as the *WUE* was initially increased (though not statistically significant) in response to drought when photosynthesis was based on a per unit of transpiration (Fig. 5.2 A & B). However, when *WUE* was based upon stomatal conductance, there was only an increased *WUE* in *P. maritima* on day 49, and in *A. maritima* the *WUE* was reduced. This implies that *P. maritima* can still photosynthesise with lower rates of stomatal conductances whilst *A. maritima* cannot photosynthesise as well with lower rates of stomatal conductance. The slightly higher rates of A over E in *P. maritima* could be due to the fact as this species accumulates mainly C-based compatible solutes (rather than nitrogenous solutes, as in *A. maritima*) (Stewart *et al.* 1979) as it will need higher rates of photosynthesis to maintain the C required for the *de novo* synthesis of sorbitol during drought. This would in turn improve the plant water relations as cell turgor could be maintained (Munns *et al.* 1983).

It was also hypothesised that the *WUE* and A/C_i ratio would be reduced over time as the activity and/or concentration of Rubisco acclimate to the long-term effects of drought and other physiological factors, such as altered stomatal conductance and density. There were slight increases in the *WUE* of *P. maritima* and *A. maritima* when expressed as a function of transpiration (Fig. 5.2), which was then reduced to rates similar to that of well-watered plants. When *WUE* was expressed as a function of stomatal conductance, the *WUE* was not reduced over time in *P. maritima*. However, the *WUE*

was reduced in *A. maritima*, suggesting that photosynthetic acclimation to drought had occurred and that responses such as reduced chlorophyll could be a major response to drought in *A. maritima* but not in *P. maritima*. This result is consistent with the results in chapter 3 showing that photosynthetic capacity, along with a reduction in chlorophyll concentration, was reduced in droughted *A. maritima* plants compared to well-watered plants (chapter 3.5.1 & 3.5.2). The A/C_i ratio did not decrease over time in droughted plants, suggesting that photosynthetic acclimation to the drought (e.g. reduced Rubisco) did not occur, otherwise the internal intercellular CO_2 concentration would increase since the CO_2 was not being assimilated.

Plant biomass and water concentrations

Finally, it was hypothesised that changes in the photosynthetic parameters and water potential would ultimately change the biomass and water concentration of the plant. Interestingly though, *P. maritima* had a more pronounced response to drought in terms of reduced biomass and water concentration than *A. maritima* (Fig. 5.6). Although *P. maritima* had higher *WUE*, and did not significantly alter its stomatal conductance and transpiration rate during drought, the biomass and water concentrations were significantly reduced in droughted plants. However, in *A. maritima*, the biomass and water concentrations were not significantly affected by drought even though the plants would be experiencing greater water loss, as shown by higher rates of photosynthesis, transpiration and *WUE*. The fact that *A. maritima* was not affected by reduced water availability may explain why it is this species that is dominant over *P. maritima* in the drier coastal cliff-top habitats, as reported in Goldsmith (1967). Passioura (1982) proposes two strategies that plants can use for dealing with a limited water supply. The first strategy is 'conservative', where other competing plants do not diminish water supply, such as for plants growing in isolation and so stomatal conductance is reduced over time. The other strategy may explain why *A. maritima* increases rates of A, g_s and E, this is termed 'prodigal' and is appropriate for plants competing for a limited water supply or subjected to mild droughts of short duration. The stomatal conductance and transpiration rates are high and so the water supply is used rapidly (Schulze & Hall 1982) until it is gone, at which point the plant tries to take out as much water from the soil so as to survive until the next rainfall. If the drought is going to be long then the conservative approach is appropriate as *WUE* is increased without affecting the water lost through transpiration. If drought periods are frequent and short, (which could be

the case in cliff-top habitats in temperate regions, such as in this study) then the prodigal strategy may be more appropriate as although *WUE* is reduced, the rain water may infiltrate the dry soil more quickly, so reducing the evaporative water loss from the soil.

5.3 Results: four-week drought and elevated CO_2 study

5.3.1 Photosynthetic parameters of *Plantago maritima* and *Armeria maritima* with decreasing water availability and elevated CO_2

In *P. maritima*, the net photosynthetic rate (A) of well-watered plants was initially increased by elevated CO_2 (day 7, $F = 20.911_{1,5}$ $P = 0.006$). However, the A was lower on day 21 and the same on day 28 when compared to A in plants grown at ambient CO_2 (Fig. 5.7 A). A conformable increase in A was significant in droughted plants on days 7 and 14 ($F = 55.102_{1,5}$ $P = 0.001$ and $F = 84.111_{1,5}$ $P = 0.000$ respectively) compared plants grown at ambient CO_2 (Fig. 5.7 B). This was due to a significantly lower A in droughted plants grown at ambient CO_2 compared to well-watered plants at ambient CO_2 (day 7, $F = 6.089_{1,6}$ $P = 0.049$ and at day 21, $F = 9.736_{1,4}$ $P = 0.036$) (Figs. 5.7 A & B).

The stomatal conductance (g_s) and transpiration rates (E) had a response to elevated CO_2 similar to that of photosynthesis. The g_s and E were higher in well-watered plants exposed to elevated CO_2 for the first 14 days ($F = 7.523_{1,5}$ $P = 0.041$ and $F = 8.027_{1,5}$ $P = 0.037$ respectively) (Figs 5.7 C & E). Thereafter, the g_s and E rates were lower than those in plants grown at ambient CO_2. However, in droughted plants, the g_s and E rates were significantly higher in plants exposed to elevated CO_2 up until day 21 (Figs. 5.7 D & F), where rates were similar to those in plants exposed to ambient CO_2 (day 7 g_s, $F = 42.338_{1,5}$ $P = 0.001$; day 7 E, $F = 39.780_{1,5}$ $P = 0.001$; day 14 g_s, $F = 15.879_{1,5}$ $P = 0.010$ and day 14 E, $F = 24.243_{1,5}$ $P = 0.004$). The g_s and E were lower in droughted plants exposed to ambient CO_2, compared to well-watered plants at ambient CO_2 (Figs. C & D; E & F). However, the g_s and E in droughted plants that were exposed to elevated CO_2 were slightly higher than well-watered plants exposed to elevated CO_2, though none of these differences were statistically significant. The *P. maritima* plants that were exposed to drought at ambient CO_2 all died between days 21 and 28.

The rates of A, g_s and E were all lower in *A. maritima* compared to *P. maritima* (compare figures 5.7 and 5.8). Only small fluctuations in the A, g_s or E occurred in *A. maritima* in response to elevated CO_2 or drought treatments (Figs. 5.8 A – F). There was a significant increase of A in response to elevated CO_2 in well-watered plants on day 14, compared to well-watered plants grown at ambient CO_2 ($F = 6.062_{1,6}$ $P = 0.049$) (Fig. 5.8 A).

The water-use-efficiency (*WUE*), in *P. maritima*, whether based on stomatal conductance (g_s) or transpiration (E), was higher in plants exposed to elevated CO_2 (Figs. 5.9 A & C) and this increase was significant on day 28 (Fig. 5.9 C) ($F = 8.684_{1,4}$ $P = 0.042$), compared to plants at ambient CO_2. However, in droughted plants the increase of *WUE* only occurred at day 21 (Figs. 5.9 B & D) (*WUE* A/g_s, $F = 18.175_{1,4}$ $P = 0.013$ and *WUE* A/E, $F = 42.154_{1,4}$ $P = 0.003$). Drought did not significantly alter the *WUE*, compared to well-watered plants (Figs. 5.9 A & B).

In *A. maritima*, the *WUE* as a function of stomatal conductance and transpiration was higher in well-watered plants exposed to elevated CO_2, compared to plants at ambient CO_2 (Fig. 5.10 A & C). Significant increases of *WUE* (A/g_s) occurred on days 14 and 28 ($F = 13.453_{1,6}$ $P = 0.010$ and $F = 9.163_{1,4}$ $P = 0.039$) and *WUE* (A/E) on days 14 and 28 ($F = 10.030_{1,6}$ $P = 0.019$ and $F = 9.290_{1,4}$ $P = 0.038$). However, there was only a significant increase of *WUE* in droughted plants exposed to elevated CO_2 on day 21 (Figs. 5.10 B & D) (*WUE* A/g_s, $F = 11.577_{1,4}$ $P = 0.027$ and *WUE* A/E $F = 15.878_{1,4}$ $P = 0.016$) compared to droughted plants at ambient CO_2. The *WUE* was increased by drought (Figs. 5.10 A & B; C & D), especially in plants at ambient CO_2 when compared to well-watered plants at ambient CO_2. The increases in *WUE* were significantly increased by drought in plants at ambient CO_2 on day 14 (A/g_s, $F = 14.103_{1,6}$ $P = 0.009$; A/E, $F = 11.690_{1,6}$ $P = 0.014$) and day 21 (A/g_s, $F = 41.758_{1,4}$ $P = 0.003$; A/E, $F = 56.201_{1,4}$ $P = 0.002$) compared to well-watered plants at ambient CO_2.

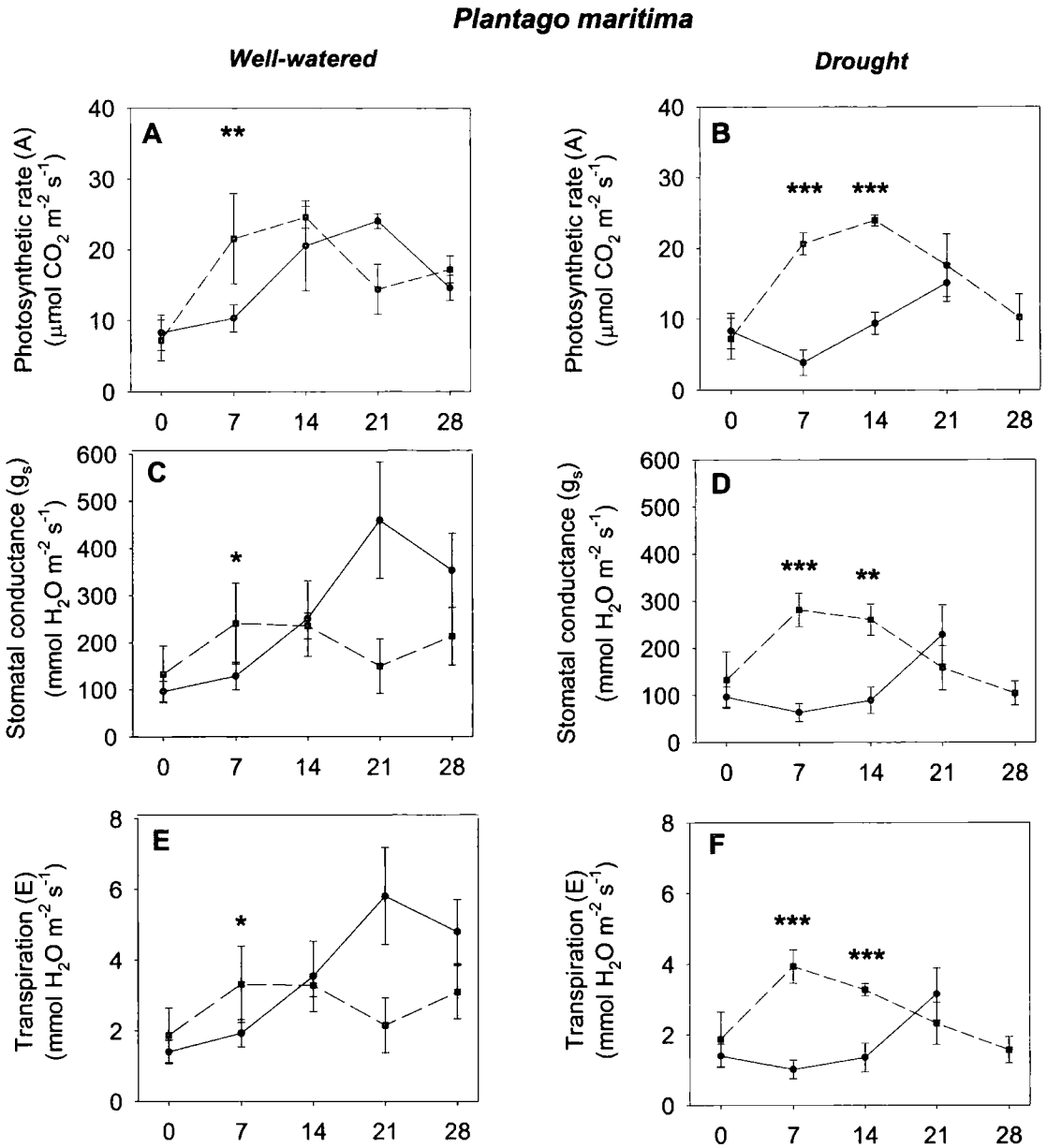


Figure 5.7. Net photosynthesis (A), stomatal conductance (g_s) and transpiration (E) of *Plantago maritima* exposed to either ambient CO_2 (solid line) (360 μmol mol^{-1}) or elevated CO_2 (dashed line) (600 μmol mol^{-1}) grown under well-watered (100 % FC) (A, C, E) or drought (20 % FC) (B, D, F) conditions over a period of 28 days. Data represent mean ± SE. n = 3-4. Significant effects of elevated CO_2 are indicated by * = $P \leq 0.05$ ** = $P \leq 0.01$ *** = $P \leq 0.001$.

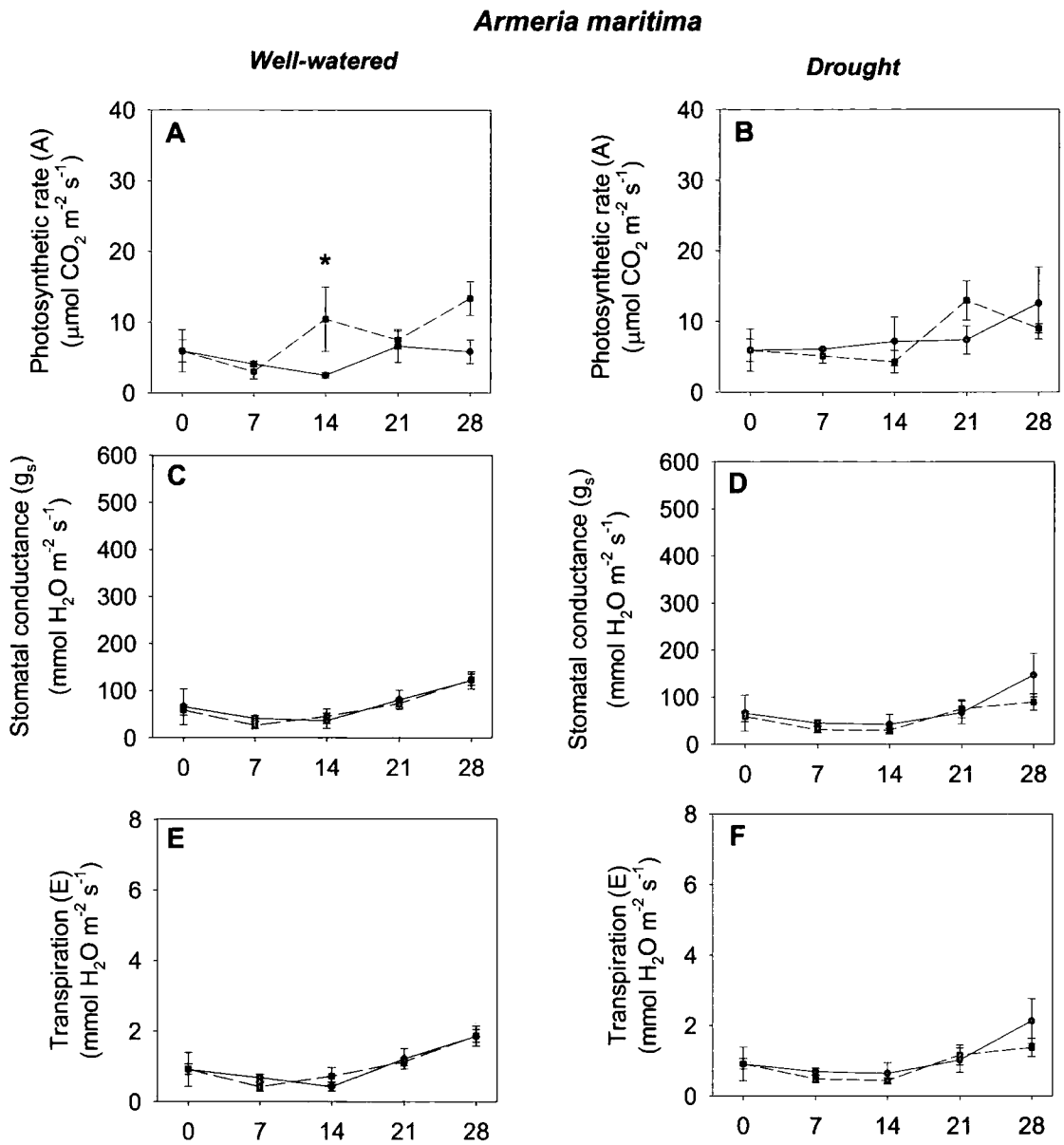


Figure 5.8. Net photosynthesis (A), stomatal conductance (g_s) and transpiration (E) of *Armeria maritima* exposed to either ambient CO_2 (solid line) (360 μmol mol^{-1}) or elevated CO_2 (dashed line) (600 μmol mol^{-1}) grown under well-watered (100 % FC) (A, C, E) or drought (20 % FC) (B, D, F) conditions over a period of 28 days. Data represent mean ± SE. n = 3-4. Significant effects of elevated CO_2 are indicated by * = $P \leq 0.05$.

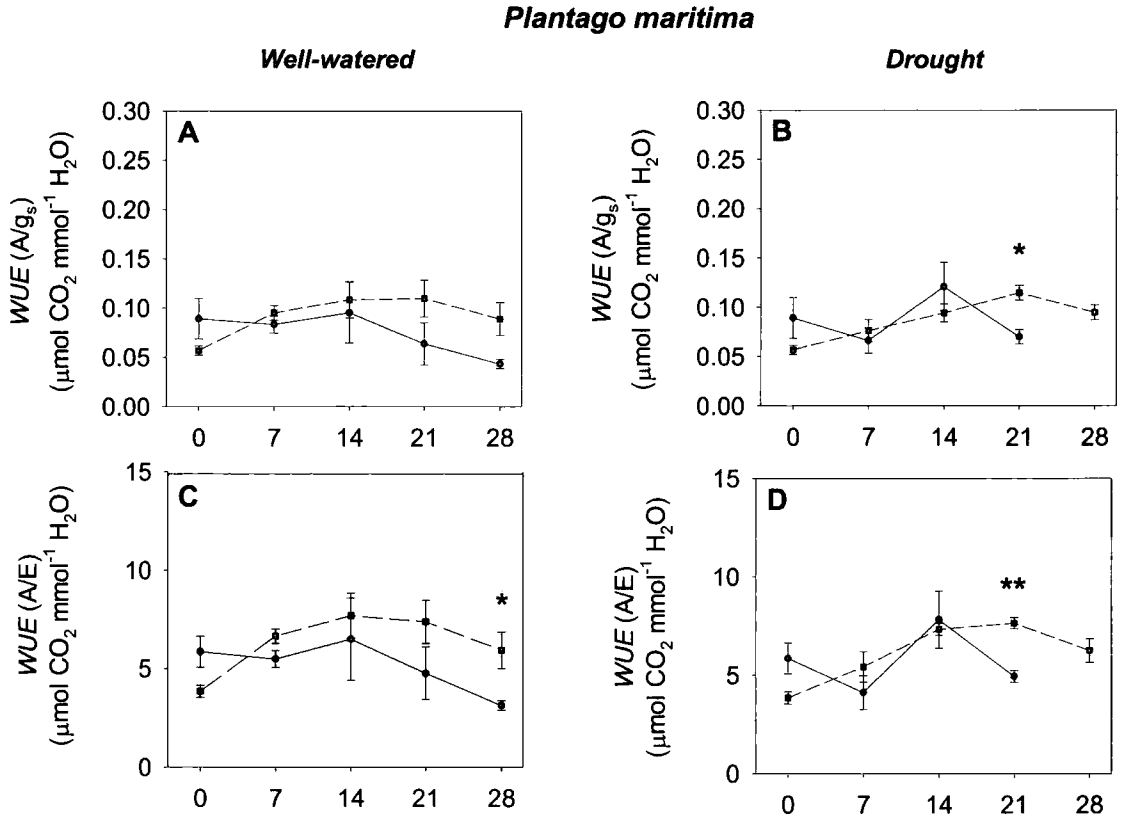


Figure 5.9. Water-use efficiency based on stomatal conductance (*WUE* A/g_s) (A, B) and on transpiration (*WUE* A/E) (C, D) of *Plantago maritima* exposed to either ambient CO_2 (solid line) (360 µmol mol^{-1}) or elevated CO_2 (dashed line) (600 µmol mol^{-1}) grown under well-watered (100 % FC) (A, C) or drought (20 % FC) (B, D) conditions over a period of 28 days. Data represent mean ± SE. n = 3-4. Significant effects of elevated CO_2 are indicated by * = $P \leq 0.05$ ** = $P \leq 0.01$.

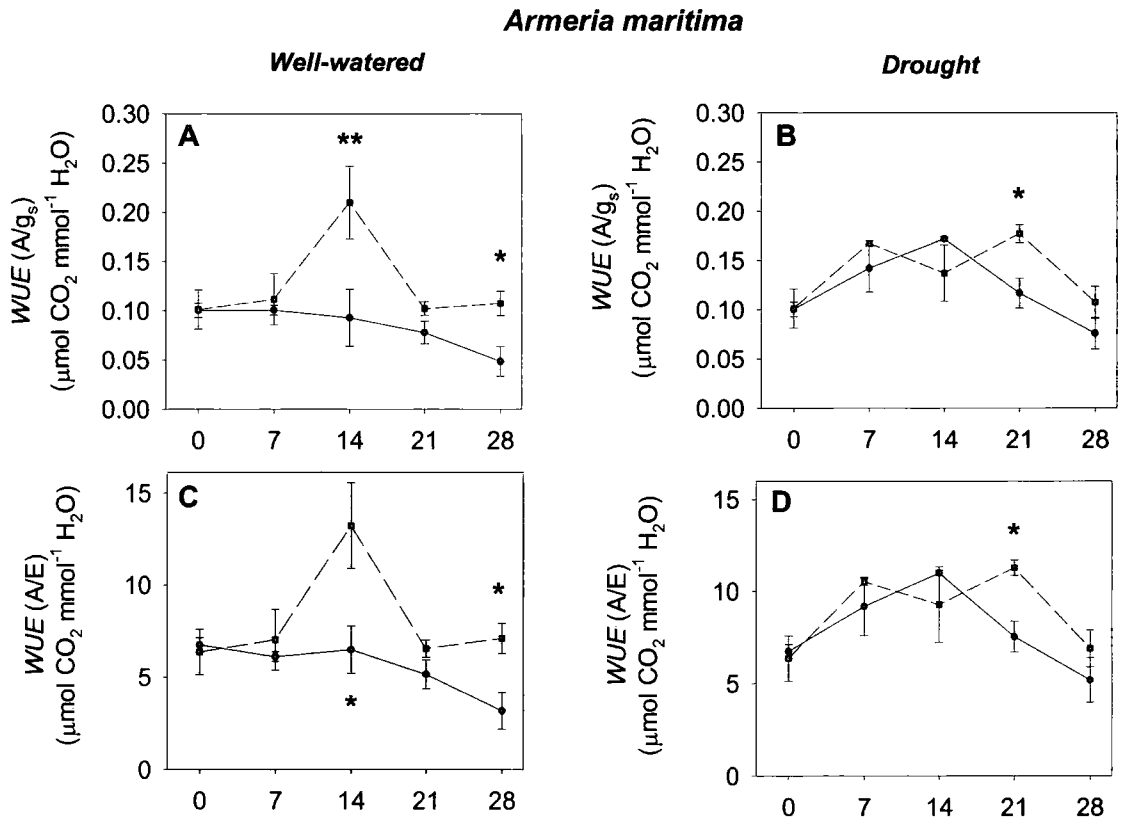


Figure 5.10. Water-use efficiency based on stomatal conductance (*WUE* A/g_s) (A, B) and on transpiration (*WUE* A/E) (C, D) of *Armeria maritima* exposed to either ambient CO_2 (solid line) (360 µmol mol^{-1}) or elevated CO_2 (dashed line) (600 µmol mol^{-1}) grown under well-watered (100 % FC) (A, C) or drought (20 % FC) (B, D) conditions over a period of 28 days. Data represent mean ± SE. n = 3-4. Significant effects of elevated CO_2 are indicated by * = $P \leq 0.05$ ** = $P \leq 0.01$.

5.3.2 Leaf water potential

In *P. maritima*, the shoot water potential was not significantly altered by elevated CO_2 (Figs. 5.11 A & B), though there was a non-significant trend towards higher water potential in well-watered plants grown at elevated CO_2. However, the root water potential was lowered (more negative) (Figs. 5.11 C & D) in plants exposed to elevated CO_2 (apart from day 21 in plants that were droughted, Fig. 5.11 D). Drought did not significantly affect the shoot or root water potential in plants grown at either ambient or elevated CO_2 (Figs. 5.11 A & B; C & D). Drought caused a significant transient increase in the shoot water potential of plants grown at ambient CO_2 on day 7 ($F = 7.879_{1,5}$ $P = 0.038$) when compared to well-watered plants at ambient CO_2 (Figs. 5.11 A & B).

In *A. maritima*, the shoot water potential was lowered significantly (made more negative) by elevated CO_2 on day 14 in well-watered plants ($F = 9.770_{1,4}$ $P = 0.035$) and on day 21 in droughted plants ($F = 11.898_{1,4}$ $P = 0.026$) (Figs. 5.12A B). In the roots of well-watered plants there was a significant increase in the water potentials of plants exposed to elevated CO_2 compared to plants grown at ambient CO_2 on day 7 ($F = 126.75_{1,2}$ $P = 0.008$) (Fig. 5.12 C). However, although not significant, the opposite occurred in droughted plants, as elevated CO_2 caused a lowering of the water potential, compared to plants grown at ambient CO_2 (Fig. 5.12 D). It is unlikely that positive MPa values occurred within the plant (Fig. 5.11 D & 5.12 D) as this would imply full cell turgor and for the cells to contain pure water. Therefore, this is probably due to slight errors in the equipment. Droughting the plants caused a significant reduction in the shoots of plants grown at ambient CO_2 on day 14 and at elevated CO_2 on day 21 ($F = 29.275_{1,4}$ $P = 0.006$ and $F = 20.947_{1,4}$ $P = 0.010$ respectively), compared to well-watered plants at ambient CO_2 (Fig. 5.12 A & B). The root water potential was not significantly altered by drought, compared to well-watered plants (Fig. 5.12 C & D).

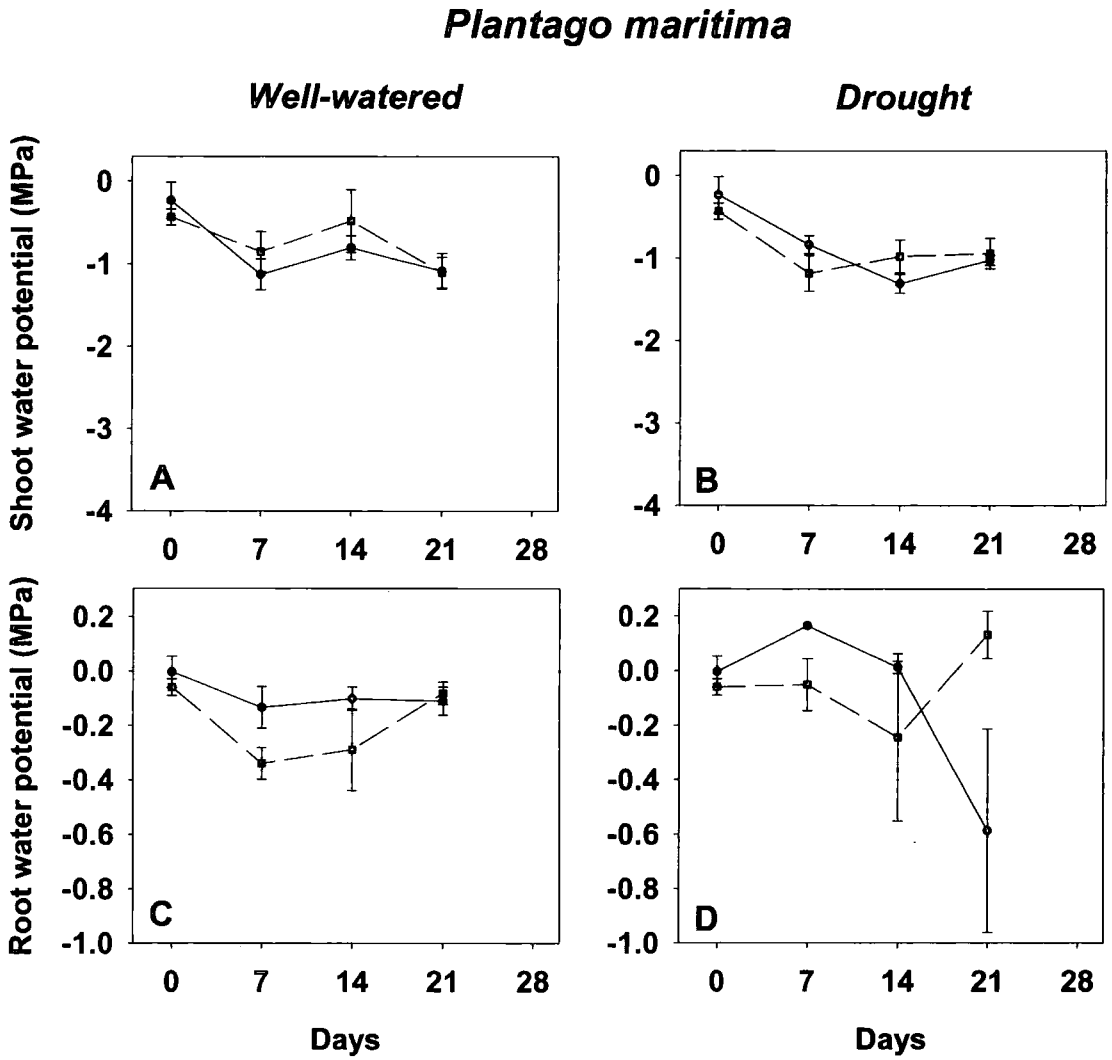


Figure 5.11. Shoot and root water potential of *Plantago maritima* exposed to either ambient CO_2 (solid line) (360 μmol mol^{-1}) or elevated CO_2 (dashed line) (600 μmol mol^{-1}) grown under well-watered (100 % FC) (A, C) or drought (20 % FC) (B, D) conditions over a period of 21 days. n = 3. Note differences of scale for root water potentials. Data represent mean ± SE.

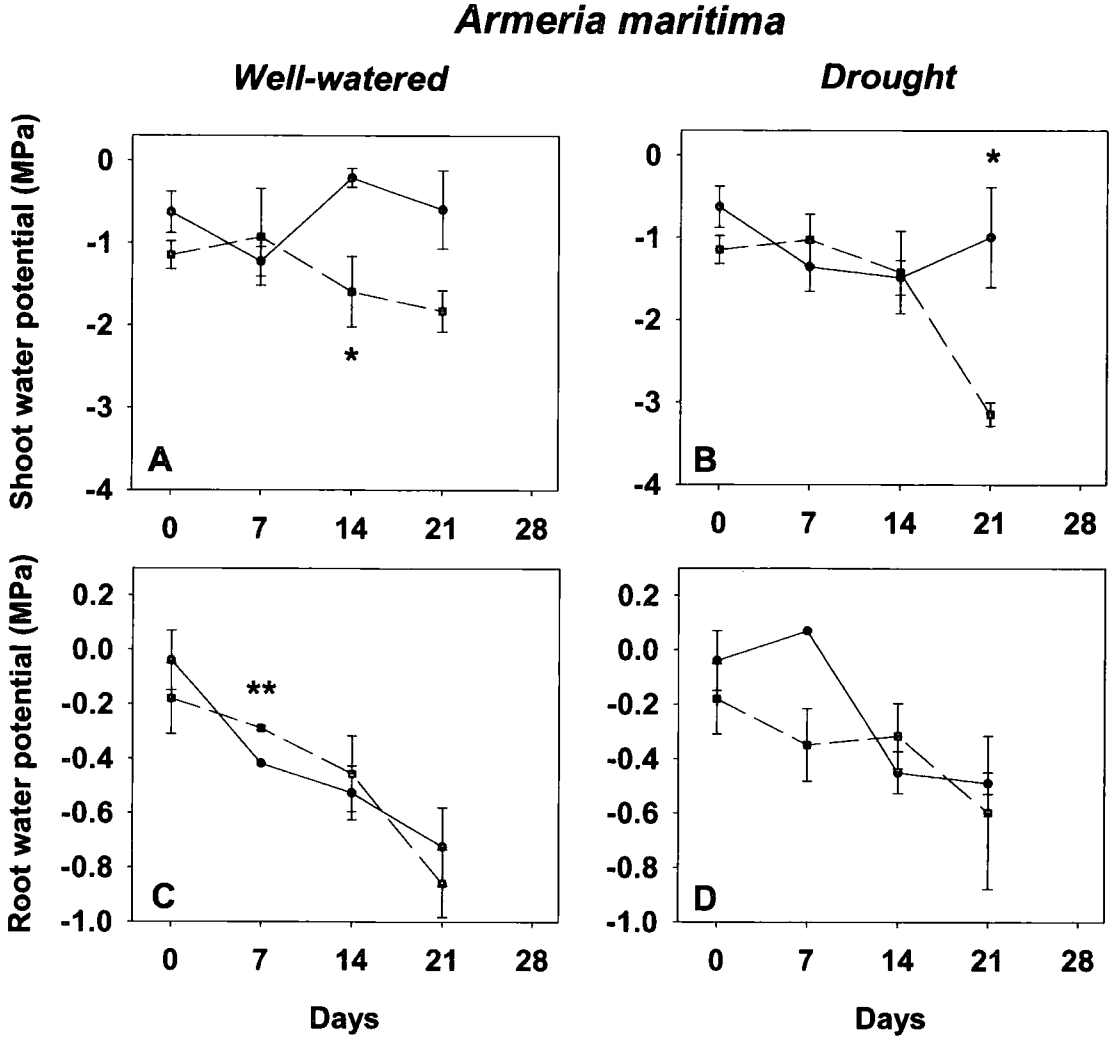


Figure 5.12. Shoot and root water potential of *Armeria maritima* exposed to either ambient CO_2 (solid line) (360 µmol mol^{-1}) or elevated CO_2 (dashed line) (600 µmol mol^{-1}) grown under well-watered (100 % FC) (A, C) or drought (20 % FC) (B, D) conditions over a period of 21 days. Note differences of scale for root water potentials. Data represent mean ± SE. n = 3. Significant effects of elevated CO_2 are indicated by * = $P \leq 0.05$; ** = $P \leq 0.01$.

5.3.3 Plant biomass and tissue water concentrations

Elevated CO_2 and drought did not significantly alter the shoot or root biomass in well-watered or droughted *P. maritima*, compared to plants grown at ambient CO_2 (Figs. 5.13 A – D). A significant decrease in the root dry weight occurred in well-watered plants exposed to elevated CO_2 on day 14 compared to plants grown at ambient CO_2 ($F = 9.784_{1,5}$ $P = 0.026$) (Fig. 5.13 C). The shoot water concentration was lower in plants exposed to elevated CO_2 with significant reductions on days 14 and 21 in well-watered plants ($F = 9.701_{1,6}$ $P = 0.021$ and $F = 14.913_{1,6}$ $P = 0.008$) (Figs. 5.13 E & F). The root water concentrations were not affected by elevated CO_2 (Figs. 5.13 G & H). Drought caused a transient lowering of the shoot water concentration in plants grown at ambient CO_2 on day 14 ($F = 8.961_{1,6}$ $P = 0.024$) (Figs. 5.13 E & F), compared to well-watered plants. However, drought did cause significant reductions in the root water concentration on day 21 in plants exposed to ambient and elevated CO_2, ($F = 8.294_{1,6}$ $P = 0.028$ and $F = 11.327_{1,6}$ $P = 0.015$ respectively) and after day 28 in plants exposed to elevated CO_2 ($F = 14.192_{1,4}$ $P = 0.020$), compared to their respective well-watered plants (Figs. 5.13 G & H).

In *A. maritima* the biomass was lower in plants exposed to elevated CO_2 (Figs. 5.14 A – D) with shoot biomass being significantly lower on day 0 ($F = 29.549_{1,6}$ $P = 0.002$) and on the root biomass in droughted plants on day 21 and 28 ($F = 17.104_{1,4}$ $P = 0.014$ and $F = 10.026_{1,4}$ $P = 0.034$ respectively). The difference on day 0 was due to bigger plants being randomly assigned to this treatment at the start and not a true effect of elevated CO_2. There was a transient increase in the root biomass of well-watered plants on day 7 compared to plants grown at ambient CO_2 ($F = 10.066_{1,4}$ $P = 0.034$) (Fig. 5.8 C). The shoot or root biomass was not significantly altered in plants that were exposed to drought (Figs. 5.14 A & B; C & D), compared to well-watered plants. The water concentrations were slightly reduced in *A. maritima* exposed to elevated CO_2 (Figs. 5.14 E – F) with significant reductions at day 0, again, being due to larger plants randomly assigned to the start group ($F = 15.022_{1,6}$ $P = 0.008$ and $F = 6.510_{1,6}$ $P = 0.043$ for the shoot and root). However, transient increases in the root water concentration did occur in droughted plants exposed to elevated CO_2 compared to plants exposed to ambient CO_2 on day 7 and day 28 ($F = 8.844_{1,4}$ $P = 0.041$ and $F = 11.316_{1,4}$ $P = 0.028$ respectively). Drought did not affect the shoot water concentration, compared to well-watered plants. However, there were significant reductions in the root water concentrations in droughted plants grown at ambient and elevated CO_2 on day 21

($F = 15.252_{1,4}$ $P = 0.017$ and $F = 12.829_{1,4}$ $P = 0.023$) and a reduction in plants grown at ambient CO_2 on day 28, when compared to well-watered plants ($F = 21.225_{1,4}$ $P = 0.010$) (Figs. 5.14 G & H).

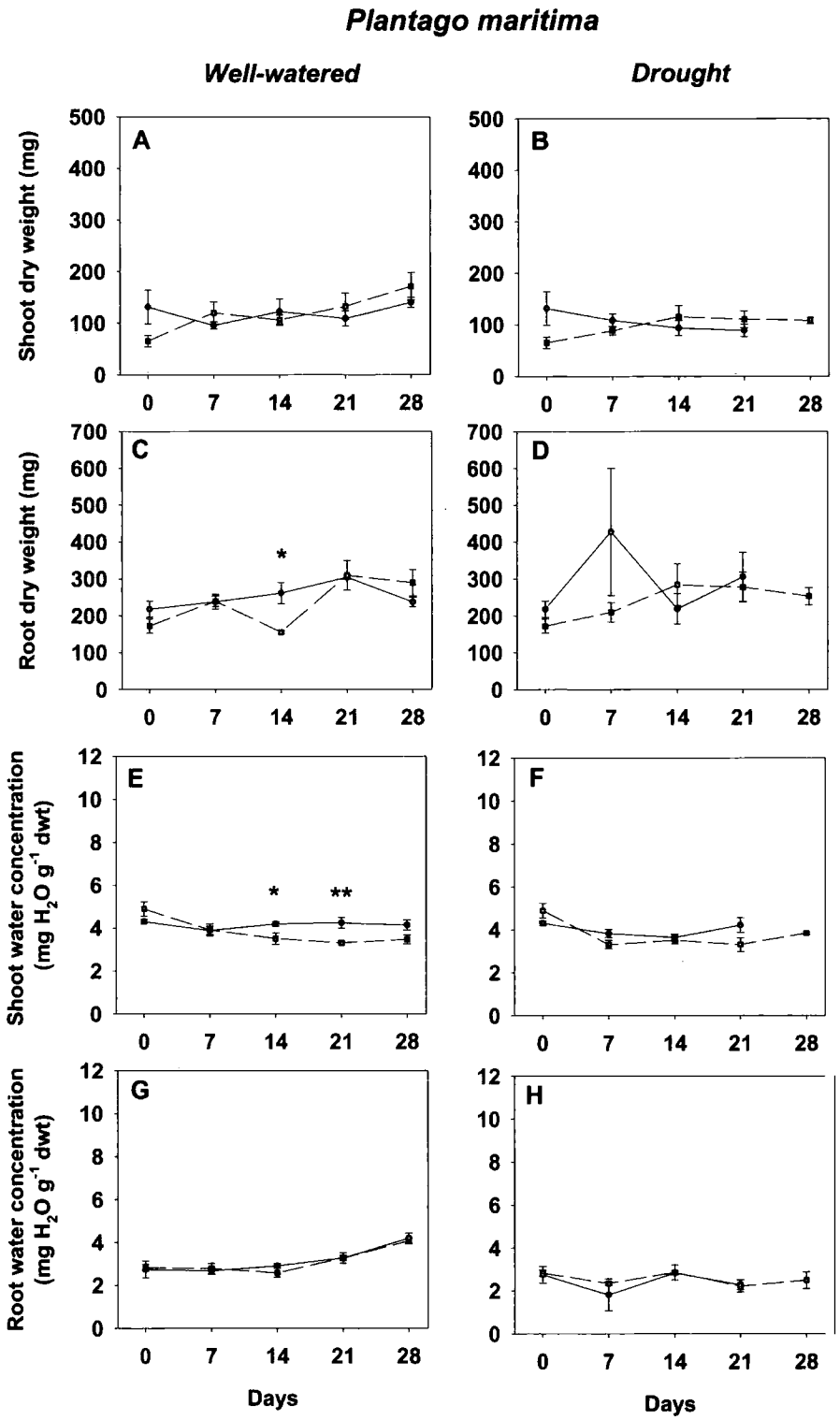


Figure 5.13. Shoot and root biomass and water concentration of *Plantago maritima* exposed to either ambient CO_2 (solid line) (360 µmol mol^{-1}) or elevated CO_2 (dashed line) (600 µmol mol^{-1}) grown under well-watered (100 % FC) (A, C, E, G) or drought (20 % FC) (B, D, F, H) conditions over a period of 28 days. Data represent mean ± SE. n = 3-4. Significant effects of elevated CO_2 are indicated by * = $P \leq 0.05$; ** = $P \leq 0.01$.

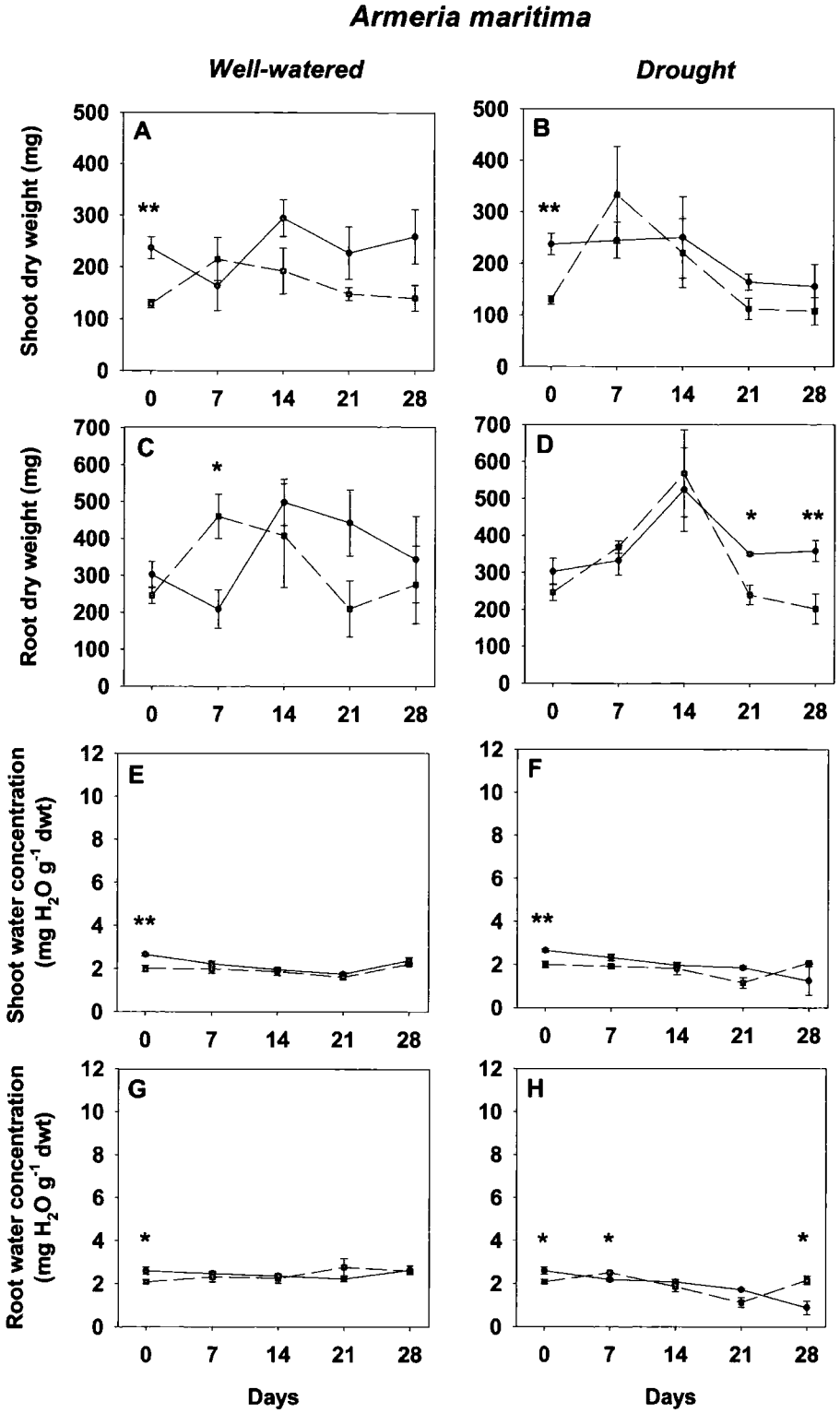


Figure 5.14. Shoot and root biomass and water concentration of *Armeria maritima* exposed to either ambient CO_2 (solid line) (360 µmol mol^{-1}) or elevated CO_2 (dashed line) (600 µmol mol^{-1}) grown under well-watered (100 % FC) (A, C, E, G) or drought (20 % FC) (B, D, F, H) conditions over a period of 28 days. Data represent mean ± SE. n = 3-4. Significant effects of elevated CO_2 are indicated by * = $P \leq 0.05$; ** = $P \leq 0.01$.

5.3.4 Conclusions and discussion: four-week drought and elevated CO_2 study

Plant photosynthesis, transpiration and water-use-efficiency

The aim of the study within the second section of this chapter was to test the hypothesis that reduced g_s, E and increased *WUE* occur in *P. maritima* and *A. maritima* under increased CO_2 availability, which in turn reduces the compatible solute concentrations, increases the water potential and water concentration in the study plants. However, in *P. maritima*, g_s and E were actually higher in well-watered plants exposed to elevated CO_2 for the first 14 days (Figs 5.7 C & E). Thereafter, g_s and E rates were lower than those in plants grown at ambient CO_2. In droughted plants though, g_s and E rates were significantly higher in plants exposed to elevated CO_2 up until day 21 (Figs. 5.7 D & F), where rates were similar to those in plants exposed to ambient CO_2, and this is consistent with other plant species (Liang & Maruyama 1995). This means that elevated CO_2 actually prolonged the time period of increased transpirational water loss in droughted *P. maritima*. Therefore, elevated CO_2 did not seem to have ameliorative effects on transpiration water loss in droughted plants when compared to plants grown at ambient CO_2, though there were signs of increased water conservation by reduced stomatal conductance and transpiration in well-watered plants (Fig. 5.7). The net photosynthetic rate (A) had a response to elevated CO_2 similar to that of stomatal conductance (g_s) and transpiration rates (E). The increased net photosynthetic rates resulting from elevated CO_2 in droughted *P. maritima* were similar to a study by Clark *et al.* (1999). They showed that the photosynthetic rates of *Plantago lanceolata* were increased by about 50 % by elevated CO_2 when well-watered but there was an increase of about 300 % when soil water was also decreased. Only small fluctuations in the A, g_s and E occurred in *A. maritima* in response to elevated CO_2 or drought treatments (Figs. 5.8 A – F). Therefore the hypothesis is refuted that changes in the compatible solute concentrations are linked to changes in transpirational water loss by low stomatal conductance. Water use efficiency (*WUE*) is usually higher in plants exposed to elevated CO_2 (Wolfe *et al.* 1998), which was the case in well-watered *P. maritima* (Figs. 5.9 A & C). It is commonly concluded that such a response to elevated CO_2 will help plants withstand drought (Eamus 1991; Tuba *et al.* 1998; Pospíšilová & Čatský 1999) though *WUE* might only be important ecologically if plant water loss is either reduced or remains unchanged. However, the *WUE*'s were the same in droughted *P. maritima* at ambient CO_2 as they were in droughted plants at elevated CO_2

(Figs. 5.9 B & D), therefore this would not cause a major ameliorative effect of drought in an elevated CO_2 atmosphere. However, despite the seemingly non-responsiveness to elevated CO_2 and drought in the A, g_s and E of *A. maritima*, the *WUE* as a function of stomatal conductance and transpiration was higher in well-watered plants exposed to elevated CO_2, compared to plants at ambient CO_2 (Fig. 5.10 A & C). However, there was only a significant increase of *WUE* in droughted plants exposed to elevated CO_2 on day 21 (Figs. 5.10 B & D) compared to droughted plants at ambient CO_2. As for *P. maritima*, the *WUE* in droughted *A. maritima* grown at elevated CO_2 was similar to droughted plants at ambient CO_2 therefore causing little benefit to the plant. The only possible benefit was that the *WUE* remained higher for a longer period of time in droughted plants exposed to elevated CO_2 (21 days) compared to droughted plants at ambient CO_2 where the *WUE* at ambient CO_2 in droughted plants began to drop after 14 days.

It was hypothesised reductions in transpiration and stomatal conductances are partly responsible for the alterations in the compatible solute concentrations in plants exposed to drought and elevated CO_2. Unfortunately, it was not possible to quantify the compatible solutes in the samples obtained for this study so the photosynthetic results from this study are compared to the concentrations of compatible solutes reported in a separate study in chapter four. It is possible that the reductions in sorbitol concentrations in *P. maritima* exposed to elevated CO_2 after 5 weeks are due to the reduced rates of transpiration and stomatal conductance as shown in Fig. 5.7. However, this does not explain why the betaine and proline concentrations in *A. maritima* were reduced when exposed to elevated CO_2, as the rates of transpiration and stomatal conductance were not altered (Fig. 5.8). Although the *WUE* was increased in *A. maritima*, this was due to increases of photosynthesis at elevated CO_2 rather than a lowering of rates of transpiration and stomatal conductance (Fig. 5.10). Hypothetically, elevated CO_2 could increase drought tolerance by increasing soluble sugars for osmotic adjustment so lowering osmotic potential (Tognetti *et al.* 2000 b; Johnson *et al.* 2002; Wullschleger *et al.* 2002). Increased osmotic adjustment will help water enter cells and stop water from leaving them, so maintaining turgor for cell expansion (Hare *et al.* 1998). However in the current study there were no changes in the soluble carbohydrate concentrations in droughted or elevated CO_2-treated plants (data not shown).

Plant water potentials

The hypothesis that reduced g_s and E, would lead to increased water potential (less negative) when compared to droughted plants grown under ambient CO_2 concentrations held true in *P. maritima*, as the leaf water potential was slightly higher in plants exposed to elevated CO_2 in well-watered plants. However, this did not occur in droughted plants (Fig. 5.11). In the roots, the trend was actually towards a lower water potential in response to elevated CO_2 in well-watered plants at days 7 and 14. However, in droughted plants at day 21 the water potential was higher in plants exposed to elevated CO_2, as the water potential in droughted plants at ambient CO_2 was reduced. This shows some ameliorative effects of elevated CO_2, as water potentials are maintained in droughted plants at elevated CO_2. However, the transpiration rates on days 7 and 14 were higher in *P. maritima* grown at elevated CO_2 (Fig. 5.7). This may explain why the root water potential was lower in the roots in response to transpirational water loss (Weatherley 1982). Also, on day 21 the transpiration rates at elevated CO_2 were lower than those at ambient CO_2 and this coincided with higher root potentials at day 21 in plants grown at elevated CO_2. This result accepts the hypothesis that elevated CO_2 alters water potential when transpiration rates are reduced. If the water potentials of the roots are lowered in *P. maritima* growing at elevated CO_2, they may be better able to extract soil water, and increased *WUE* will also increase carbon acquisition without increasing stomatal conductance in plants grown at elevated CO_2. This result is consistent with another member of the *Plantago* family as the root water potential of *Plantago media* was more negative in plants grown at elevated CO_2 than in those grown at ambient CO_2. It was concluded that this was due to an increase in cell expansion at elevated CO_2 and hence 'cell-wall loosening', i.e., cell wall properties were altered (Passioura 1982; Ferris & Taylor 1994). The opposite response to elevated CO_2, compared to *P. maritima*, occurred in *A. maritima* as the shoot water potentials were significantly lowered by elevated CO_2 in both well-watered and droughted plants (Fig. 5.12). This refutes the hypothesis that altered g_s and E would change the water potential; the shoot water potential was still reduced although the g_s and E were not altered by elevated CO_2 (Fig. 5.8). It could be that as the g_s and E were not altered by elevated CO_2 there may be increases in the compatible solute concentration that would lower the water potential, though this is in disagreement with results reported for compatible solute concentrations in chapter 4. However, other compounds, such as phenolics and organic acids could contribute to the lowering of plant water potentials

(Peltier *et al.* 1997; Wullschleger *et al.* 2002). Although caution should be given to simple timepoint changes, the root water potential was actually increased on day 7 in well-watered plants in *A. maritima*. However, this response was the opposite in droughted plants as the root water potential at elevated CO_2 was lower than that at ambient CO_2.

These results are consistent with a study by Ferris & Taylor (1995) who also found that *WUE* increased with elevated CO_2 in the chalk land plant *Sanguisorba minor*. They also reported that the leaf water potential was more negative in plants grown under elevated CO_2, in droughted and well-watered plants, compared to plants grown at ambient CO_2. However, in its neighbouring species, *Anthyllis vuleraria*, elevated CO_2 did not affect the water potential. The results from the current study are not in agreement with the review of papers that show, in general, elevated CO_2 leads to less negative water potentials with lower stomatal conductance (Wall 2001; Wullschleger *et al.* 2002). In some circumstances the stomatal conductance can be higher and not lower in plants exposed at elevated CO_2 (Wullschleger *et al.* 2002). This response occurred in *P. maritima* (Fig. 5.7) during the first 14 days of growth and this would mean that soils would become drier more quickly and so plants are subjected to higher levels of drought and associated plant water stress. A lower root water potential at elevated CO_2 would allow better extraction of water from the soil (soils have water potentials of around –0.02 and –2.0 MPa, Beadle *et al.* 1993), as the water potential of soil would be reduced in droughted soils. The plant may die as it risks depleting the soil water at a quicker rate (Serraj & Sinclair 2002).

Plant biomass and water concentrations

It was hypothesised that changes in g_s and E caused by elevated CO_2 may ultimately affect the water content and growth of the plant. The shoot biomass of *P. maritima* was marginally higher in plants exposed to elevated CO_2, even in plants exposed to drought, which shows that there is little ameliorative effect of elevated CO_2 in droughted plants (Fig. 5.13). This could be due to the increased photosynthetic rates at the start of the experiment (Fig. 5.7). However, the root biomass was not affected by elevated CO_2. Despite the lower transpiration rates after day 14 the shoot water concentration was significantly lower in well-watered plants exposed to elevated CO_2 (Fig. 5.13). However, the shoot water content was not significantly lowered in droughted plants by elevated CO_2. This means that elevated CO_2 maintained water concentrations in the

shoot of droughted plants, which may explain why the shoot water potential was not affected by elevated CO_2 during drought. The root water content was not affected by elevated CO_2. The plants of *P. maritima* that were exposed to drought at ambient CO_2 all died between days 21 and 28, however, the droughted plants that were exposed to elevated CO_2 all survived so providing evidence that elevated atmospheric CO_2 could help the survival of plants experiencing long-term drought.

Interestingly, the *A. maritima* shoot and root biomass were eventually reduced by elevated CO_2 over time despite increased *WUE* caused by increased photosynthetic rates (Figs. 5.10 and 5.14). Although the shoot water potentials were more negative in plants exposed to elevated CO_2, this did not alter the shoot water concentrations (Fig. 5.14). However, the root water concentrations were actually higher in droughted plants exposed to elevated CO_2 and this also corresponded with lower root water potential (Fig. 5.12). It is also possible that the fine root area was increased so maintaining water uptake (Wullschleger *et al.* 2002). Therefore, there are some benefits to increased CO_2 in droughted *A. maritima*. This means there is potential to alter the competitive relationships, in terms of tissue water relations, amongst plant species that are frequently exposed to drought (Tognetti *et al.* 2000 a).

5.4 General conclusions

A: Increased soil water deficit:

1. increased shoot water potential in *P. maritima* but does not alter it in *A. maritima*
2. increased water-use-efficiencies in both species due to higher rates of photosynthesis over transpiration
3. reduced the biomass and water concentration in *P. maritima* but not in *A. maritima*

B: Elevated atmospheric CO_2:

1. initially increased, then decreased the stomatal conductance and transpiration in *P. maritima*, with transpiration rates remaining higher for a longer period of time
2. did not affect photosynthesis, stomatal conductance or transpiration rates in *A. maritima*
3. increased *WUE* but did not increase the *WUE* any further in droughted plants for both species, though the increased *WUE* remained higher for a longer period
4. slightly increased the shoot water potential and lowered the root water potential in well-watered *P. maritima*
5. lowered the shoot water potential and caused a transient increase in the root water potential of well-watered *A. maritima*, though the root water potential was lowered in droughted plants
6. did not significantly alter the biomass of *P. maritima* but did reduce the shoot and root biomass of *A. maritima* despite increased photosynthetic rates and *WUE*
7. reduced the shoot water concentration in well-watered *P. maritima* but not in droughted shoots or the roots
8. did not affect the shoot water concentration but increased the root water concentration in droughted *A. maritima*

Chapter 6: Long-term responses to elevated CO_2: a test of the protein competition model of phenolic allocation in *Plantago maritima* and *Armeria maritima*

Aims

The aim of work reported in this chapter was to investigate the effects of long-term increased atmospheric CO_2 on the biomass, photosynthesis, total protein and phenolic concentrations of *Plantago maritima* and *Armeria maritima*.

6.1 Introduction

As atmospheric CO_2 concentrations are predicted to rise from 360 to 550-1000 μmol CO_2 mol^{-1} over the next 100 years (IPCC 2001) it is important to understand the physiological impacts this will have on plant primary and secondary metabolism (Farrar & Williams 1991; Peñuelas *et al.* 1997). One of the major components of secondary metabolism is the production of carbon (C)-based phenolic compounds, which are vital in many plant functions such as defence against abiotic and biotic stress (Dixon & Paiva 1995). The two main models to predict responses of plant phenolics to changes in atmospheric CO_2 are the carbon-nutrient balance model (CNB) (Bryant *et al.* 1983) and the growth-differential balance model (GDB) (Herms & Mattson 1992), which postulates that C-based phenolics are determined by the balance between C and nutrient availability. The basic hypothesis of these models is that if the plant, through increased photosynthesis acquires more C, a greater proportion of C can be allocated to secondary metabolism, and hence C-based phenolics. This allocation of C to phenolics in plants grown under elevated CO_2 is predicted to increase when other factors, such as nitrogen (N) availability, is limited. These models have provided a sound hypothetical basis for many experiments on predicting the effects of elevated CO_2 on plant secondary metabolism (Peñuelas & Estiarte 1998). However, these models are over-simplified and do not include the biochemical mechanisms that might be underlying the C allocation to phenolic synthesis, which also needs to be taken into account if a clearer understanding of the changes in metabolism are to be understood in a wide range of plant species. Therefore, other models to improve the CNB and GDB models need to be devised and tested (Hamilton *et al.* 2001; Koricheva 2002; Lerdau & Coley 2002; Nitao *et al.* 2002). The protein competition model (PCM) for predicting phenolic allocation has been proposed as alternative to the CNB and GDB models (Jones & Hartley 1999) but to date

it has been little tested (Bezemer *et al.* 2000). The PCM uses the fact that both protein and phenolic synthesis utilise the same precursor, the amino acid phenylalanine (PHE), which can either be acted on by the enzyme phenylalanine ammonium lyase (PAL; EC 4.3.1.5) to generate cinnamic acid for phenylpropanoid and related phenolic synthesis or be incorporated into protein synthetic pathways (Margna 1977). However, although the PCM does not claim that PAL is *the* pivotal point in the control of C- and N- metabolism it is based on research showing C allocation to phenolic synthesis is linked to the PHE allocation to the total protein demand. As cell PHE concentrations may be a limiting substrate for protein and phenolic production, an inverse relationship between its incorporation into proteins and phenolics would be expected to occur. By understanding the protein demands within a plant in a given environmental condition it is possible to predict the C allocation to phenolic synthesis and the total phenolic concentration (Fig. 6.1), which may ultimately affect how plants respond to environmental stress and insect and pathogen attack (Jones & Hartley 1999).

The PCM is split into 12 components (Fig. 6.1), which are explained and referenced in detail in Jones & Hartley (1999). The first component of the model considers the fact that the first committed step in phenylpropanoid synthesis is when PAL catalyses the conversion of PHE to *trans*-cinnamic acid. Up to this point, the PHE, irrespective of its origin, is available to enter protein synthesis. Therefore, when rates of protein synthesis are high, rates of phenylpropanoid related phenolic synthesis should be low and *vice versa*.

The demands for protein in the PCM are described as supporting *i, growth* for cell division and non-photosynthetic primary metabolism, especially during plant development; *ii, carbon fixation* for carboxylation via ribulose-1,5-bisphosphate (RuBP) and *iii, homeostatic* protein demand for cellular repair, maintenance and defence. The former two, in most cases, comprise the greatest protein demand and are ultimately controlled by gross genetic traits (e.g. fast-growing plants should have a greater growth and C-fixing protein demand than slow-growing plants) and biotic and abiotic environmental factors (availability of resources such as N, CO_2), which can be taken into account within the PCM.

The total phenolic demand in the PCM of the plant takes into account the allocation of totally or partially PHE-derived phenolic compounds via the PAL enzyme. These compounds include phenylpropanoids, lignans, lignin, flavonoids and most tannins.

However, the model cannot predict the response to individual classes of phenolics as other rate-limiting factors can determine the concentration of each individual class. Jones & Hartley (1999) assume that there is no indication that elevated CO_2 should affect PAL expression, and lead to altered phenolic synthesis, as there is no direct injury incurred on the plant. This is contrary to other evidence (Hartley *et al.* 2000; Lavola *et al.* 2000), which has shown increases in PAL activity in plants exposed to elevated CO_2. These and other factors which change in plants exposed to elevated CO_2, mainly altered photosynthetic rates, biomass accumulation and non-C resource limitations, may alter the protein and phenolic demands within the plant.

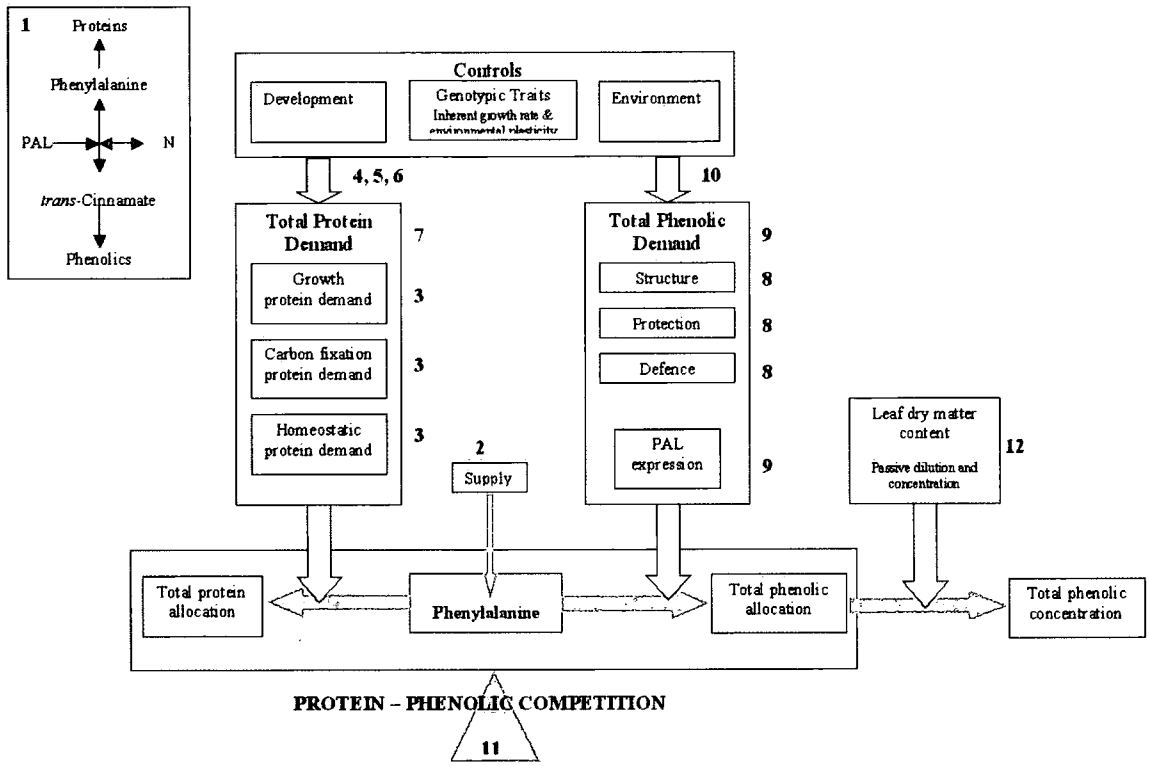


Figure 6.1. The protein competition model of phenolic allocation. Phenylalanine (PHE) is committed either to phenylpropanoid synthesis, via the phenylalanine ammonia lyase (PAL) step or to protein synthesis (1). PHE demand for proteins or phenolics exceeds PHE supply (2), resulting in process level competition and an inverse relationship between total protein allocation and total phenolic allocation (11). Allocation is determined by the balance between total protein demand (7) and total phenolic demand (9). Total protein demand is comprised of component protein demands (3) for growth, C fixation and homeostasis. Total phenolic demand for structure, protection and defence (8) is integrated via PAL expression (9). Total demands are controlled by development, by genetic traits associated with inherent growth rate and environmental plasticity, and by the environment (4-6, 10). Passive dilution or concentration of phenolics by dry matter content (12) is used to translate total phenolic allocation into total phenolic concentration, expressed as the total concentration of PHE-derived phenolics. Figure and text taken from Jones & Hartley (1999).

One of the main strengths of the PCM is that, unlike other models of C allocation to secondary metabolism, altered photosynthetic rates (C fixation demand) is taken into account as down-regulation of photosynthetic carboxylation by Rubisco can occur in plants grown under elevated CO_2. The short-term (hours to days) response to elevated CO_2 usually consists of an increase in photosynthetic rates as ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco; E.C. 4.1.1.39) is a primary rate-limiting step of photosynthesis. Since Rubisco is not saturated at the current atmospheric CO_2 concentration, any increase in the CO_2 substrate will increase photosynthetic rates in the short-term (Bowes 1991). Increased rates of photosynthesis are usually not sustainable, as other resources become limiting, such as the ability to regenerate RuBP, and this leads to photosynthetic acclimation of photosynthetic capacity (photosynthetic acclimation) (Stitt 1991; Stitt & Krapp 1999). Photosynthetic acclimation to elevated CO_2 in plants over a longer-term (days to weeks) can dramatically alter the whole plant response to increased C availability, particularly if plants are grown with a reduced N supply (Baxter *et* al. 1997; Harmens *et al.* 1998; Wolfe *et al.* 1998).

Although the underlying mechanisms are not fully understood, the hypothesis is that much of this acclimation is attributed to an increase in the efficiency of the C fixation per unit enzyme of the Rubisco protein so causing increased rates of net photosynthesis (Drake *et al.* 1997). Then, as the efficiency of the C fixation increases, the quantity of Rubisco enzyme can decrease. This reduction in the Rubisco content, and possibly activity due to inhibitory feedback mechanisms, is measured as a lower maximum velocity of carboxylation by Rubisco (*in vivo* carboxylation activity; $V_{c,max}$), compared to plants grown at ambient CO_2 (Sage *et al.* 1989). This ($V_{c,max}$) is derived from the initial slope of the response of CO_2 assimilation (A) to intercellular CO_2 concentration (ci). The electron transport-limited rate of regeneration of RuBP (J_{max}) can also be determined from this A/ci response curve (Farquhar & von Caemmerer 1982; Long & Hällgren 1993; Wullschleger 1993). *In vivo* carboxylation activity ($V_{c,max}$) is a product of Rubisco content and activity, so changes in $V_{c,max}$ can be due to either decreases in Rubisco content or activity. Therefore, the central argument is that a reduction of Rubisco protein would allow N, which would otherwise be allocated to Rubisco synthesis (considering that Rubisco usually consists of between 30-50 % of the total leaf soluble-protein content) to be reallocated to other non-photosynthetic functions, usually growth, thus resulting in a negative correlation between leaf N (or protein) and Rubisco activity. This reduction in Rubisco protein can be as much as 60 % (Bowes 1991; Cheng & Fuchigami 2000), however, increased net rates of photosynthesis can still be

observed in plants with 50 % less Rubisco due to changes in Rubisco activity (Stitt *et al.* 1991; Sicher *et al.* 1994).

As well as the protein demand for C-fixation, the protein demand for growth is considered in the PCM due to the possible increase of growth of plants exposed to elevated CO_2, especially if an adequate supply of N is available (Saralabai *et al.* 1997). Therefore, the PCM states that there are four possible physiological responses to elevated CO_2, which are all dependent on the level of photosynthetic acclimation (acclimation of photosynthetic capacity) and growth in response to elevated atmospheric CO_2. The four outcomes are; *i*, No photosynthetic acclimation (photosynthesis increases under elevated CO_2), increased growth; *ii*, No photosynthetic acclimation, no increase in growth; *iii*, Full photosynthetic acclimation (photosynthesis remains unchanged at elevated CO_2), increase in growth and *iv*, Full photosynthetic acclimation, no increase in growth. Generally, under elevated atmospheric CO_2, the model hypothesises that non- acclimated plants will favour protein synthesis for growth, over phenolic synthesis, and an increase of phenolic allocation in fully acclimated plants. Although many experiments show increased photosynthesis and growth, fewer studies have observed photosynthetic acclimation to elevated CO_2 with or without increases in growth (Jones & Hartley 1999). The PCM was based upon predicting phenolic concentrations in leaves as they have been well studied, however, the authors claim that it can be applied to other plant tissues.

The aim of the work reported in this chapter was to investigate the effect of long-term increased atmospheric CO_2 on the biomass, photosynthesis and total protein and phenolic contents in *Plantago maritima* and *A. maritima maritima*. It was hypothesised that both species will exhibit different responses to elevated CO_2 because *P. maritima* accumulates the C-based polyhydric alcohol, sorbitol, as a major end product of photosynthesis (Chapter 4; Walsh 2000) whilst *A. maritima* accumulates high levels of nitrogenous compatible solutes (betaine and proline) even under non-stressed conditions (Chapter 3) (Stewart *et al.* 1979). Therefore, this study will enhance the understanding of different metabolic responses of both these species to environmental change by enabling a clearer understanding of C and N partitioning within these plants under different availabilities of C.

The PCM was tested by assessing whether measured changes in the shoot and root phenolic concentrations in response to elevated CO_2 are the same as the hypothesised phenolic response (after taking into account the biomass and photosynthetic acclimation

response to elevated CO_2). The V_{cmax} and J_{max} measurements will give an indication of acclimation of photosynthetic capacity; therefore it was possible to test the hypothesis that acclimation of photosynthetic capacity is related to a reduction in the shoot soluble-protein concentration. The PCM was originally based upon leaves of fast-growing, mainly crop species. Therefore, by applying it to the leaves and roots of native species will provide a more rigorous test of the model.

6.2 Methods

Clonal shoots of *P. maritima* and *A. maritima* were collected from a coastal cliff-top in Northumberland, UK in July 2001 and transferred into seed trays containing acid-washed silica sand at the CEH Solardome facility. Plants were watered daily and a $1/4^{th}$ strength Long-Ashton solution modified to contain 28 mg l^{-1} N-NH_4NO_3 was added to each seed tray once per week. After four weeks, 64 similar-sized plants were individually placed into 500 cm^3 deep pots filled with 800 g of dry acid-washed silica sand. Plants were equally divided between four Solardomes within which the atmosphere was regulated to either ambient (360 μmol mol^{-1}) or elevated (600 μmol mol^{-1}) atmospheric CO_2 over a period of 12 months from July 2001 to 2002 (section 2.2.3). Plants were watered daily using an internal watering system and 50 ml $1/4^{th}$ strength Long-Ashton nutrient solution containing 28 mg l^{-1} N-NH_4NO_3 was added once per week from July to December 2001 and February to July 2002 and once a month from December 2001 to February 2002. After 12 months the tissues were either stored frozen at -80°C for total soluble protein (section 2.12) and total soluble phenolic (section 2.13) determination in fully expanded leaves or placed in an oven for dry weight determination. The phenolic assay quantifies most classes of PHE-derived compounds, however, it was not possible to quantify lignin for this study. The maximum rate of carboxylation by Rubisco ($V_{c,max}$) and the efficiency of RuBP regeneration by electron transport (J_{max}) of fully expanded leaves were determined by plotting photosynthetic rates (A) at different intercellular CO_2 (ci) concentrations (section 2.4.2). The rate of triose phosphate utilisation (TPU), which indicates the availability of inorganic P for the Calvin cycle, was not reported as the A/ci curves were not fully saturated (ie. did not plateau at high ci) for most cases. A Pearson correlation test was employed to give an indication of the relationship between the phenolic and protein concentrations and to test whether the carboxylation rates of were related to a reduction of the protein, and hence Rubisco, pool.

6.3 Results

6.3.1 Biomass and Gas exchange measurements

Elevated CO_2 significantly increased both shoot and root biomass of *P. maritima* (Tables 6.1, 6.2). In *A. maritima*, elevated CO_2 did not alter root biomass and even resulted in significantly lower shoot biomass (Table 6.1, 6.2).

The carboxylation rate of Rubisco ($V_{c,max}$) and the rate of electron transport affecting RuBP regeneration (J_{max}) was lower in *P. maritima* grown under elevated CO_2 (Table 6.3; Fig. 6.2 A), compared to the ambient CO_2 control plants. This implies that the plants were slightly negatively acclimated (photosynthetic capacity has been reduced) to the elevated CO_2. The $V_{c,max}$ remained unaltered in *A. maritima* but the J_{max} was slightly higher in plants exposed to elevated CO_2 (Table 6.3; Fig. 6.2 B). This implies that the plants were not acclimated (photosynthetic capacity was not reduced) to the elevated CO_2. However, none of the photosynthetic changes in *P. maritima* or *A. maritima* were statistically significant ($P > 0.05$).

Table 6.1. Shoot, root and total biomass (dry weight) of *Plantago maritima* and *Armeria maritima* exposed to either ambient (360 μmol CO_2 mol^{-1}) or elevated (600 μmol CO_2 mol^{-1}) CO_2 for 12 months. Data represent mean ± SE. n = 8-12. Significant differences are indicated by ns = not significant; * = $P < 0.05$; ** = $P < 0.01$. Arrows indicate general response to elevated CO_2 treatment; ↑ = increase; ↓ = decrease; ≈ = no change.

		Total biomass (g)		
		Atmospheric CO_2 concentration (μmol CO_2 mol^{-1})		
Species	Organ	360	600	
Plantago maritima	Shoot	1.51 ± 0.31	3.37 ± 0.50*	↑
	Root	1.65 ± 0.17	2.28 ± 0.20*	↑
	Total	2.97 ± 0.40	5.65 ± 0.60**	↑
Armeria maritima	Shoot	3.05 ± 0.59	1.69 ± 0.17*	↓
	Root	1.64 ± 0.14	1.38 ± 0.09ns	≈
	Total	4.69 ± 0.66	3.07 ± 0.22*	↓

Table 6.2. Results from a oneway ANOVA analyses to test for significant effects of elevated CO_2 on shoot, root and total biomass measurements of *Plantago maritima* and *Armeria maritima* after 12 months growth. Significance level is set at $P \leq 0.05$.

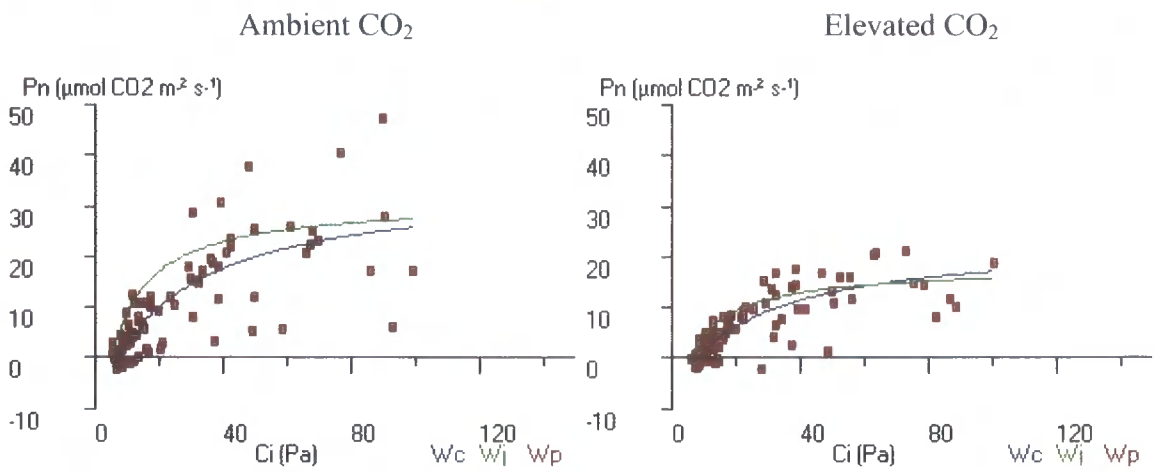
		Biomass data			
Species	Organ	df between groups	df within groups	*F*-value	*P*
Plantago maritima	Shoot	1	17	**7.019**	**0.017**
	Root	1	18	**5.144**	**0.036**
	Total	1	18	**10.900**	**0.004**
Armeria maritima	Shoot (LN)	1	18	**5.938**	**0.025**
	Root	1	18	2.845	0.109
	Total (LN)	1	18	**7.115**	**0.016**

(LN) = data were natural log transformed to increase homogeneity of variance.

Table 6.3. Maximum rate of carboxylation by Rubisco ($V_{c,max}$) and the electron transport limited rate of carboxylation (J_{max}) of *Plantago maritima* and *Armeria maritima* exposed to either ambient (360 μmol CO_2 mol^{-1}) or elevated (600 μmol CO_2 mol^{-1}) CO_2 for 12 months. Data represent mean ± SE. n = 8. Arrows indicate general response to elevated CO_2 treatment; ↑ = increase; ↓ = decrease; ≈ = no change.

	Maximum rate of carboxylation by Rubisco ($V_{c,max}$; μmol m^{-2} s^{-1})		Electron transport limited rate of carboxylation (J_{max}; μmol m^{-2} s^{-1})	
	Atmospheric CO_2 concentration (μmol CO_2 mol^{-1})			
Species	360	600	360	600
Plantago maritima	43.3 ± 6.2	32.2 ± 2.9 ↓	178.9 ± 44.1	105.6 ± 8.8 ↓
Armeria maritima	59.2 ± 7.7	60.6 ± 5.0 ≈	199.1 ± 34.4	238.2 ± 20.6 ↑

A: *Plantago maritima*



B: *Armeria maritima*

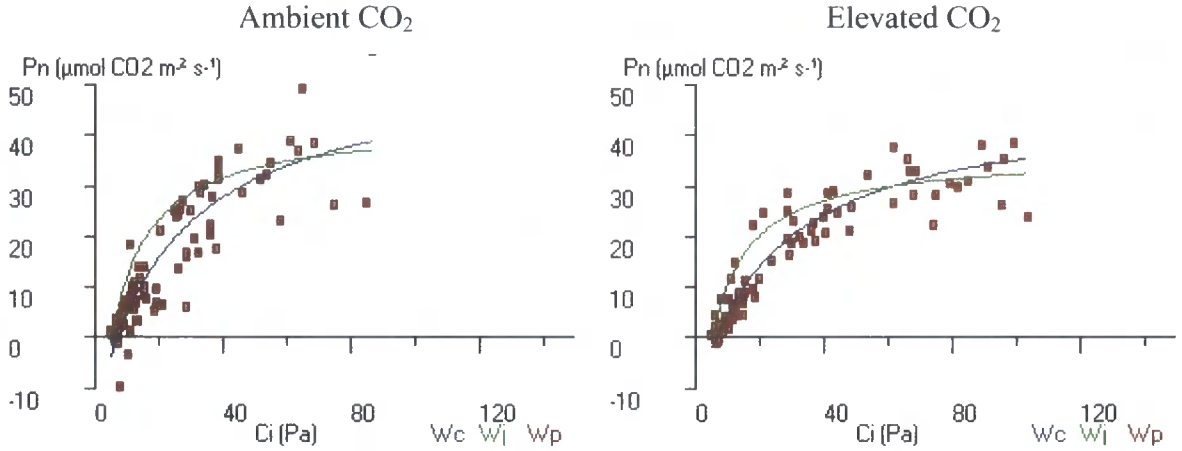


Figure 6.2. A/c_i curves for the determination of the maximum rate of carboxylation by Rubisco ($V_{c,\,max}$, blue curves) and electron transport limited rate of carboxylation (J_{max}, green curves) of *Plantago maritima* (A) and *Armeria maritima* (B) exposed to ambient (360 µmol CO_2 mol^{-1}) or elevated (600 µmol CO_2 mol^{-1}) CO_2 for 12 months. n = 8.

6.3.2 Total soluble protein and phenolic concentrations

For *P. maritima*, there were minor increases (although not significant, Tables 6.4 and 6.5) in the total phenolic concentration in the roots, but not in the leaves in response to elevated CO_2 for one year (Table 6.4). The total soluble protein concentration was significantly reduced in both leaves and roots, at elevated CO_2 causing an increase in the phenolic:protein ratio (Tables 6.4, 6.5).
Negative correlations were shown between the protein and phenolic concentrations in the leaves, which were significant in plants grown under ambient CO_2 (Fig. 6.3A; Table 6.6) but not in those grown under elevated CO_2. In the roots there was no correlation of protein and phenolic concentrations in plants grown under ambient CO_2 but a positive trend occurred in the plants exposed to elevated CO_2 (although non-significant) (Fig. 6.3B; Table 6.6).

For *A. maritima*, there were small reductions in the total phenolic concentrations, in the leaves, in plants exposed to elevated CO_2 for one year, compared to those grown under ambient CO_2 (Tables 6.4 and 6.5). However, the soluble protein concentration was higher in plants exposed to elevated CO_2; and this increase was significant in the roots (Tables 6.4 and 6.5). The phenolic:protein ratio was therefore reduced in plants grown under elevated CO_2, compared to those in the ambient CO_2 control (Tables 6.4 and 6.5). There was a positive correlation in the phenolic and protein concentrations in leaves of *A. maritima* grown under both ambient and elevated CO_2 (Fig. 6.3C; Table 6.6). However, if the outlier in the leaves of the plants grown under elevated CO_2 is omitted (as it is only this data point which strongly alters the correlation), a significant negative correlation occurs (Table 6.6). The opposite occurred in the roots (Fig. 6.3D; Table 6.6) as a negative correlation occurred in the plants grown under ambient CO_2 and a significant positive correlation occurred in plants grown under elevated CO_2 (Table 6.6).

Table 6.4. Total phenolic and protein concentrations, and the respective ratios, in the leaves and roots of *Plantago maritima* and *Armeria maritima* exposed to either ambient (360 μmol CO_2 mol^{-1}) or elevated (600 μmol CO_2 mol^{-1}) CO_2 after 12 months. Data represent mean ± SE. n = 6 –12. Significant differences are indicated by ** = $P < 0.01$ and *** = $P < 0.001$. Arrows indicate general response to elevated CO_2 treatment; ↑ = increase; ↓ = decrease; ≈ = no change.

		Total Phenolics (mg g^{-1} dwt)			Total Protein (mg g^{-1} dwt)			Phenolic:Protein ratio		
		Atmospheric CO_2 concentration (μmol CO_2 mol^{-1})								
Species	Organ	360	600		360	600		360	600	
Plantago maritima	Shoot	45.0 ± 10.1	39.6 ± 5.0	≈	41.2 ± 4.2	9.7 ± 1.9 ***	↓	1.26 ± 0.36	5.98 ± 1.41**	↑
	Root	65.3 ± 15.6	98.0 ± 13.1	↑	19.3 ± 4.3	7.0 ± 0.6 ***	↓	4.28 ± 1.19	15.16 ± 2.00***	↑
Armeria maritima	Shoot	83.8 ± 9.8	73.0 ± 8.6	↓	20.1 ± 4.7	27.2 ± 6.9	↑	5.85 ± 1.15	4.49 ± 1.26	↓
	Root	104.3 ± 9.9	106.7 ± 9.7	≈	17.8 ± 2.1	41.3 ± 5.9 **	↑	6.51 ± 0.99	3.11 ± 0.45 **	↓

Table 6.5. Results from oneway ANOVA analyses to test for significant effects of elevated CO_2 on leaf and root soluble phenolics and protein concentrations and the respective ratios in *Plantago maritima* and *Armeria maritima* after 12 months. Significance level is set at $P \leq 0.05$.

		Total Phenolics (mg g^{-1} dwt)		Total Protein (mg g^{-1} dwt)		Phenolic:Protein ratio	
Species	Organ	*F*- value (df)	*P*	*F*- value (df)	*P*	*F*- value (df)	*P*
Plantago maritima	Shoot	0.299 $_{1,16}$	0.592	**58.750** $_{1,18}$	**0.000**	**9.763** $_{1,15}$	**0.007 LN**
	Root	2.519 $_{1,16}$	0.132	**28.777** $_{1,18}$	**0.000 LN**	**16.200** $_{1,16}$	**0.001**
Armeria maritima	Shoot	0.677 $_{1,18}$	0.421	0.583 $_{1,18}$	0.455	0.567 $_{1,18}$	0.461
	Root	0.028 $_{1,17}$	0.869	**9.812** $_{1,18}$	**0.006**	**11.886** $_{1,17}$	**0.003**

LN = data were natural log transformed.

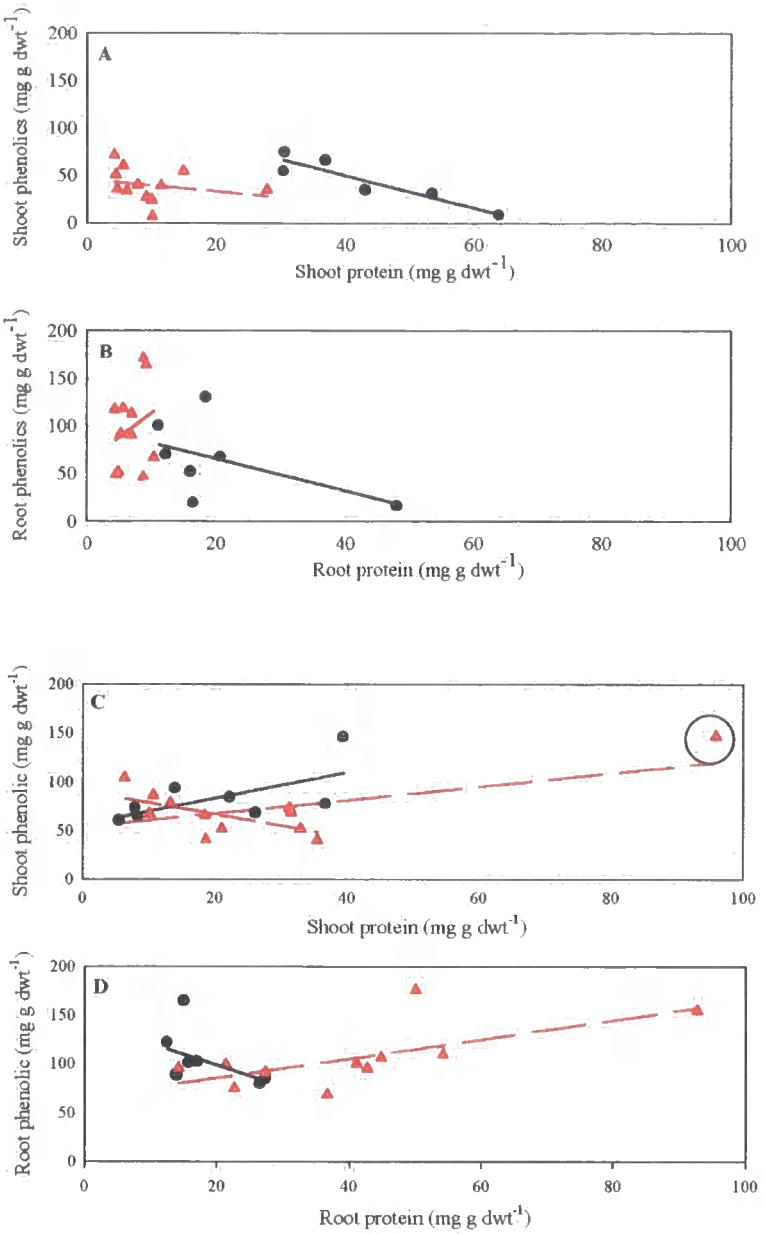


Figure 6.3. Correlations between the soluble phenolic and protein concentrations in the leaves and roots of *Plantago maritima* (A, B) and *Armeria maritima* (C, D) after 12 months exposure to either ● (solid line) ambient (360 µmol CO_2 mol^{-1}) or ▲(dashed line) elevated (600 µmol CO_2 mol^{-1}) CO_2. Encircled data point is a possible outlier.

Table 6.6. Results from the linear regression equation and Pearson correlation analyses to test for correlations (r) between phenolic and protein concentrations in the leaves and roots of *Plantago maritima* and *Armeria maritima* after 12 months exposure to either ambient (360 µmol CO_2 mol^{-1}) or elevated (600 µmol CO_2 mol^{-1}) CO_2. Significance level is set at $P \leq 0.05$.

		Atmospheric CO_2 concentration (µmol CO_2 mol^{-1})							
		360				**600**			
Species	Organ	n	Regression equation	r	P	n	Regression equation	r	P
Plantago	Shoot	**6**	y = 118.24 + -1.70x	**-0.923**	**0.009**	12	y = 45.42 + -0.60x	-0.230	0.471
maritima	Root	7	y = 99.61 + -1.68x	-0.517	0.235	11	y = 63.35 + 5.10x	0.248	0.463
Armeria	Shoot	8	y = 56.90 + 1.34x	0.643	0.085	**11**	y = 90.74 + -1.17x	**-0.626**	**0.039**
maritima	Root	8	y = 143.36 + -2.20x	-0.460	0.251	**11**	y = 66.38 + 0.99x	**0.661**	**0.027**

6.3.3 $V_{c,max}$ and shoot protein concentrations

There was a significant positive correlation between the shoot protein concentration and the maximum rate of carboxylation ($V_{c,max}$) in *P. maritima* (Fig. 6.4; Table 6.7). The relationship between shoot protein and the maximum rate of carboxylation ($V_{c,max}$) in *A. maritima*, although positive according to figure 6.4, was not statistically significant (Table 6.7).

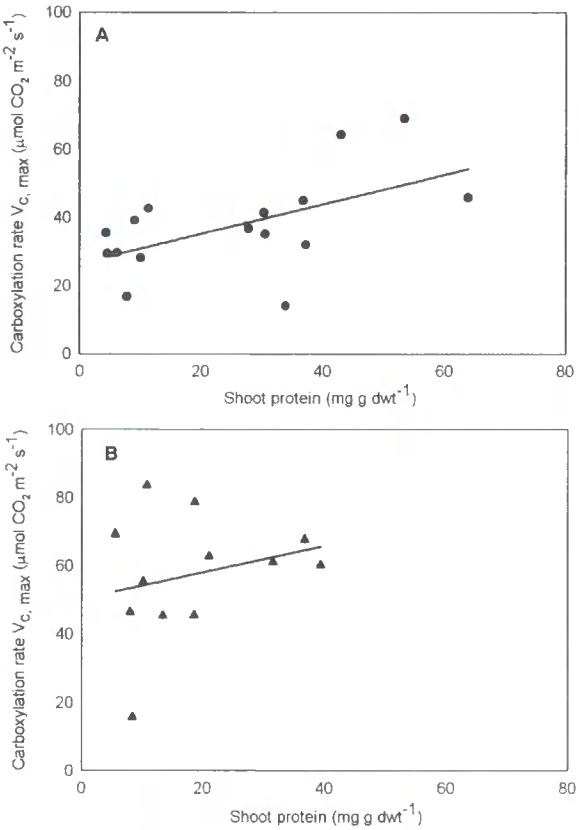


Figure 6.4. Correlations between the soluble protein concentrations and the carboxylation rate of (A) *Plantago maritima* (●) and (B) *Armeria maritima* (▲) after 12 months exposure to ambient (360 μmol CO_2 mol^{-1}) and elevated (600 μmol CO_2 mol^{-1}) CO_2.

Table 6.7. Results from the linear regression equation and Pearson correlation analyses to test for correlations (r) between the maximum rate of carboxylation by Rubisco ($V_{c,max}$) and protein concentrations (mg g^{-1} dwt) in the leaves of *Plantago maritima* and *Armeria maritima* after 12 months exposure ambient (360 μmol CO_2 mol^{-1}) and elevated (600 μmol CO_2 mol^{-1}) CO_2. Significance level is set at $P \leq 0.05$.

			Pearson correlation	
Species	*N*	Regression equation	*r*	*P*
Plantago maritima	**16**	y = 26.64 + 0.43x	**0.536**	**0.023**
Armeria maritima	12	y = 50.46 + 0.39x	0.249	0.436

6.4 Conclusion and Discussion

There were contrasting responses in biomass accumulation to elevated CO_2 between the two study species, with increased shoot and root biomass in *P. maritima* (Table 6.1) and decreased shoot biomass in *A. maritima* (Table 6.1). A contrast between the species was also found in the level of photosynthetic acclimation to elevated CO_2, as *P. maritima* showed some acclimation of photosynthetic capacity (reduced $V_{c,max}$) and *A. maritima* did not (no change in $V_{c,max}$) (Table 6.3). Also, the accumulation of both soluble phenolics and proteins in response to elevated CO_2 differed between the two species. In *P. maritima*, there were small increases in the phenolic concentrations, albeit only in the root (Table 6.4) and large reductions in the soluble protein concentrations within the leaves and roots (Table 6.4). Conversely, in *A. maritima*, minor reductions in the phenolic concentrations occurred in the leaves and large increases in the soluble protein occurred, particularly in the roots at elevated CO_2 (Table 6.4).

The results showed an increase in the C allocation to growth in *P. maritima* and not in *A. maritima*. Increased plant biomass as a result of elevated CO_2 has been observed in other *Plantago* species (den Hertog *et al.* 1998; Klus *et al.* 2001), however, this is the first known recording of the biomass changes in these two species when exposed to elevated CO_2. The results for *P. maritima* support the theory that increased C supply will increase plant biomass despite some down-regulation of photosynthetic capacity; however, the growth response of *A. maritima* did not support this theory despite no down-regulation of photosynthetic capacity. Photosynthetic acclimation can be triggered by a decrease in the plant sink strength for C assimilates causing an increase in carbohydrate accumulation (Saralabai *et al.* 1997) and can cause negative feedback on the transcription of Rubisco mRNA (*rbcS/L*) (Cheng *et al.* 1998). Soluble-carbohydrate accumulation has not been observed in either *P. maritima* or *A. maritima* (Chapter 4) in plants exposed to elevated CO_2; therefore it is unlikely that such a feedback mechanism is the signal for reduced Rubisco production in *P. maritima*. Results by Harmens *et al.* (2000) show in *Dactylis glomerata* that increases in non-structural carbohydrate can occur without causing reductions in photosynthetic capacity.

Testing the PCM

The PCM states that there are four possible physiological responses to elevated CO_2, which are all dependent on the level of photosynthetic acclimation and growth in response to elevated CO_2. The four outcomes are; *i*, No photosynthetic acclimation (photosynthesis increases under elevated CO_2), increased growth; *ii*, No photosynthetic acclimation, no increase in growth; *iii*, Full photosynthetic acclimation (photosynthesis remains unchanged at elevated CO_2), increase in growth and *iv*, Full photosynthetic acclimation, no increase in growth. The *P. maritima* data best fitted the category of '*full acclimation and increase in growth*'. Under these conditions the PCM predicts that there would be a small increase in the total phenolic concentration, mainly due to a 'concentration effect' caused by reduced dry matter storage during increased growth. Thus, the total protein demand for growth would *increase* and the protein demand for carboxylation would *decrease* (as the plant has reduced its photosynthetic capacity). *Armeria maritima* best fitted the category of '*no acclimation and no increase in growth*'. A fifth category of '*no acclimation and decreased growth*' would have been more appropriate but this was not included in the PCM. For *A. maritima* the model predicted that there would be a decrease in the total phenolic concentration possibly caused by dilution effects caused by an increase in the percent dry weight. In addition, as there is not a clear positive relationship between photosynthesis and growth, a resource other than C would be limiting growth. Therefore, the growth, carboxylation and total protein demand would remain unchanged in plants exposed to elevated CO_2.

Phenolic concentration

The increase of phenolic compounds with a reduction in protein concentration is a common occurrence in plants (Booker & Maier 2001). The fact that the ratio of phenolics to protein accumulation increased in *P. maritima* (Table 6.4) at elevated CO_2 means that although only a slight increase of phenolics occurred, the allocation of PHE to phenolic metabolism was higher; otherwise the phenolic concentration would decrease along with the reduction in protein concentration. Growth and phenolic concentrations both increased in *P. maritima*. It is suggested that this occurs when there is no internal competition for C (Lavola *et al.* 2000). The increased growth and phenolic concentration over protein may also be due to efficient N recycling within the plant as after PHE is converted to *trans*-cinnamic acid by PAL, the ammonium ion can be reincorporated into amino acid metabolism via glutamine synthetase (Razal *et al.*

1996; Riipi *et al.* 2002). Although PHE was not measured in this study it is hypothesised that this recycling would ultimately allow a relatively constant pool of PHE to be available for phenolic or protein synthesis, particularly if N availability is limited.

The phenolic concentrations in *A. maritima* did not significantly alter between ambient and elevated CO_2 treatments (Table 6.4). This has been attributed to the fact that as growth did not increase and there was no acclimation of photosynthetic capacity in plants exposed to elevated CO_2, then another resource must be limiting growth, such as N. Therefore, most of the PHE is allocated to proteins involved in growth rather than phenolic accumulation (Jones & Hartley 1999). This may be the case as there was a substantial pool of protein in the roots of plants exposed to elevated CO_2, causing the phenolic to protein ratio to be significantly reduced.

Dry matter effects causing 'passive' dilution and concentration of compounds

The PCM suggests that decreases in the dry matter in *P. maritima* grown under elevated CO_2 would cause a 'passive' concentration effect of the phenolic compounds already present in the plant. There was no change in the dry matter content of the leaves but there was a 28 % reduction in the dry matter content of the root, which may have contributed to the measured increase in the root phenolic concentrations (data not shown). However, a similar reduction (23 %) of root dry matter was measured in *A. maritima* (although the PCM predicted an increase of percent dry matter), but did not result in an increase of the phenolic concentrations. Therefore, the assumption that changes in the dry matter will alter the phenolic concentrations in a predictive manner should be approached with caution.

Protein concentration

A reduction in the protein content of leaves is a common response to elevated CO_2 (Poorter *et al.* 1997). The reduction in the soluble protein content of the leaves found in *P. maritima* would be expected, as photosynthetic acclimation is usually associated with a reduction in the Rubisco protein, which can make up 30 – 50 % of the soluble protein concentration (Bowes 1991; Drake *et al.* 1997). Therefore, the PCM was correct in hypothesising that the C-fixing demand would be reduced in photosynthetically acclimated plants. The reduction in leaf protein was significantly correlated to a

reduction in the maximum carboxylation rate of Rubisco ($V_{c,max}$) in *P. maritima* (Fig. 6.4A; Table 6.7). This suggests that the carboxylation rate was highly dependent on the quantity of protein rather than an increase in the activation state of the enzyme, assuming that the protein:Rubisco ratio did not change. Although the Rubisco protein concentration was not measured in this study, other research (Cook *et al.* 1998; Sims *et al.* 1998) has shown that a concurrent reduction in the Rubisco protein content occurs with a reduction in the Rubisco carboxylation rate. The N that would have otherwise been allocated to Rubisco would have been available for reallocation to other N-demanding processes within the plant. It is unlikely that the N was reallocated to other proteins involved in photosynthetic electron transport in *P. maritima* as J_{max} was reduced implying that the capacity to regenerate RuBP was reduced (Rey & Jarvis 1998). It is likely that the reduction in Rubisco has allowed reallocation of N into growth, as increased biomass was measured in *P. maritima*, so allowing the plant to take advantage of the increased C availability.

The significant reduction of protein concentration in the roots of *P. maritima* cannot be explained by the model hypothesis. Reduced root protein concentrations in *P. maritima* are consistent with other studies in that the reduction in root protein concentrations is less than the reduction in shoot protein concentration in plants exposed to elevated CO_2 (Cotrufo 1998). This reduced root soluble protein concentration could be explained by the increased growth of the plant binding proteins into structural components (growth protein demand) so reducing the soluble protein content. However, another plausible explanation is that nitrate reductase activity was inhibited. In a study by Matt *et al.* (2002), the small Rubisco subunit (*RbcS*) synthesis was reduced by using Tobacco plants with antisense *RbcS*. The authors show that a reduction in the Rubisco enzyme concentration caused an inhibition of nitrate reductase activity, thus a decrease in the soluble protein and free amino acid content of the tobacco plant. They also showed that the concentration of C-based secondary compounds was slightly reduced. However, the opposite occurred in the current study as root phenolic concentrations slightly increased (Table 6.4).

In *A. maritima*, the increase of soluble proteins (Table 6.4) as a response to elevated CO_2 was not predicted as it was assumed that the plant would be N limited as there was no increase in growth. An unchanged shoot soluble protein content would be expected in a plant that had not down-regulated photosynthetic capacity, such as *A. maritima* in this study, as it is usually the increase in Rubisco enzyme activation state rather than

content that causes the increased photosynthetic rates at elevated CO_2 compared to ambient CO_2. In the case of *A. maritima* it is possible that the rate of carboxylation (V_{cmax}) was independent of protein content (Fig. 6.3B) and that the enzyme activation state of Rubisco was changed rather than any increase of Rubisco protein. Such a response was observed in a similar study on *Solanum tuberosum* (Sage *et al.* 1989). The maintenance of an increased photosynthetic capacity at elevated CO_2 could be due to the ability of *A. maritima* to slightly increase the regeneration of RuBP, as indicated by the subtle increase (although not significant) in J_{max} (whereas *P. maritima* showed a reduced RuBP regeneration rate) (Table 6.3). As *A. maritima* maintained protein concentrations comparable to those measured at ambient CO_2 (Table 6.4), it is possible that soluble proteins, including Rubisco, were being used as storage compounds for N (Cheng & Fuchigami 2000). This is plausible since *A. maritima* accumulates high levels of nitrogenous compatible solutes during periods of osmotic stress (Chapter 4) (Stewart *et al.* 1979). A similar response has been proposed in orange tree (*Citrus aurantium*) leaves exposed to elevated CO_2 (Idso *et al.* 2001). They (Idso *et al.* 2001) reported that the concentrations of three major soluble proteins were increased in plants exposed to elevated CO_2 for most of the year, however, these proteins decreased in concentration as new growth occurred in the spring. This enabled the orange tree to use a N source other than Rubisco protein should the demand arise.

Relationship between phenolic and protein concentrations

The PCM states that there will be an inverse relationship of the PHE allocation to phenolics and protein. This held true for the relationship between phenolic and protein concentrations in *P. maritima* when plants were grown under ambient CO_2 (Figs. 6.3A and 6.3B). However, when the plants were grown under elevated CO_2 this correlation was not significant in the leaves, with a positive correlation determined in the roots. The inverse relationship of the PHE allocation to phenolics and protein did not hold true for the relationship between phenolic and protein concentrations in *A. maritima*, the exception being the shoot concentrations in plants exposed to elevated CO_2, if the outlier data point was omitted (Fig. 6.3C), and in the roots of plants grown under ambient CO_2 (Fig. 6.3D). An increase in the phenolic concentrations together with increased protein concentrations suggests no metabolic competition for the PHE substrate. However, from the phenolic and protein measurements made in this study, it was impossible to separate compounds arising from *de novo* synthesis from those that

had been stored for some period of time, which may have affected the measured phenolic and protein ratio.

Mechanisms of carbon and nitrogen sensing and control

The PCM was based upon research showing the regulation of phenolic and protein synthesis are tightly linked (Weaver and Hermann 1997) and that cells do not appear to be capable of simultaneously allowing high rates of protein and phenolic synthesis (Jones and Hartley 1999). However, one of the authors of the PCM recently published results showing that although PAL activity increased in plants exposed to elevated CO_2 this did not equate to significant increases in the total phenolic concentration of the plant (Hartley *et al.* 2000). This implies that the biochemical mechanisms involved in the model do not always relate to processes in actual plants. Therefore, other C and N sensing mechanisms may control C allocation to phenolic synthesis rather than just a simple upregulation of PAL. There is now much known about the influence of C- and N- sensing mechanisms on the allocation of C and N to metabolites in plants that needs to be considered when testing such models (Stitt and Krapp 1999).

The controls of C and N metabolism have been unravelled in recent years and a complex picture is now emerging where C and N regulation is dependent upon cell-type, development, metabolic and environmental conditions (Coruzzi and Zhou 2001). The main compounds in which the plant monitors its N and C status are sucrose, glucose, glutamine, glutamate, nitrate and ammonium (Kang and Turano 2003). The presence of sugar sensing mechanisms enables plants to turn off photosynthesis when C skeletons are abundant whilst N sensing mechanisms enables plants to turn nitrate uptake and reduction when levels of reduced or organic N are high. The two processes are linked in that when C skeletons are abundant, genes for N assimilation are activated, and to stop N assimilation when photosynthate is low (Coruzzi and Bush 2001; Coruzzi and Zhou 2001).

The main sensor of C status in plants is the enzyme Hexokinsae. Hexokinase is a glucose sensor that modulates carbohydrate status, and hormone signalling pathways and was shown to have a direct effect on the expression of photosynthetic genes (Jang and Sheen 1997; Coruzzi and Zhou 2001). High levels of glucose will control the expression of the genes important for hormonal control of the plants, such as abscisic acid, which in turn can inhibit growth (Coruzzi and Zhou 2001; Cheng *et al.* 2002). As

this enzyme can regulate the expression of photosynthetic genes, it is likely that photosynthetic acclimation in plants grown at elevated CO_2 is the result of carbohydrate repression (Rolland *et al.* 2002).

Nitrate is the key compound for nitrogen sensing in plants. Nitrates can be used as a signal for inorganic-N status whereas compounds derived from nitrates, such as ammonium, glutamate and glutamine can act as signals for reduced or organic-N status. Nitrate induces genes encoding high and low affinity nitrate transporter systems, nitrate reductase, nitrite reductase and ammonium assimilation, all of which increases rates of nitrate uptake, nitrate reductase protein and activity and glutamine synthetase (Stitt 1999; Coruzzi and Bush 2001). There is recent evidence that it is the glutamate receptor genes (AtGLR1.1) that are highly involved in the control of C and N metabolism. In *Arabidopsis thaliana* with antisense AtGLR1.1, there was decreased accumultion of glutamate receptors together with decreases in key C and N metabolic enzymes (such as 6-phosphogluconate dehydrogenase, isocitrate dehydrogenase, glutamine synthetase and cytosolic asparate aminotransferase) and their corresponding transcripts together with decreases in the hexokinase transcript (Lam et al. 1998; Kang and Turano 2003). As well as AtGLR1.1 glutamate receptors, the PII protein (a protein allosteric effector originally named from a component of the N regulatory system in *Escherichia coli*, encoded by the glutamine synthetase, *gln*B, gene) regulates glutamine synthetase at the transcriptional and post-transcriptional level in response to nitrogen availability. The protein in effect can perceive the C and organic N status of the plant. When plants are nitrogen deficient, the PII protein activates glutamine synthetase activity, whilst under excess N the PII protein can cause inactivation of the glutamine synthetase enzyme (Hsieh *et al.* 1998). The C and N sensing processes are also linked in that nitrate assimilation requires the synthesis of organic acids, all of which requires diversion of carbon away from carbohydrate synthesis. Therefore nitrate assimilation causes decreases in the starch synthesis enzyme ADP-glucose pyrophosphorylase to divert C towards the N-assimilatory pathway (Stitt 1999; Coruzzi and Bush 2001).

The information on C- and N- sensing in plant already indicate that such factors will play a more important role in the allocation of N and C to proteins and phenolics rather than single, pivotal control points in plant metabolism.

Test of the Protein Competition Model

In the present study, the PCM has worked reasonably well in predicting the phenolic concentrations of the two species in relation to the plants growth response and photosynthetic acclimation to elevated CO_2, particularly for *P. maritima*. However, the response does seem to be organ specific; something that the model does not take into account. As many studies have concentrated on above-ground responses to elevated CO_2, it is possible that important alterations in the phenolic metabolism and C allocation of the plant as a whole have been underrepresented. The PCM has certainly given a clearer prediction of phenolic concentrations in plants exposed to elevated CO_2 when compared to the other CNB and GDB models, which would have predicted a general increase of plant phenolics in plants exposed to elevated CO_2 without considering photosynthetic acclimation and protein demand. However, limitations of the PCM and the disagreements with the predicted phenolic and protein outcome have already become evident in this study. Considering the fact that hexokinase and nitrate are heavily involved in C and N regulation it is likely that such factors will have more influence on C allocation to protein and phenolics than a supply of Phenylalanine and PAL activity. These C- and N- sensing data should be incorporated in future alterations of the PCM.

Therefore, it appears in this study that the potential benefits of increased atmospheric CO_2 are offset by photosynthetic acclimation and growth limitations. However, whether the acclimation will ultimately benefit the plants in terms of increased protection by increases in phenolics in *P. maritima* and the ability to accumulate protein in *A. maritima* remains unanswered.

Chapter 7. Response of individual phenolic metabolites to changes in resource availability

Aim

The aim of work reported in this chapter was to investigate in detail the effects of long term increased atmospheric CO_2 on the phenolic secondary metabolism and the vasculature of *Plantago maritima*.

7.1 Introduction

Plants can use an increased carbon (C) source either for growth, such as cellulose or lignin or for defence and storage compounds such as phenolics and starch (Farrar & Williams 1991; Lindroth *et al.* 1993). The two major hypotheses for predicting the allocation of C in plants exposed to elevated CO_2 are incorporated into the carbon-nutrient balance model (CBM) (Bryant *et al.* 1983) and the growth-differentiation balance (GDB) model (Herms & Mattson 1992). These models predict that, if the plant through increased photosynthesis acquires more C, a greater proportion of C can be allocated to secondary metabolism, and hence C-based natural compounds. This allocation of C to secondary metabolites in plants grown under elevated CO_2 is usually enhanced when another factor, such as nitrogen (N) availability, is limited. Of particular interest is the effect of alterations in C allocation on the phenylpropanoid pathway, due to its central importance in lignification and the synthesis of bioactive phenolics (Dixon & Paiva 1995). Such phytochemical and physiological alterations provide useful insights into resource allocation and the longer-term ecophysiological consequences of biotic and abiotic stress during climate change.

The literature examining the effect of elevated CO_2 on phenolic metabolism in plants has given rise to conflicting results. For example, increases of up to 14 % of phenolic compounds in plants exposed to elevated CO_2 have been reported (Peñuelas *et al.* 1997), whereas other studies have showed no change and even reductions in plant phenolic concentrations, with much of the current literature testing either crop plants or tree species (Bezemer *et al.* 2000; Hartley *et al.* 2000). Although difficult to quantify, and many studies have concentrated on senesced leaves only, similar variable results have been described for the total lignin content in plants exposed to elevated CO_2 (Poorter *et al.* 1997; Hartley *et al.* 2000).

Many studies quantify gross 'total soluble phenolics' when assessing the effect of elevated CO_2 on plant secondary metabolism. Although this approach gives some indication as to the C allocation to secondary compounds, it does not quantify variations of individual phenolic compounds under elevated CO_2. There is increasing evidence that the response of phenolic compounds to environmental change varies at the individual metabolite level and that this needs to be taken into account when testing predictive models of C-allocation (Koricheva *et al.* 1998; Peñuelas & Estiarte 1998; Hamilton *et al.* 2001). In the case of soluble phenolics, studies on deciduous trees (Lavola *et al.* 2000; Veteli *et al.* 2002) and wheat (Estiarte *et al.* 1999) have shown that elevated CO_2 causes changes in individual secondary metabolites. Phenylpropanoid, flavonoid and tannin concentrations in plants have all shown to alter under elevated CO_2, compared to plants grown under ambient CO_2 (Coviella & Trumble 1999; Lavola *et al.* 2000). Any alterations in the concentration of phenylpropanoids may change the amount of monolignols available for lignin polymerisation (Humphreys & Chapple 2002).

The aim of the work described in this chapter was to study the effect of elevated CO_2, N availability and drought on the phenolic secondary metabolism in *Plantago maritima*. *Plantago maritima* already has a relatively well-defined metabolic response to abiotic stress at ambient CO_2, accumulating sorbitol as a compatible solute in response to stress treatments (Ahmed *et al.* 1979; Jefferies *et al.* 1979). Therefore, this study will complement previous chapters on how C and N are allocated to growth and compatible solutes in *P. maritima* in response to changes in environmental conditions. A combination of metabolite profiling to identify specific changes in phenolic metabolism and detailed structural analysis of the patterns of lignified tissues were employed to study the response to both short-term exposure to elevated CO_2, reduced N availability and drought and long-term exposure to elevated CO_2. Initial studies on the changes of the individual phenolic metabolites in *Armeria maritima* were undertaken. However, later studies concentrated on *P. maritima* as this species had a clearer response to the applied treatments.

7.2 Materials and methods

The experimental set-up and harvesting have been reported previously in Chapters 3.2.1 & 6.2 and for analytical methods in Chapter 2.

7.3 Results: Separation and identification of phenolic compounds

7.3.1 Analytical Thin-Layer Chromatography (TLC) of phenolic compounds

Leaf tissues of *P. maritima* and *A. maritima* were extracted in methanol:acetone. Hydrolysed (cellulase-digested) extracts (procedure 2.14.1) were reduced to dryness and resuspended in 20 µl ethyl acetate, which was loaded onto the TLC plate. Phenolic aglycones were separated by a chloroform:methanol (9:1 v/v) solvent system and viewed under UV light at a wavelength of 302 nm.

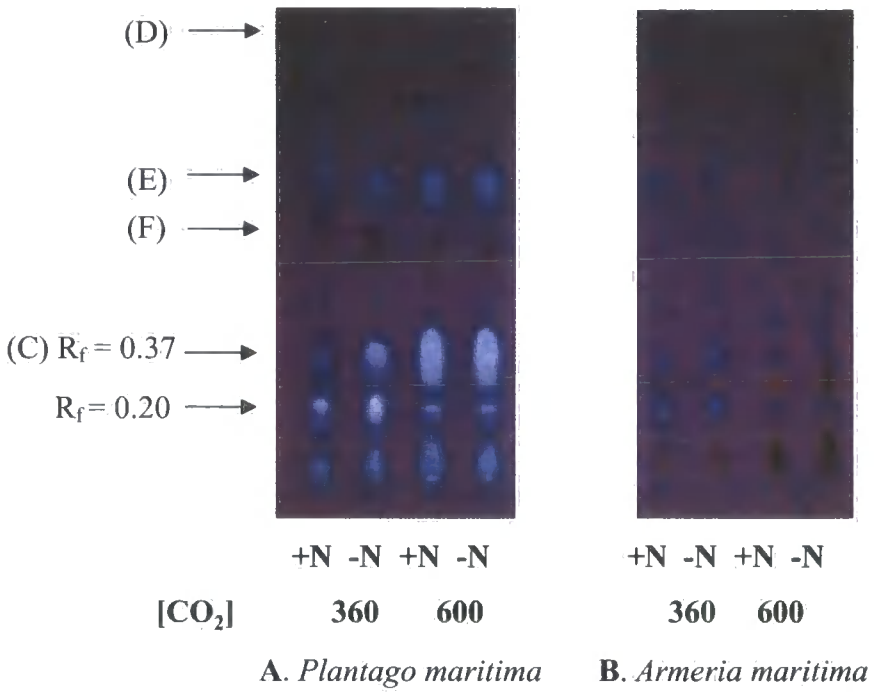


Figure 7.1. Analytical thin-layer chromatography (TLC) of cellulose-digested leaf extracts of **A**, *Plantago maritima* and **B**, *Armeria maritima*. Plants were exposed to either ambient (360 µmol mol^{-1}) or elevated (600 µmol mol^{-1}) CO_2 concentrations, under which plants were treated with either 28 mg l^{-1} N (+ N) or 2.8 mg l^{-1} N (-N) nutrient solution for five weeks. TLC plates were viewed under UV light at a wavelength of 302 nm. C, D, E and F correspond to HPLC chromatograms of PTLC eluted compounds in fig. 7.3. Although compound D does not show well in the above image it was visible under UV light.

The TLC plates separated six distinctive UV-absorbing and fluorescing compounds in *P. maritima* (Fig. 7.1A). There was an increase in the white fluorescent compound with a ratio of solute to solvent front (R_f) value of 0.37 from shoot extracts from plants that were grown under elevated CO_2 compared to the extract from plants grown under ambient CO_2. Low N treatments only altered the quantity of one compound (R_f = 0.20) under ambient CO_2 conditions; this did not occur in plants exposed to both elevated CO_2 and low N.

Armeria maritima did not produce a significant amount of UV fluorescing compounds though seven UV-absorbing compounds were separated (Fig. 7.1B). Elevated CO_2 was not associated with the appearance of any unique metabolites, though minor increases in some of the polar UV-absorbing material was observed. As *P. maritima* had a clearer response to N and elevated CO_2 treatments, it was decided to concentrate this study on identifying and quantifying which compounds were altered in this species under varied CO_2, N and drought treatments, rather than *A. maritima*.

7.3.2 Preparative Thin-Layer Chromatography (PTLC) of phenolic compounds

Preparative TLC (PTLC) was used to concentrate the compounds of interest for further analyses by UV-spectral analysis, HPLC and mass spectrometry. Ten separated compounds were eluted from a PTLC plate loaded with hydrolysed shoot extract from plants grown under ambient CO_2 and five compounds were eluted from a PTLC plate loaded with extract from plants grown under elevated CO_2 for five weeks. The compounds eluted from plants grown under elevated CO_2 were also present in the extract from plants grown under ambient CO_2. The compound separated with the R_f value of 0.37 was particularly prominent on the PTLC plates (Fig. 7.1A).

7.3.3 UV-spectral analyses of PTLC eluted compounds

The UV-spectra of the compounds eluted from the PTLC plates were obtained. The metabolite with an R_f of 0.37 had a clear UV-spectrum (absorbance maximum λ_{max} 279, 304sh; Fig. 7.2), which was diagnostic of a phenylpropanoid (single band) rather than a flavonoid, which would usually show distinctive double absorbance maxima.

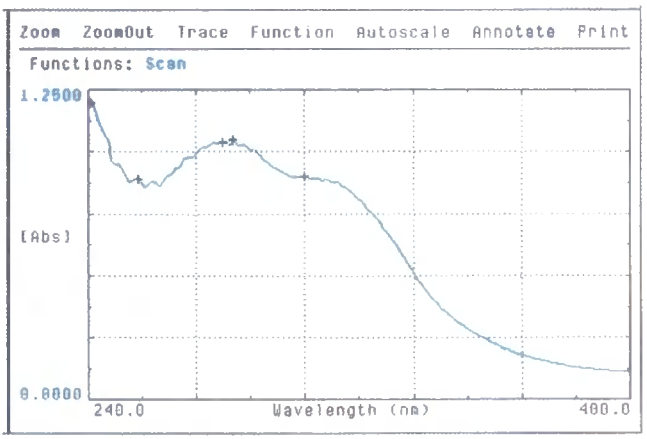


Figure 7.2. UV-spectrum of a UV-fluorescing compound in the shoot of *Plantago maritima* as eluted by PTLC. Compound was later identified to be caffeic acid.

7.3.4 HPLC analyses of PTLC eluted compounds

The compounds purified from the PTLC plates were analysed by HPLC to see whether their retention times (R_t) matched those of the hydrolysed extract injected directly into the HPLC column (Fig. 7.3B). From the 10 compounds eluted from the PTLC sheets, four compounds matched retention times from those compounds separated by HPLC (Figs. 7.3C-F). When the non-cellulase digested extract was separated by HPLC two major compounds were identified (Fig. 7.3A). When analysed by TLC, the same unhydrolysed extract contained six compounds, which could be separated, two of which had similar R_f values and colouration to the hydrolysed extract. It was concluded that two major UV-absorbing metabolites in *P. maritima* were resistant to hydrolysis by cellulose but that the other compounds were present as a mixture of β-D-glycosides.

It was decided to concentrate on the two major peaks that were present in the aglycone form (R_t 6.4 and R_t 10.8 minutes) and the metabolite eluting at 7.6 minutes as these compounds were readily detectable by both HPLC and PTLC. In addition, as the study concentrated on the effect of elevated CO_2 on the accumulation of phenolic moieties rather than conjugating sugars, advantage was taken of the increased sensitivity and accuracy of compound determination following cellulase treatment to identify and quantify the respective aglyca. For preparative purposes hydrolysed extract was loaded into the HPLC column and the three compounds collected for further analyses by UV-spectrometry and mass-spectrometry.

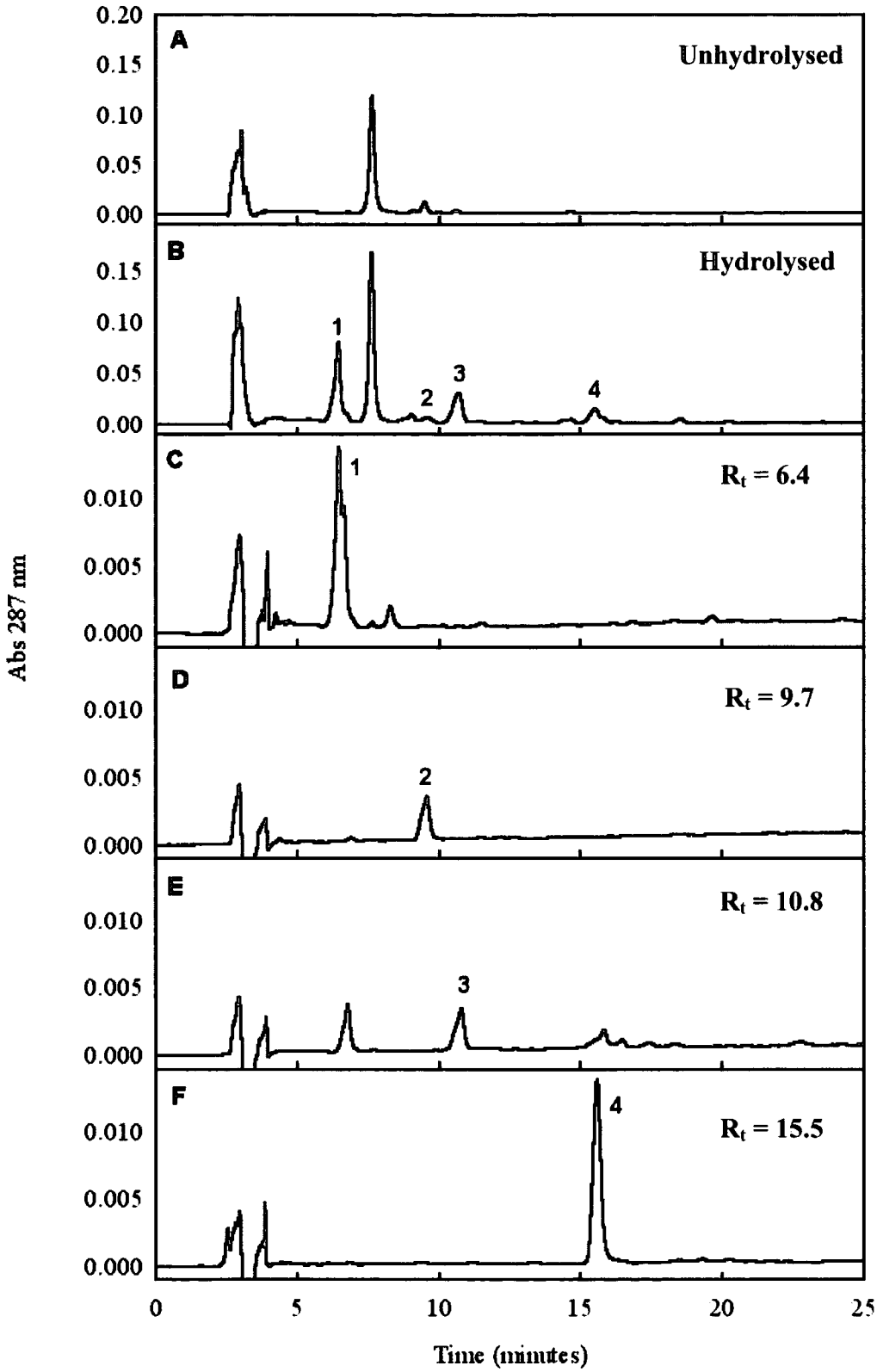


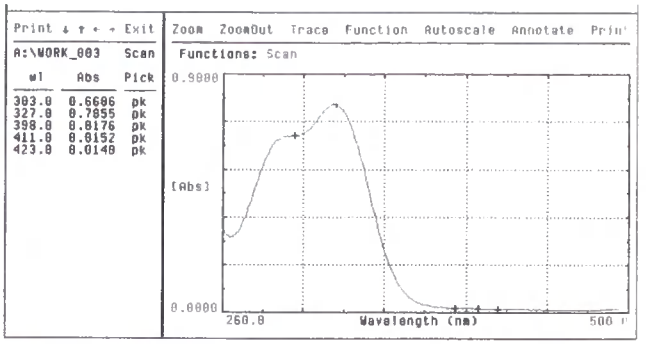
Figure 7.3. HPLC chromatograms of A, unhydrolysed (non-cellulase-digested) shoot extract from *Plantago maritima* grown at ambient CO_2; B, hydrolysed (cellulase-digested) shoot extract; C-F, eluted compounds from hydrolysed extract loaded onto a PTLC plate (see figure 7.1). Peak numbers on chromatogram B correspond to matching peak retention times from compounds eluted by PTLC.

7.4 UV-spectral analyses and mass-spectrometry of HPLC eluted compounds

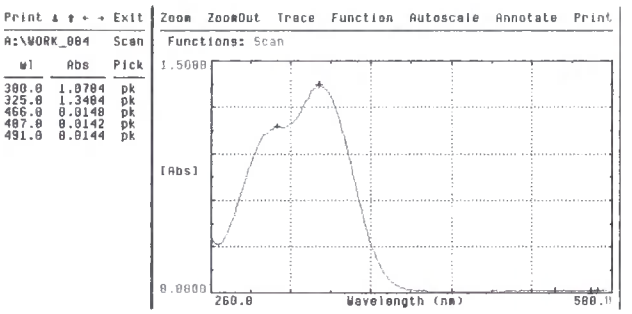
The three compounds collected via preparative HPLC separation produced clearer UV-spectra (Figs. 7.4.A; 7.5.A; 7.6.A). Compounds were directly injected into the mass-spectrometer and analysed using electrospray time-of-flight mass-spectrometry (ES TOF-MS) in the negative mode. Mass ions (*m/z*) of 179 for R_t 6.4 minutes; 623 for R_t 7.6 minutes and 193 for R_t 10.8 minutes were detected (Figs. 7.4.C; 7.5.B; 7.6.C).

The distinctive shape of the UV-spectra and the molecular mass ions suggested that the compounds were phenylpropanoid in origin. Using reference compounds, the UV-spectra of the compound eluting at 6.4 minutes was very similar to that of caffeic acid whilst the compound at 10.8 minutes resembled ferulic acid. By using an isotope-model program, within the MS-MassLynx software, it was possible to confirm the exact mass of caffeic acid and ferulic acid (Figs. 7.4.D; 7.6.D). Comparing the mass of 624 (623 + H) obtained for the peak R_t 7.6 minutes with other phenolic compounds found in *Plantago* species, gave an exact match with the phenylethanoid verbascoside (Fig. 7.5C). Verbascoside is a caffeic acid conjugate hence producing a UV-spectrum similar to that of other phenylpropanoids.

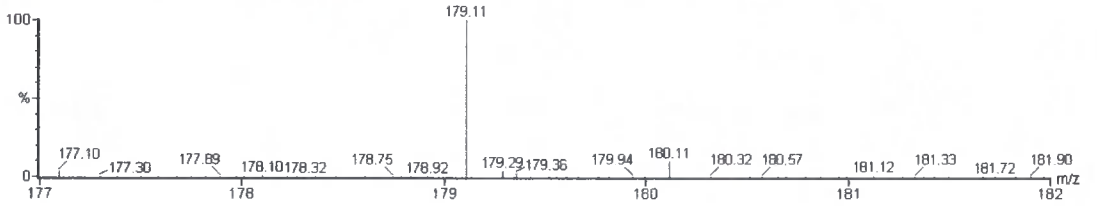
A.



B.



C.



D.

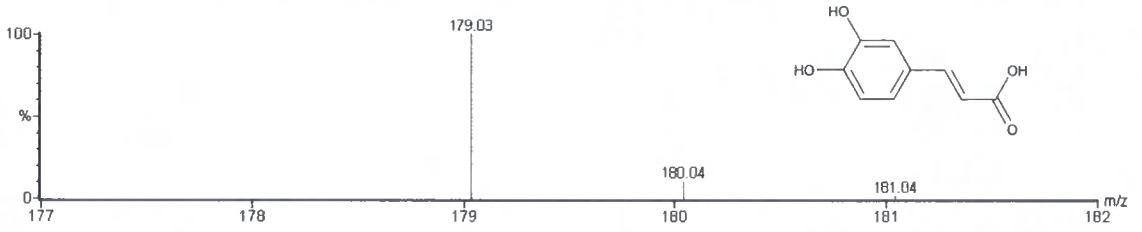
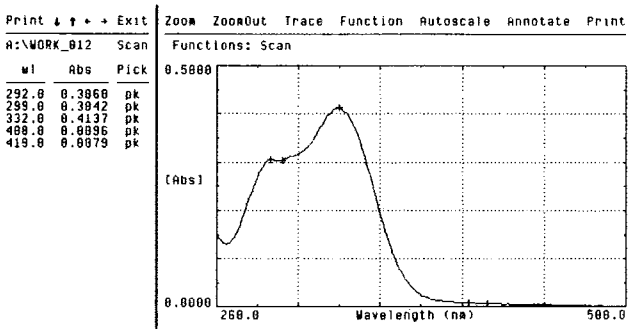
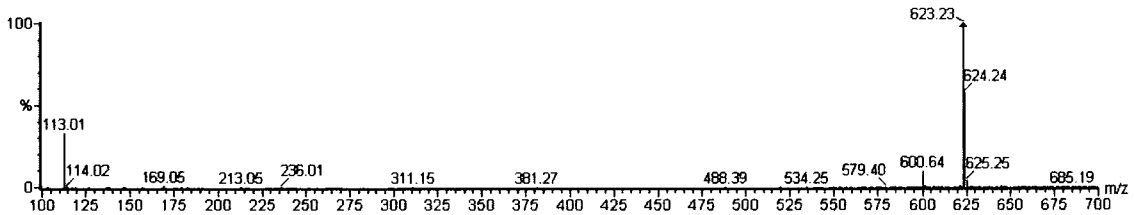


Figure 7.4. A, UV-spectrum of unidentified compound; B, UV-spectrum of caffeic acid; C, mass spectrum of unidentified compound eluted after 6.4 minutes by HPLC showing a mass ion (*m/z*) of 179.09 (180.11) and D, isotope model of caffeic acid ($C_9H_8O_4$).

A.



B.



C.

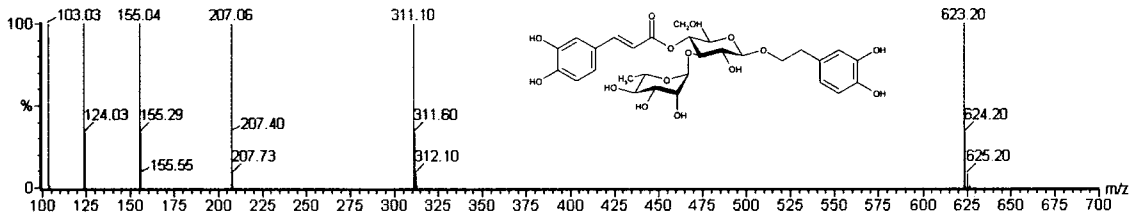
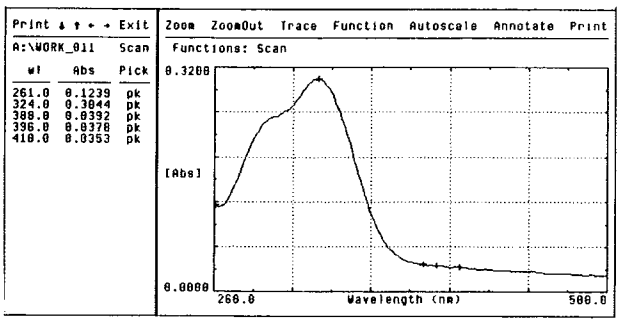
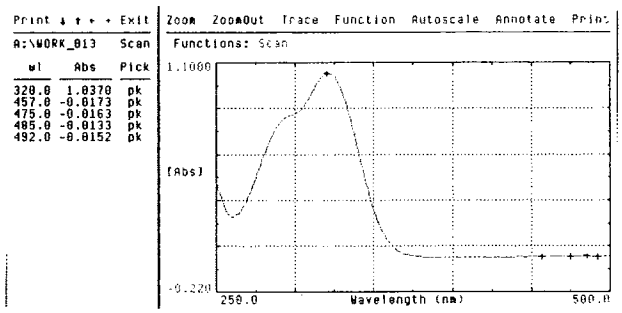


Figure 7.5. A, UV-spectrum of unidentified compound; B, mass spectrum of unidentified compound eluted after 7.6 minutes by HPLC showing a mass ion (*m/z*) of 623.23 (624) and C, isotope model of verbascoside ($C_{29}H_{36}O_{15}$).

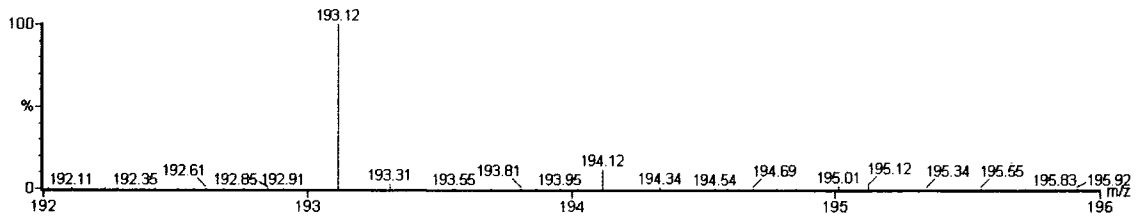
A.



B.



C.



D.

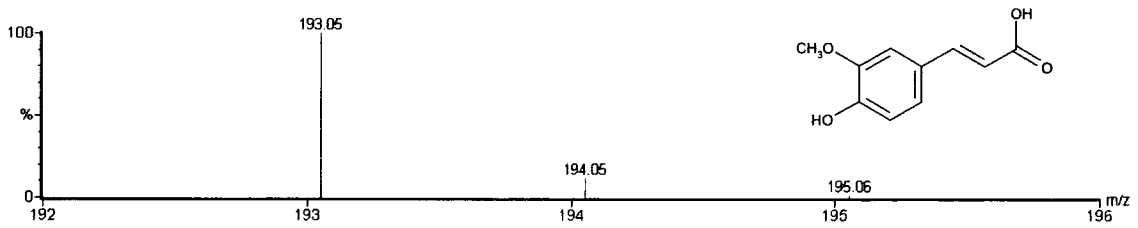


Figure 7.6. A, UV-spectrum of unidentified compound; B, UV-spectrum of ferulic acid; C, mass spectrum of unidentified compound eluted after 10.8 minutes by HPLC showing a mass ion (*m/z*) of 193.12 (194) and D, isotope model of ferulic acid ($C_{10}H_{10}O_4$).

As the UV-spectra and mass-spectrometry data revealed that two of the compounds were probably caffeic and ferulic acid, co-chromatography of the compounds was carried out by HPLC. The HPLC eluted compounds (R_t 6.4 and R_t 10.8) and the caffeic and ferulic acid reference compounds were analysed alone followed by analysing both HPLC eluted and reference compounds together. A verbascoside reference was not available for testing. The HPLC profiles showed that the eluted compounds had the same retention times as the reference compounds (Fig. 7.7).

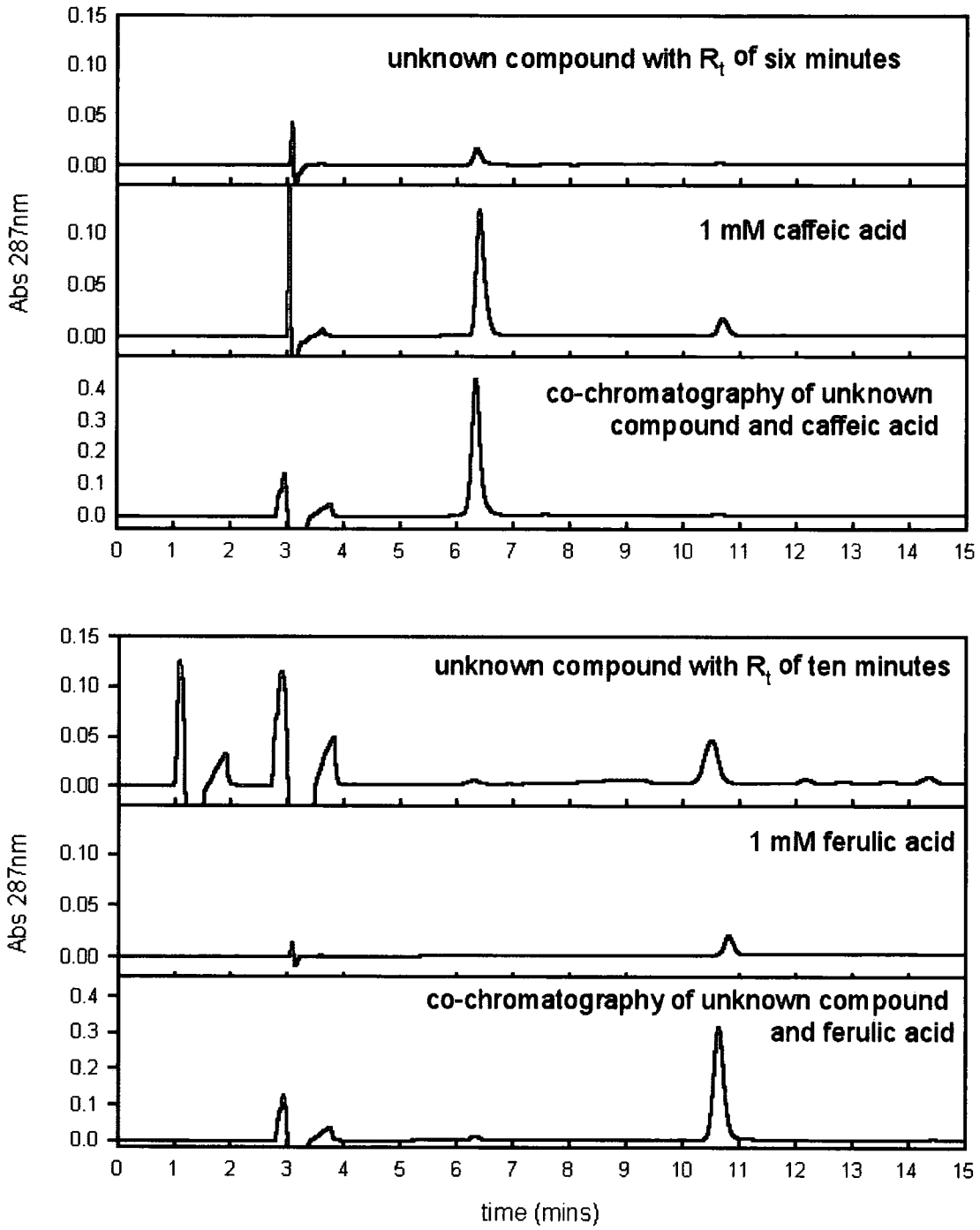


Figure 7.7. HPLC profiles of unidentified HPLC eluted compounds (R_t 6.4 and R_t 10.8); caffeic and ferulic acid reference compounds and co-chromatography of unidentified and reference compounds.

Using the detection methods detailed above it was concluded that the two major compounds were caffeic acid and ferulic acid and that it was highly likely that the third major compound was verbascoside.

To locate the presence of caffeic and ferulic acid in their glycosylated form, the solvent front at the start of the HPLC run (injection peak R_t 2-4 minutes) was collected and subjected to cellulase digestion. Peaks at the same R_t as caffeic and ferulic acid were detected after the hydrolysed solvent front was analysed by HPLC (data not shown). This implied that the glycosylated compounds were present in the injection peak due to increased solubility. Further characterisation of these glycosylated compounds was not undertaken.

By utilising a more advanced identification technique, it was possible to characterise and identify more phenolic compounds in the shoot and root tissue of *P. maritima*.

7.5 Metabolite profiling by combined HPLC-PDA-MS

The HPLC metabolite profiles separated six major compounds from the shoot and root tissue of *P. maritima*. Similar PDA/UV profiles were attained in both the Waters Alliance 2790 LC (Fig. 7.8.A) and Beckman System Gold 125P HPLC (Fig. 7.8B) and this allowed direct comparisons between the two HPLC systems used.

UV-spectra and molecular mass (*m/z*) of the HPLC eluted compounds were obtained using the PDA detector and MS respectively (Fig. 7.9). The same unhydrolysed extract was analysed by HPLC-PDA-MS where 10 compounds were separated, two of which had similar retention times to the hydrolysed extract. This confirmed that the majority of the compounds separated from the hydrolysed extract by HPLC-PDA-MS were aglycones.

The UV-spectra and molecular mass of the unknown compounds indicated that the compounds were simple phenolic compounds (Fig. 7.9). Commercial reference compounds injected into the HPLC-PDA-MS system were confirmed in terms of their retention times (at 287 nm), UV-spectra and molecular mass spectra. This confirmed that four of the compounds were the phenylpropanoid aglycones *p*-coumaric acid, caffeic acid, ferulic acid and the flavone, luteolin (Fig. 7.10).

The caffeoyl phenylethanoid glycosides, verbascoside and plantamajoside, were also identified, being formerly identified in other *Plantago* species (Rønsted *et al.* 2000). Verbascoside (peak 3) was the major UV-absorbing peak present and was constantly associated with a slightly later running peak 3a, which had an identical mass ion to verbascoside. It was concluded that 3A was a MS labile derivative of verbascoside, probably an ester, which yielded parent verbascoside on ESI. To further confirm their identities the UV λ_{max} were compared to published UV maxima (Grayer & de Kok 1998; Ravn & Brimer 1988) and verbascoside was ionised at a higher cone voltage (90 V) to initiate the classic fragmentation of the conjugates, producing two ions with a *m/z*⁻ of 461 and 161 (Ryan *et al.* 1999) (Figs. 7.10.3a,b). All data are summarised in Table 7.1.

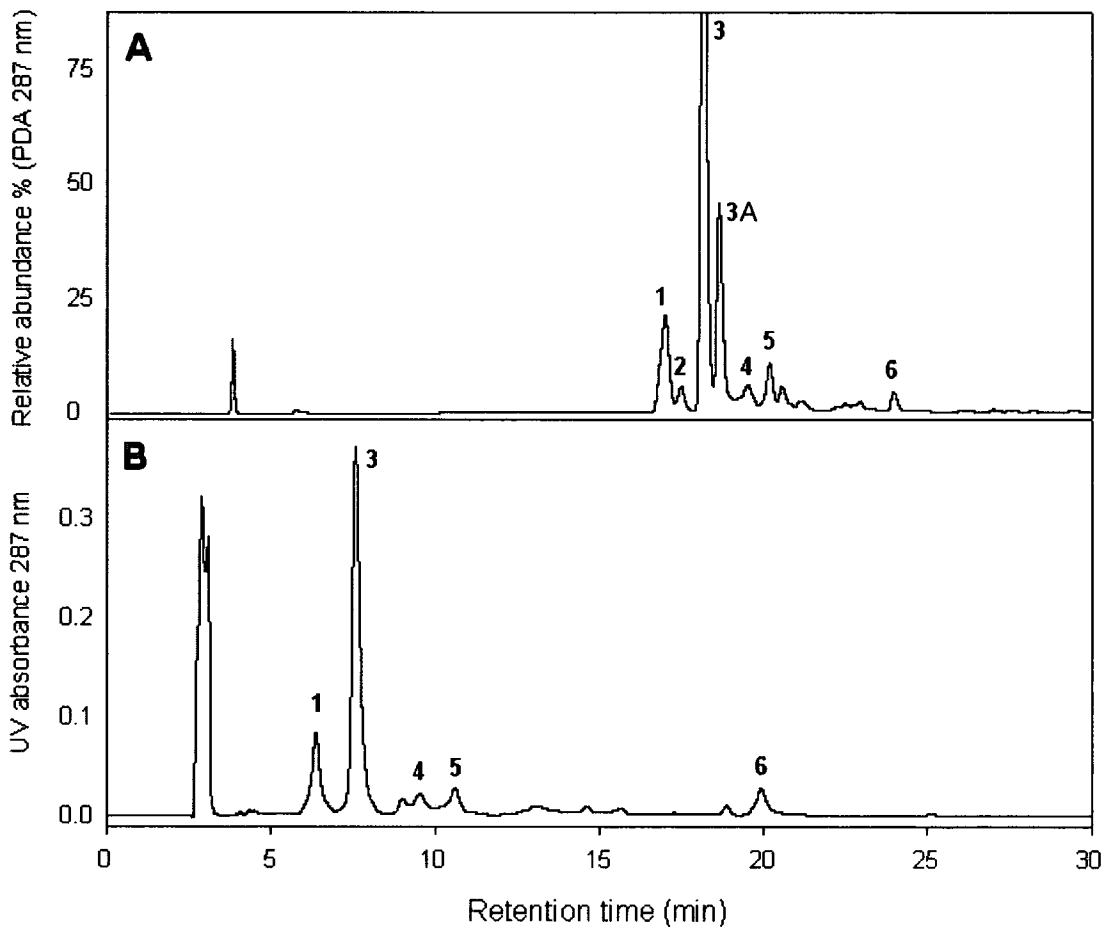


Figure 7.8. Typical HPLC chromatograms of shoot extract from *Plantago maritima*. (**A**) Peaks detected at 287 nm using the LC-PDA Waters Alliance 2790 LC, Phenomenex Synergi™ 4um POLAR-RP column, connected to a Waters 996. PDA. (**B**) Peaks detected at 287 nm using Beckman System Gold 125P HPLC, Phenosphere 5 um ODS2 column. Plantamajoside is not shown, as this peak was not always detected on the Beckman HPLC. Peaks were finally identified as (**1**) caffeic acid, (**2**) plantamajoside, (**3**) verbascoside, (**3A**) verbascoside derivitive (**4**) *p*-coumaric acid, (**5**) ferulic acid and (**6**) luteolin.

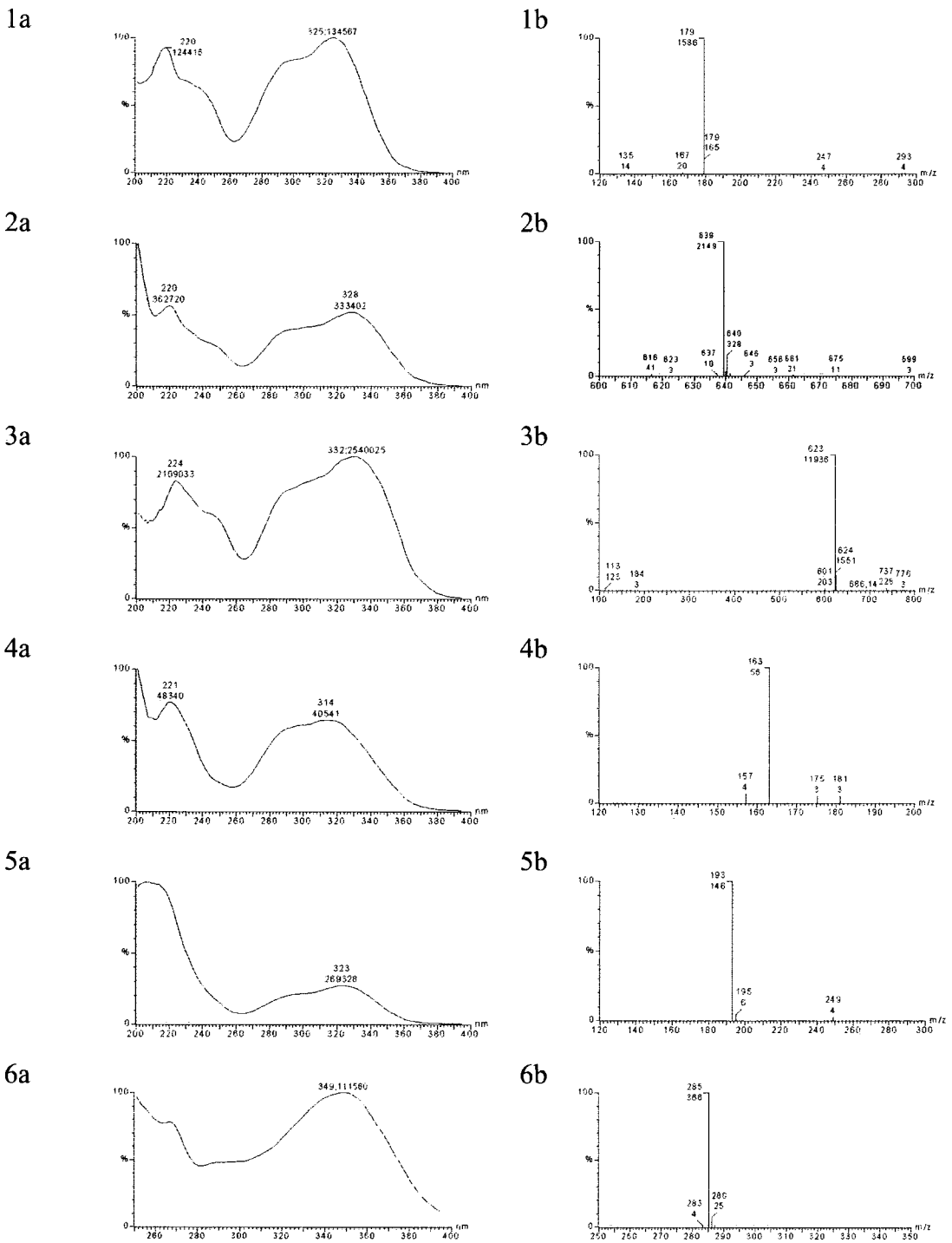


Figure 7.9. UV-spectra (1-6a) and mass profiles (1-6b) of compounds separated and analysed by HPLC-PDA-MS. Peaks were finally identified as (**1**) caffeic acid, (**2**) plantamajoside, (**3**) verbascoside, (**4**) *p*-coumaric acid, (**5**) ferulic acid and (**6**) luteolin.

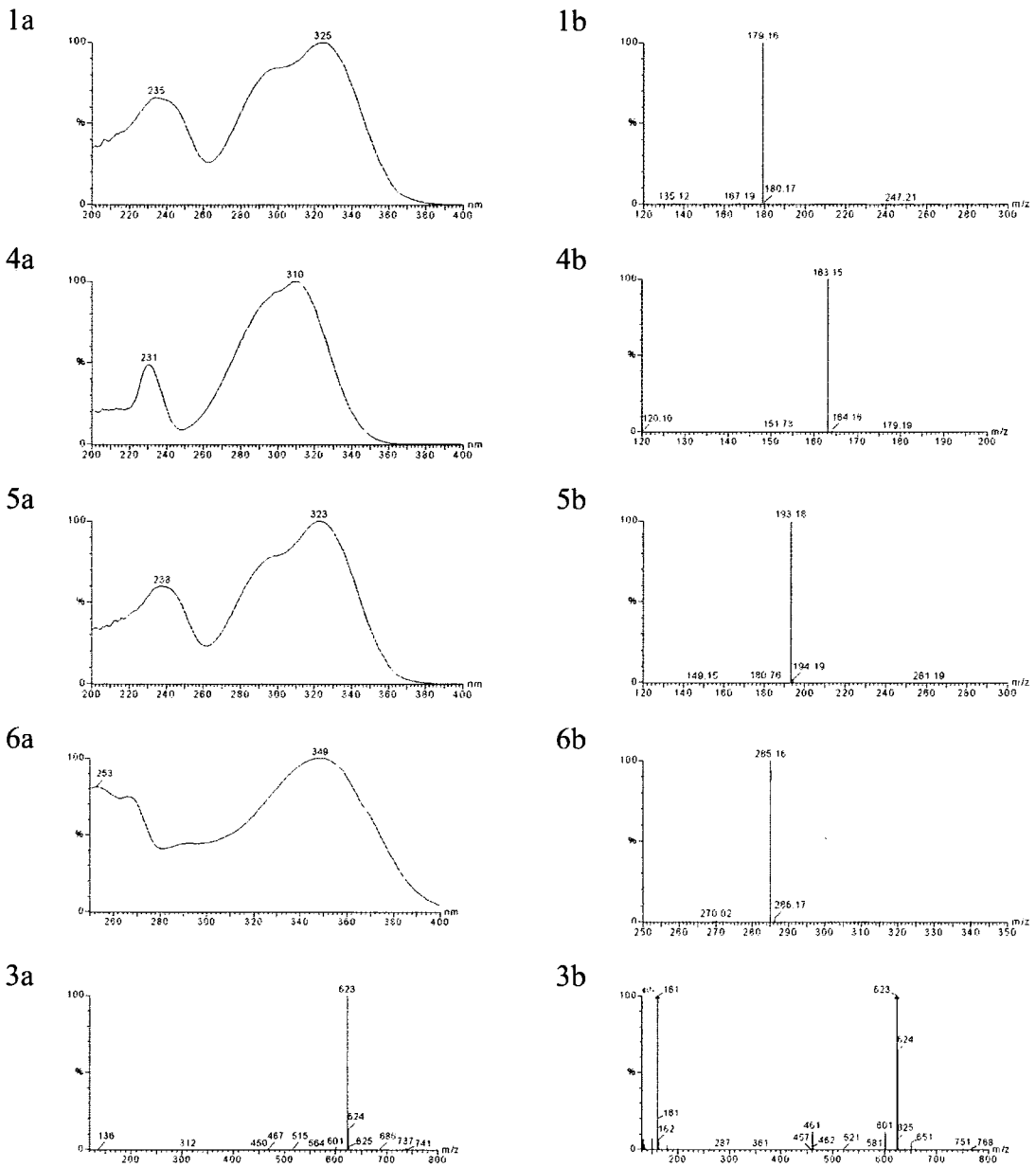
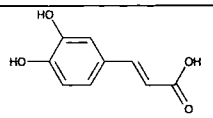
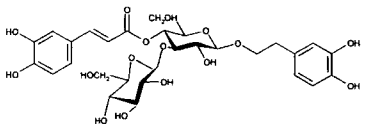
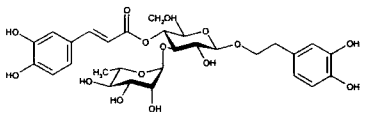
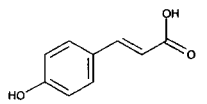
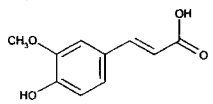
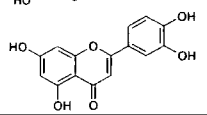


Figure 7.10. UV-spectra (a) and mass profiles (b) of reference compounds separated by HPLC-PDA-MS. (**1**) caffeic acid, (**4**) *p*-coumaric acid, (**5**) ferulic acid and (**6**) luteolin. Compound (verbascoside) fragmentation by increased MS cone-voltage (**3a**), 20 volts and (**3b**), 90 volts.

Table 7.1. Elucidation of phenolic metabolites of *Plantago maritima* compounds by molecular mass ionisation (m/z^-), UV-spectral maxima (PDA) and retention time (R_t).

Compound number	Compound	Obtained molecular mass ion m/z^-	Reference mass ion m/z^-	PDA UV λ_{max} (nm)	Reference PDA UV λ_{max} (nm)	LC-MS R_t (min) (††)	Compound structure
1	Caffeic acid	179.13	179.16	324	324	17.0 (6.4)	
2	Plantamajoside	639	-	329	332 ‡	17.5 (7.4)	
3	Verbascoside	623.3	-	331	330 †	18.2 (7.6)	
3A	Verbascoside derivative	623.3	-	331	330 †	18.7	
4	*p*-Coumaric acid	163.16	163.15	313	310	19.5 (9.7)	
5	Ferulic acid	193.18	193.18	323	323	20.2 (10.8)	
6	Luteolin	285.18	285.16	349	349	24.0 (20.8)	

† = UV λ_{max} from Grayer & de Kok (1998). ‡ UV λ_{max} from Ravn & Brimer (1988).

(††) = R_t on Beckman System Gold 125P HPLC.

7.6 Quantification of major metabolites

Once the phenolic compounds were identified, it was possible to quantify each compound in *P. maritima* grown with different availabilities of C and N to the plant as well as under well-watered and drought conditions. *p*-Coumaric acid, caffeic acid, ferulic acid and verbascoside were identified as the major phenolic compounds and were quantified using commercial standards. Verbascoside was quantified as caffeic acid equivalents. Luteolin and plantamajoside were usually below the level of reliable detection and so were not routinely quantified. To monitor recoveries, samples were spiked with a known amount (280 nmol) of the flavonol quercetin at the beginning of the extraction. The mean recovery of the flavonoid spike was determined to be 98 % $\pm$ 6 % (n =3).

7.6.1 Five and ten week study: Effect of elevated atmospheric CO_2

The most abundant compounds in all samples were verbascoside, followed by caffeic acid, ferulic acid then *p*-coumaric acid. In plants that were well-watered, there were significant increases in the ferulic acid concentrations in plants exposed to elevated CO_2 at both high and low availabilities of N ($F_{1,4}$ = 39.08; P = 0.003 and $F_{1,4}$ = 19.468; P = 0.012 respectively) after five weeks. However, this significant increase in ferulic acid in plants exposed to elevated CO_2 in well-watered plants was not replicated in drought-stressed plants (Tables 7.2, 7.3). A significant reduction of *p*-coumaric acid occurred in drought-stressed plants at low N after five weeks ($F_{1,4}$ = 8.027; P = 0.047) and in well-watered, high N plants after ten weeks ($F_{1,4}$ = 9.57; P = 0.036) when exposed to elevated CO_2 compared to concentrations under ambient CO_2 (Tables 7.2; 7.3).

7.6.2 Effect of low N availability

There were selective increases in the phenylpropanoid concentrations in plants that were treated with low N availability, however these responses were only observed in plants exposed to ambient CO_2, and not elevated CO_2. This increase was significant for caffeic and ferulic acid in well-watered plants exposed to ambient CO_2 after five weeks ($F_{1,4}$ = 11.184; P = 0.029 and $F_{1,4}$ = 30.462; P = 0.005 respectively) and in droughted plants under ambient CO_2 for caffeic acid after ten weeks ($F_{1,4}$ = 7.791; P = 0.049) (Tables 7.2; 7.4).

7.6.3 Effect of drought-stress

The phenylpropanoid concentrations were selectively increased in plants that were drought-stressed. However, these responses were only observed in plants exposed to ambient CO_2, and not elevated CO_2. This increase was significant for ferulic acid in plants exposed to ambient CO_2 with high N availability after five weeks ($F_{1,4} = 24.179$; $P = 0.008$) and for caffeic acid in plants exposed to ambient CO_2 with low N availability after ten weeks ($F_{1,4} = 10.541$; $P = 0.031$) (Tables 7.2; 7.5).

7.6.4 Interactions of treatments

An ANOVA GLM univariate analysis was carried out to test for significant interactions among the CO_2, N and drought treatments (Table 7.6). After five weeks exposure, the ANOVA GLM analyses detected significant effects of CO_2 and N availability on ferulic acid concentrations (also confirmed by Oneway ANOVA; Tables 7.3; 7.4), and a significant interaction of water availability and CO_2 concentration. This significant interaction was due to significant increases in ferulic acid in well-watered plants exposed to elevated CO_2, which was not found in drought-stressed plants. A significant interaction of water availability and CO_2 concentration treatments also occurred for caffeic acid. This interaction arose because the combination of elevated CO_2 and drought caused a reduction in the caffeic acid concentrations, which did not occur in response to elevated CO_2 or drought alone.

After ten weeks exposure, the ANOVA GLM analyses detected significant interactions of watering and CO_2 in *p*-coumaric acid. Similar to ferulic acid after five weeks, this treatment interaction was brought about by a significant reduction of *p*-coumaric acid in well-watered plants exposed to elevated CO_2, which was not found in plants exposed to elevated CO_2 and drought. There was also a significant three-way interaction of CO_2 concentration, N availability and water availability for caffeic acid. This can be explained by the fact that significant effects of drought and N availability occur in plants only grown under ambient CO_2 and not in plants exposed to elevated CO_2.

Table 7.2. Concentrations of the major identified phenolic compounds *p*-coumaric acid, caffeic acid, ferulic acid and verbascoside in the leaves of *Plantago maritima* after five and ten weeks growth. Plants were exposed to either ambient (360 μmol mol^{-1}) or elevated (600 μmol mol^{-1}) atmospheric CO_2. Data represent leaf phenolic concentrations under treatments; well-watered and high N availability; well-watered with low N availability; drought with high N availability and drought with low N availability. Data represent mean ± one SE. n = 3. Significant differences between CO_2 treatments are indicated by * ($P \leq 0.05$); ** ($P \leq 0.01$). All concentrations are expressed as mg g^{-1} dry weight apart from verbascoside (mg caffeic acid equivalents g^{-1} dry weight).

Compound		Five weeks		Ten weeks	
		Atmospheric CO_2 concentration (μmol CO_2 mol^{-1})			
	Treatment	360	600	360	600
***p*-Coumaric acid**	Watered + N	0.08 ± 0.02	0.14 ± 0.04	0.14 ± 0.07	0.06 ± 0.02 (*)
mg g^{-1} d.wt	Watered – N	0.25 ± 0.21	0.28 ± 0.12	0.26 ± 0.18	0.06 ± 0.03
	Drought + N	0.11 ± 0.03	0.18 ± 0.15	0.09 ± 0.05	0.06 ± 0.02
	Drought - N	0.22 ± 0.05	0.05 ± 0.03 (*)	0.11 ± 0.05	0.09 ± 0.03
Caffeic acid	Watered + N	0.45 ± 0.25	1.48 ± 0.52	1.83 ± 0.93	1.62 ± 0.28
mg g^{-1} d.wt	Watered – N	1.39 ± 0.12	2.33 ± 0.80	0.62 ± 0.41	1.65 ± 0.41
	Drought + N	1.27 ± 0.65	0.90 ± 0.28	0.95 ± 0.43	1.67 ± 0.53
	Drought - N	2.60 ± 1.22	0.97 ± 0.25	3.19 ± 0.68	1.54 ± 0.57
Ferulic acid	Watered + N	0.02 ± 0.02	0.28 ± 0.04 (**)	0.33 ± 0.14	0.34 ± 0.10
mg g^{-1} d.wt	Watered – N	0.18 ± 0.02	0.36 ± 0.03 (*)	0.29 ± 0.09	0.30 ± 0.03
	Drought + N	0.17 ± 0.02	0.17 ± 0.06	0.20 ± 0.06	0.30 ± 0.13
	Drought - N	0.23 ± 0.09	0.22 ± 0.04	0.40 ± 0.07	0.26 ± 0.03
Verbascoside	Watered + N	0.78 ± 0.54	3.73 ± 2.93	2.77 ± 1.46	2.46 ± 0.68
mg caffeic acid	Watered – N	3.75 ± 1.76	6.07 ± 1.55	5.63 ± 2.64	4.97 ± 2.24
equivalents	Drought + N	2.92 ± 1.16	2.41 ± 1.33	4.19 ± 2.30	1.84 ± 0.55
g^{-1} d.wt	Drought - N	4.27 ± 1.22	2.87 ± 1.00	2.83 ± 1.01	2.96 ± 1.46

Table 7.3. Results from a Oneway ANOVA analyses to test for significant effects of **elevated CO_2** among shoot phenolic concentrations in *Plantago maritima* after five and ten weeks. All values have degrees of freedom of 1 between groups and 4 within groups. Significance level is set at $P \leq 0.05$.

Five weeks		*p*– Coumaric acid		Caffeic acid		Ferulic acid		Verbascoside	
Growth conditions		*F*-value	*P*	*F*-value	*P*	*F*-value	*P*	*F*-value	*P*
Well-watered	High N	1.452	0.295	3.130	0.152	**39.083**	**0.003**	1.394	0.303 LN
	Low N	0.012	0.919	1.356	0.309	**19.468**	**0.012**	0.975	0.379
Drought	High N	1.806	0.250 LN	0.277	0.626	0.000	0.993	0.084	0.787
	Low N	**8.027**	**0.047**	1.714	0.261	0.010	0.927	0.789	0.425

Ten weeks		*p*– Coumaric acid		Caffeic acid		Ferulic acid		Verbascoside	
Growth conditions		*F*-value	*P*	*F*-value	*P*	*F*-value	*P*	*F*-value	*P*
Well-watered	High N	**9.573**	**0.036**	0.045	0.842	0.001	0.977	0.036	0.858
	Low N	1.214	0.332	3.226	0.147	0.016	0.905	0.036	0.858
Drought	High N	0.401	0.561	1.119	0.350	0.533	0.506	0.997	0.374
	Low N	0.103	0.765	3.476	0.136	3.896	0.120	0.006	0.942

LN = data were natural log transformed.

Table 7.4. Results from a Oneway ANOVA analyses to test for significant effects of **nitrogen** availability among shoot phenolic concentrations in *Plantago maritima* after five and ten weeks. All values have degrees of freedom of 1 between groups and 4 within groups. Significance level is set at $P \leq 0.05$.

Five weeks		*p*– Coumaric acid		Caffeic acid		Ferulic acid		Verbascoside	
Growth conditions		*F*-value	*P*	*F*-value	*P*	*F*-value	*P*	*F*-value	*P*
Ambient CO_2	Well-watered	0.247	0.645 LN	**11.184**	**0.029**	**30.462**	**0.005**	2.628	0.180
	Drought	2.918	0.163	0.927	0.390	0.567	0.493	0.642	0.468
Elevated CO_2	Well-watered	1.292	0.319	0.790	0.424	2.474	0.191	0.496	0.520
	Drought	0.289	0.619 LN	0.044	0.844	0.638	0.469	0.076	0.796

Ten weeks		*p*– Coumaric acid		Caffeic acid		Ferulic acid		Verbascoside	
Growth conditions		*F*-value	*P*	*F*-value	*P*	*F*-value	*P*	*F*-value	*P*
Ambient CO_2	Well-watered	0.395	0.564	1.426	0.298	0.058	0.822	0.902	0.396
	Drought	0.056	0.825	**7.791**	**0.049**	5.087	0.087	0.297	0.615
Elevated CO_2	Well-watered	0.000	0.985	0.004	0.951	0.100	0.768	1.154	0.343
	Drought	0.560	0.496	0.028	0.874	0.106	0.761	0.521	0.510

LN = data were natural log transformed.

Table 7.5. Results from a Oneway ANOVA analyses to test for significant effects of **drought** among shoot phenolic concentrations in *Plantago maritima* after five and ten weeks. All values have degrees of freedom of 1 between groups and 4 within groups. Significance level is set at $P \leq 0.05$.

Five weeks		*p*– Coumaric acid		Caffeic acid		Ferulic acid		Verbascoside	
Growth conditions		*F*-value	*P*	*F*-value	*P*	*F*-value	*P*	*F*-value	*P*
Ambient CO_2	High N	0.837	0.412	1.357	0.309	**24.179**	**0.008**	2.807	0.169
	Low N	0.375	0.574 LN	0.984	0.377	0.348	0.587	0.059	0.820
Elevated CO_2	High N	0.077	0.795	0.973	0.380	2.834	0.168	0.169	0.702
	Low N	3.670	0.128	2.611	0.181	6.213	0.067	2.997	0.158

Ten weeks		*p*– Coumaric acid		Caffeic acid		Ferulic acid		Verbascoside	
Growth conditions		*F*-value	*P*	*F*-value	*P*	*F*-value	*P*	*F*-value	*P*
Ambient CO_2	High N	0.335	0.594	0.739	0.439	0.741	0.438	0.275	0.627
	Low N	0.654	0.464	**10.541**	**0.031**	0.945	0.386	0.984	0.377
Elevated CO_2	High N	0.004	0.955	0.007	0.936	0.048	0.837	0.511	0.514
	Low N	0.488	0.523	0.026	0.881	1.127	0.348	0.563	0.495

LN = data were natural log transformed.

Table 7.6. Results from an ANOVA GLM univariate analyses to test for significant interactions of CO_2, N availability and drought among shoot phenolic concentrations of *Plantago maritima* after five and ten weeks. Significance level is set at $P \leq 0.05$.

Five weeks	*p*– Coumaric acid (LN)		Caffeic acid		Ferulic acid		Verbascoside	
	F-value	*P*	*F*-value	*P*	*F*-value	*P*	*F*-value	*P*
CO_2	0.173	0.684	0.000	0.987	**11.394**	**0.004**	0.564	0.463
N	0.189	0.670	3.351	0.086	**8.085**	**0.012**	2.540	0.131
Water	0.023	0.881	0.003	0.960	0.173	0.683	0.172	0.684
N x water	0.747	0.402	0.046	0.832	0.826	0.377	0.614	0.445
N x CO_2	1.035	0.326	0.586	0.455	0.505	0.488	0.118	0.736
water x CO_2	1.239	0.284	**5.184**	**0.037**	**12.345**	**0.003**	2.584	0.127
CO_2 x N x water	1.171	0.298	0.447	0.513	0.308	0.587	0.003	0.956

Ten weeks	*p*– Coumaric acid (LN)		Caffeic acid		Ferulic acid		Verbascoside	
	F-value	*P*	*F*-value	*P*	*F*-value	*P*	*F*-value	*P*
CO_2	1.504	0.238	0.004	0.951	0.006	0.940	0.436	0.518
N	0.108	0.747	0.348	0.563	0.108	0.747	1.133	0.303
Water	3.805	0.069	1.065	0.317	0.175	0.681	0.688	0.419
N x water	0.057	0.815	4.292	0.055	0.832	0.375	1.352	0.262
N x CO_2	0.037	0.850	0.511	0.485	0.863	0.367	0.197	0.663
water x CO_2	**4.745**	**0.045**	1.227	0.284	0.046	0.833	0.068	0.798
CO_2 x N x water	0.037	0.850	**5.187**	**0.037**	0.962	0.341	0.348	0.563

LN = data were natural log transformed.

7.7 Exposure for one year to elevated atmospheric CO_2 concentration

Plants exposed to elevated CO_2 had increased concentrations of caffeic acid in shoots ($F_{1,6} = 17.55$; $P = 0.006$), compared to plants exposed to ambient CO_2 (Table 7.7). In the roots, there was an increase in the *p*-coumaric ($F_{1,6} = 44.94$; $P = 0.001$) and verbascoside concentrations in plants exposed to elevated CO_2, compared to plants grown in ambient CO_2. As compared with shoots, phenylpropanoid concentrations were lower in the roots while verbascoside concentrations were higher.

Table 7.7. Concentrations of the major identified phenolic compounds; *p*-coumaric acid; caffeic acid; ferulic acid and verbascoside in the shoot and root of *Plantago maritima* after one years growth. Plants were exposed to either ambient (360 µmol mol^{-1}) or elevated (600 µmol mol^{-1}) atmospheric CO_2. Data represent mean ± one SE. n = 4. Significant differences between CO_2 treatments are indicated by ** ($P \leq 0.01$) and *** ($P \leq 0.001$). All concentrations are expressed as mg g^{-1} dry weight apart from verbascoside (mg caffeic acid equivalents g^{-1} dry weight).

Compound (mg g^{-1} d.wt)	**Atmospheric CO_2 concentration (µmol mol^{-1} CO_2)**	
	360	**600**
***p*-Coumaric acid**		
Shoot	0.41 ± 0.29	0.57 ± 0.23
Root	0.09 ± 0.03	0.52 ± 0.06 (***)
Caffeic acid		
Shoot	1.08 ± 0.22	2.31 ± 0.19 (**)
Root	1.04 ± 0.14	1.25 ± 0.15
Ferulic acid		
Shoot	0.37 ± 0.10	0.51 ± 0.07
Root	0.16 ± 0.02	0.22 ± 0.03
Verbascoside		
Shoot	9.57 ± 1.62	9.24 ± 1.00
Root	17.88 ± 1.70	27.61 ± 4.15

7.8 Leaf anatomy and lignification

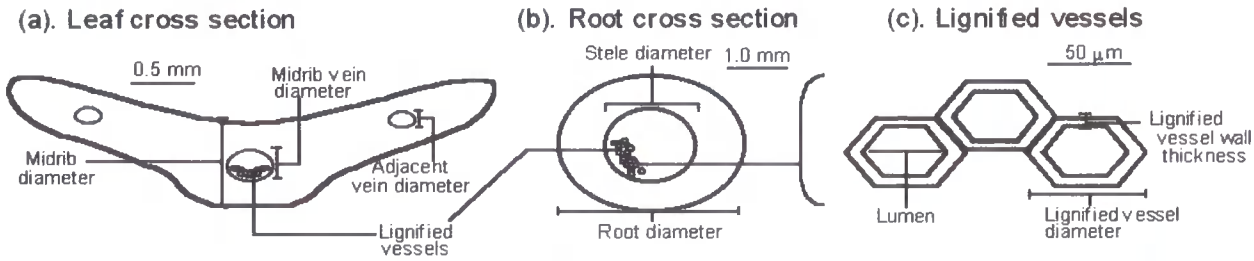
This study within this section was focussed on CO_2 effects only as N and drought treatments were not incorporated into the one year experiment. Overall shoot and root biomass was increased, 124 % and 38 % respectively, in plants exposed to elevated CO_2 (Table 7.8.f). In the leaves, this was accompanied by subtle changes in the anatomy and lignification of leaves, when compared to plants grown under ambient CO_2 (Table 7.8.d). The diameter of the midrib vein was not altered under elevated CO_2. However, when expressed as a ratio of vein diameter to midrib diameter the vein had slightly increased in size in proportion to the whole leaf, when compared to plants grown under ambient CO_2. Adjacent veins were wider in leaves exposed to elevated CO_2, though there was not an associated increase in the number of lignified vessel members. However, the diameters of the lignified vessels in the adjacent veins were increased in the middle part of the leaves exposed to elevated CO_2 ($F_{1,5} = 10.92$; $P = 0.021$). Within the proximal midrib and middle adjacent veins, the lignified vessel wall thicknesses were slightly reduced in plants exposed to elevated CO_2, compared to those exposed to ambient CO_2.

7.9 Root anatomy and lignification

The anatomy and lignification of roots were altered in response to elevated CO_2, when compared to plants grown under ambient CO_2 (Table 7.8.e). The diameter of the stele increased in roots exposed to elevated CO_2 and this enhancement was in proportion to the total width of the root. Such an increase in stele width under elevated CO_2 coincided with an increase in the number of lignified vessel members. There was a small increase in the diameter of measured vessels in roots grown under elevated CO_2. However, there was a reduction in the lignified vessel wall thickness to vessel diameter ratio in the middle and distal sections of the root in plants exposed to elevated CO_2 ($F_{1,6} = 8.50$; $P = 0.027$ and $F_{1,6} = 35.02$; $P = 0.001$ respectively), compared to those exposed to ambient CO_2. As well as a lignification of xylem vessel members, an increase in the number of lignified fibre vessels was observed in roots exposed to elevated CO_2 (Fig. 7.11).

Responses to elevated CO_2 were generally consistent throughout the proximal and middle parts of the leaf and root. Clear cross-sections of the distal sections were difficult to achieve causing low replicate numbers in some cases.

Table 7.8. Key to anatomical measurements made on cross sections of leaf (a); root (b) and lignified vessels (c) transverse cross sections of *Plantago maritima*. Quantitative changes in leaf (d) and root (e) histochemistry and (f) biomass following exposure of *P. maritima* to enhanced CO_2 for 12 months. Data represent mean ± SE. n = 3-4 for proximal and middle measurements and n = 1-4 for distal measurements n = 7-12 for biomass measurements. Significant differences between CO_2 treatments are indicated by * ($P < 0.05$); ** ($P \leq 0.01$) and *** ($P \leq 0.001$).



d: Leaf	Proximal		Middle		Distal	
	Atmospheric CO_2 concentration (µmol CO_2 mol^{-1})					
	360	600	360	600	360	600
Midrib vein diameter (µm)	255 ± 6	229 ± 12	186 ± 25	202 ± 16	153 ± 16	145 ± 10
Adjacent vein diameter (µm)	151 ± 8	180 ± 15	120 ± 13	166 ± 11	84 ± 4	117 ± 0
Midrib vein diameter/midrib diameter * 100	24 ± 2	24 ± 2	19 ± 2	25 ± 3	21 ± 2	25 ± 5
No. lignified vessels in midrib vein	21 ± 2	23 ± 1	21 ± 2	20 ± 3	17 ± 4	14 ± 2
No. lignified vessels in adjacent veins	15 ± 1	15 ± 1	11 ± 1	13 ± 1	10 ± 0	10 ± 0
Vessel diameter (µm) in midrib vein	11 ± 0.5	13 ± 0.9	12 ± 0.5	12 ± 0.5	9 ± 2.0	11 ± 0.6
Vessel diameter (µm) in adjacent veins	10 ± 0.1	11 ± 0.7	9 ± 0.5	11 ± 0.4 *	9 ± 1.8	11 ± 0.0
Lignified vessel wall thickness : vessel diameter ratio in midrib vein	0.32 ± 0.01	0.23 ± 0.01 **	0.29 ± 0.02	0.24 ± 0.01	0.30 ± 0.03	0.31 ± 0.04
Lignified vessel wall thickness : vessel diameter ratio in adjacent veins	0.33 ± 0.03	0.26 ± 0.02	0.31 ± 0.01	0.25 ± 0.02 *	0.33 ± 0.01	0.24 ± 0.00

e: Root	Proximal		Middle		Distal	
	Atmospheric CO_2 concentration (µmol CO_2 mol^{-1})					
	360	600	360	600	360	600
Stele diameter (µm)	1183 ± 255	1618 ± 150	892 ± 180	1288 ± 205	793 ± 238	1298 ± 322
No. lignified vessels per stele bundle	371 ± 95	561 ± 49	211 ± 37	472 ± 122	204 ± 55	487 ± 233
Stele diameter/root diameter * 100	42 ± 4	48 ± 1	41 ± 3	46 ± 2	41 ± 2	46 ± 2
Lignified vessel diameter (µm)	32 ± 2	34 ± 3	35 ± 2	36 ± 2	32 ± 3	37 ± 1
Lignified vessel wall thickness : vessel diameter ratio	0.24 ± 0.01	0.19 ± 0.02	0.23 ± 0.01	0.18 ± 0.02 *	0.24 ± 0.01	0.17 ± 0.01 ***

f: Total biomass (g)	Atmospheric CO_2 concentration (µmol CO_2 mol^{-1})	
	360	600
Shoot	1.51 ± 0.31	3.37 ± 0.50
Root	1.65 ± 0.17	2.28 ± 0.20

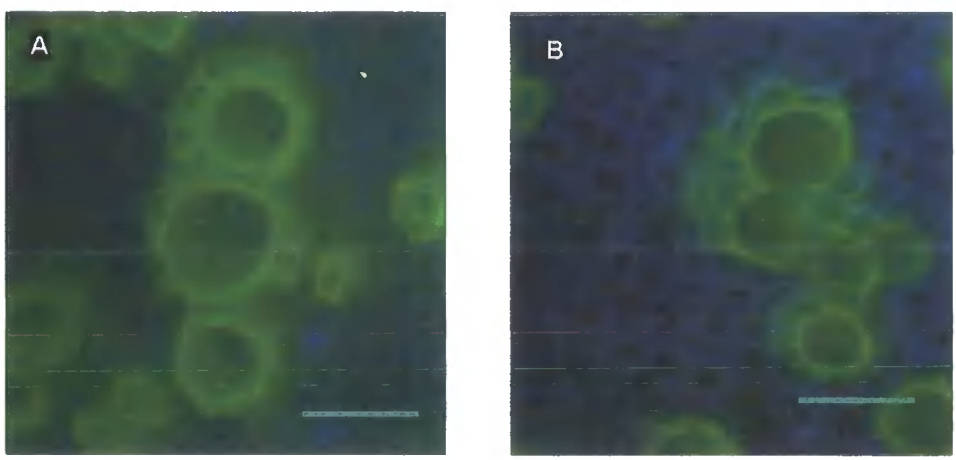


Figure 7.11. Image of transverse sections of the main root after one year's exposure to either (A) ambient (360 µmol mol^{-1}) or (B) elevated (600 µmol mol^{-1}) atmospheric CO_2 demonstrating visible changes in the width of lignified xylem vessel wall thickness (fluorescent yellow) and increased abundance of lignified fibres adjacent to xylem vessels. Bar length represents 50 µm.

7.10 Discussion and conclusions

7.10.1 Metabolite profiling

By utilising the HPLC-PDA-MS system it was possible to identify and quantify the major phenolics in *P. maritima* with a basic knowledge of the chemotaxonomy of *Plantago* species. The phenolic content of *P. maritima* was dominated by phenypropanoids, notably caffeic acid and its derivative verbascoside. Verbascoside is known to be present in other members of the Plantaginaceae family (Rønsted *et al.* 2000), though this is the first report of verbascoside in *P. maritima*. *Plantago maritima* is also known to accumulate luteolin (Moore *et al.* 1972) though in this study it was only a minor metabolite. Plantamajoside was detected in some of the samples but not in most and it is probable that its variable presence was due to its interconversion with verbascoside (Fons 1999). Interestingly, both verbascoside and plantamajoside were resistant to hydrolysis by cellulase. The hydrolysis of these conjugates has been shown to be enzyme specific in experiments by Ravn & Brimer (1988) with plantamajoside only successfully hydrolysed by β-glucuronidase and not by β-glucosidase.

7.10.2 Effect of elevated CO_2 after five and ten weeks and one years exposure

In well-watered plants, elevated atmospheric CO_2 had a subtle effect on phenylpropanoid metabolism, with the change being more prominent in the root rather than the shoot. Elevated CO_2 increased the concentration of ferulic acid in the shoots after five weeks and caffeic acid in the shoots and *p*-coumaric acid in the roots after one year. Verbascoside also increased in the roots after a one-year exposure to elevated CO_2.

The total percent C contribution these compounds represented in the plant under ambient CO_2, in well-watered plants with high N availability, was on average 0.08 % and 0.29 % in the shoots after 5 and 10 weeks and 0.65 % and 1.08 % in the shoots and roots after one year of the tissue dry weight. These C percentages were altered in plants exposed to elevated CO_2 to 0.32 %; 0.26 %; 0.72 % and 1.66 % respectively. Therefore, the C contribution was on average 321 % greater in the shoots after 5 weeks, 12 % lower in the shoots after 10 weeks and 12 % and 54 % greater in the shoots and roots, respectively, after one year compared to C concentrations in plants grown under ambient CO_2 conditions. This shows that there was on average, in the compounds that were detected, an increase of C allocation to secondary metabolism in plants exposed to

elevated CO_2. This is in agreement with the CNB/GDH models (Bryant *et al.* 1983 and Herms & Mattson 1992). An increase of verbascoside was reported in the shoots of *Plantago lanceolata* grown under low-N conditions but not in plants exposed to elevated CO_2 (Fajer *et al.* 1992). This was the case in the current study, however, the phenolic responses to elevated CO_2 in *P. maritima* were observed in the root rather than the shoot tissue. As the compound concentrations were increased under elevated CO_2 after one years growth, together with an increase in biomass, this suggests that there was no internal competition for C between growth and secondary metabolism (Peñuelas *et al.* 1997) in *P. maritima*.

7.10.3 Effect of reduced nitrogen availability

It was hypothesised that there would be a further increase in C compound concentrations under elevated CO_2 when plants were grown under low N availability, as there would be an imbalance between the increased photosynthetic rates commonly observed under elevated CO_2 and a reduction in growth rates due to a limited N supply. This would cause C compounds, usually carbohydrates, to accumulate in the plant tissue, particularly the shoot (Farrar & Williams 1991; Stitt 1991; Baxter *et al.* 1995). However, in the present study, a combined treatment of elevated CO_2 and low N did not significantly increase concentrations of the phenolic compounds. Only ferulic acid increased under elevated CO_2 combined with reduced N availability and this increase was also observed in plants exposed to elevated CO_2 with high N availability. *p*-Coumaric acid concentrations were actually reduced under elevated CO_2 and reduced N supply. A reduced N supply had more of an effect in plants grown under ambient CO_2, rather than elevated CO_2, with increases in caffeic and ferulic acid observed. This response has been observed in the phenolic concentrations of *Plantago lanceolata* (Fajer *et al.* 1992) and such an increase in secondary metabolism under N-stress has been attributed to increases in activity of the enzyme phenylalanine ammonium lyase (PAL; EC 4.3.1.5) (Stewart *et al.* 2001), which is involved in the early stages of phenylpropanoid synthesis. However, as discussed in chapter 6, protein synthesis also shares the precursor phenylalanine, which may explain the reduction of the concentration of these phenolic compounds in plants grown at elevated CO_2 as phenylalanine that would otherwise be allocated to phenylpropanoid synthesis may be allocated to protein synthesis for extra growth (Jones & Hartley 1999).

7.10.4 Effect of drought

Plantago maritima used in the study was likely to be a drought-tolerant ecotype, frequently subjected to reduced water-availability in a coastal cliff-top habitat (Goldsmith 1973 a & b; Rodwell 2000). Although drought brought about a significant increase in caffeic and ferulic acid, droughting the plants did not further increase phenolic concentrations when combined with elevated CO_2 (compared to well-watered and elevated CO_2 plants). Similar effects were found in wheat flavonoid concentrations exposed to CO_2 and drought (Estiarte *et al.* 1999). The significant increases observed in ferulic acid in well-watered plants under elevated CO_2 were not replicated in droughted plants, as the ferulic acid concentrations at ambient CO_2 were similar to those under elevated CO_2. Such subtle fluctuations suggest that the underlying mechanisms for phenylpropanoid metabolism may not only be regulated by C supply from the shikimate pathway but other abiotic factors (such as drought) might bring about similar changes in individual phenolic compounds.

7.10.5 Lignification and anatomical changes after one years exposure to elevated CO_2

Any alteration in the intermediate phenylpropanoid concentrations within shoot or root tissue may lead to changes in monolignol compound concentration and polymerisation for end-point syntheses of lignin (Peñuelas & Estiarte 1998; Anterola & Lewis 2002; Humphreys & Chapple 2002). The results showed a reduction in the lignification (vessel wall thickness to vessel diameter ratio) of leaf and root vessel members in plants exposed to elevated CO_2. However, the actual diameters of the lignified vessels were slightly increased throughout the lengths of the leaves or roots. This implies that the vessel wall surface area available for lignification was increased, and together with an increase in the number of lignified vessels, an overall increase in the lignin concentration of the plant could be expected.

The increase in the vessel diameter may be caused by increased cell expansion rates commonly observed in plants growing under elevated CO_2 (Pritchard *et al.* 1999). Therefore, the associated thinner lignified walls may be a by-product of a faster cell expansion rate caused by elevated CO_2 (and hence an increase in cell wall surface area for lignification), rather than due to minor changes in phenolic metabolism. This could explain the variable responses of lignin concentration in plants exposed to elevated CO_2 (Hartley *et al.* 2000).

In agreement with the results in this study, increases in stem vessel number and lumen area have also been observed in Oak under elevated CO_2 conditions (Atkinson & Taylor 1996) and increases in main vein diameter and root stele width were observed in *Layia platyglossa* exposed to elevated CO_2 (St. Omer & Horvath 1984).

One of the effects of modifying the accumulation of soluble phenylpropanoids would be on the associated protective activity of these compounds in protecting the plant from biotic stress. *p*-Coumaric, caffeic and ferulic acid have all shown alleochemical (Barkosky *et al.* 2000; Wu *et al.* 2000); antifungal (Daayf *et al.* 2000); and anti-oxidative properties (Kikuzaki *et al.* 2002; Son & Lewis 2002). Verbascoside and plantamajoside also have anti-herbivore/pathogen activities (Ravn & Brimer 1988). Increases in the shoot and root concentration of soluble phenylpropanoids under elevated CO_2 may have subtle effects on plant-herbivore/pathogen interactions.
As there were selective changes in the phenylpropanoid compounds under elevated CO_2, this suggests that a concurrent increase in the enzymes and/or gene transcript activity regulating the pathway and/or the related gene transcripts would be expected and therefore requires further study (Maher *et al.* 1994). An increase in phenylalanine ammonium lyase (PAL) activity has been reported in Birch (*Betula pendula*) seedlings (Lavola *et al.* 2000) and *Senecio vulgaris* (Hartley *et al.* 2000). Transcripts levels for (*pal*) have been shown to reduce in Aspen (*Populus tremuloides*) trees exposed to elevated CO2, compared to an ambient CO_2 control (Wustman *et al.* 2001). However, no such increase in PAL activity was detected in *P. maritima* in this study (data not shown), activities being just below the level of detection in all samples analysed. The fact that the relative ratios of the phenylpropanoid intermediates change in *P. maritima* exposed to enhanced CO_2 concentrations suggests that the respective pool sizes are under independent control and that factors more complex than a simple upregulation of PAL must be regulating phenolic metabolism.

7.10.6 Conclusions

1. Metabolic profiling using HPLC-PDA-MS has proved to be a useful tool in assessing subtle changes in secondary metabolism in native *P. maritima.*
2. Under elevated CO_2, there were increases in the concentrations of ferulic acid and verbascoside in the shoots after five weeks growth; caffeic acid in the shoot and *p*-coumaric acid in the roots after one years exposure, compared to ambient CO_2.
3. Reduced N availability increased concentrations of selective phenolic compounds in plants exposed to ambient CO_2 but not in plants exposed to elevated CO_2.
4. Under ambient CO_2 conditions, there was a selective increase in the phenolic concentrations in droughted plants compared to well-watered plants. Drought did not alter the phenolic response to elevated CO_2, compared to well-watered plants under elevated CO_2.
5. The lignified vessel wall thicknesses were reduced in plants exposed to elevated CO_2, compared to those grown under ambient CO_2. However, there were increases in leaf vessel and vein diameters; the number of lignified root vessels and stele width, implying an increased surface area for lignification.

Chapter 8: General discussion and conclusion

Concentrations of C and N-based compounds within plants can change in response to elevated atmospheric CO_2, particularly when soil N availability is limited (Stitt & Krapp 1999). Most literature on assimilate partitioning in response to elevated atmospheric CO_2 is based on crop species, and assesses the impact of elevated CO_2 on the major end-products of primary metabolism (Farrar & Williams 1991; Stitt 1991; Urban 2003). Such research has provided a sound basis for understanding changes in plant physiology and biochemistry, but the focus on a limited number of compounds probably results in an underestimation of the potential importance of C and N partitioning to secondary metabolism (Peñuelas & Estiarte 1998) and compounds involved in plant survival in harsh environments. One such environment is coastal cliff-tops, where plants are exposed to frequent periods of soil water-deficit and/or salinity (Goldsmith 1973 a & b; Rodwell 2000). However, few studies have concentrated on the effects of elevated CO_2 on coastal plants (Lenssen 1993; Lenssen *et al.* 1995; Gray & Mogg 2001). *Plantago maritima* and *Armeria maritima* can withstand such drought and hypersaline conditions on coastal cliff-tops. It is likely that such tolerance to drought and salinity is due in part to their ability to accumulate C and N compounds as cytoplasmic osmotica, otherwise known as 'compatible solutes' (Brown & Simpson 1972; Stewart & Lee 1974). These are the C-based polyhydric alcohol sorbitol in *P. maritima* and the N-based imino-acid proline and the quaternary-ammonium compound betaine in *A. maritima*. Jefferies *et al.* (1979) and Yeo (1983) proposed that the diversion of C and N to compatible solutes would have a detrimental effect of growth.

The primary objective of this thesis was to carry out a detailed investigation on *Plantago maritima* and *Armeria maritima* to assess responses of growth, photosynthesis and resource allocation to changes in resource availability, in particular to atmospheric CO_2. The contrasting metabolic responses to abiotic stress in *P. maritima* and *A. maritima* (C-accumulating versus N-accumulating) was hypothesised to cause contrasting responses to changes in resource availability. This final chapter summarises and integrates the findings that are discussed extensively in the preceding results chapters.

8.1 Compatible solute accumulation

Since Brown & Simpson (1972) first described the role of organic-solute accumulation in maintaining cellular water relations, it has been shown that many plants accumulate compatible solutes during drought. The results presented in chapter 4 show some level of 'compatible solute accumulation' in response to drought. However, as mentioned in chapter 4, although these species accumulate such compounds in high concentrations, the intensity of increase in concentration in response to drought is unlike that measured by Ahmed *et al.* (1979) where a 100 fold increase in sorbitol was measured in the roots of *P. maritima* exposed to high external concentrations of NaCl. This implies that the level of water deficit used in the experiment was not of a magnitude to cause a severe drought response in the plants. There is also recent evidence that compounds such as sorbitol are accumulated regardless of growth conditions and that the compound is used as high energy source instead of osmoregulation being its primary role (Walsh 2000). Other studies have shown that during a typical response to drought, concentrations of betaine are two- to ten-fold higher (Hanson *et al.* 1995), proline are 3- to 10-fold higher (Delauney & Verma 1993) and sorbitol are 2- to 10-fold higher (Popp and Smirnoff 1995). In the current study there was roughly a 2-fold increase in betaine, 7-fold increase in proline (root) and a 2-fold and a 7-fold increase in the shoot and root sorbitol. However, these were found to be dependent on the concentrations of C and N available to the plant (section 4.3). For example, in response to elevated CO_2, concentrations of betaine and proline in *A. maritima* increased when supplied with sufficient water and decreased when droughted. Sorbitol concentrations were also lower in droughted *P. maritima* when grown under elevated CO_2. However, the fact that gross changes in compatible solutes were not observed, it is highly likely that the changes observed in plants exposed to elevated CO_2 and N treatments, although statistically significant, are not of an order of magnitude to impose serious changes in the growth of the plant.

The only other known experiments on C-partitioning to sorbitol at elevated CO_2 showed increased sorbitol concentrations in the leaves of apple trees (*Malus domestica*) (Pan *et al.* 1998; Wang *et al.* 1999). However, one surprising result was that *P. maritima* accumulated less sorbitol when exposed to elevated atmospheric CO_2 (section 4.3.1), even though *P. maritima* and *Malus* have similar concentrations of sorbitol at ambient CO_2. The reduction of sorbitol in elevated CO_2 plants could be due to an increased rate

of sorbitol catabolism as C is needed elsewhere either for storage, such as starch, or growth (Escobar-Gutiérrez & Gaudillère 1997; Pan *et al.* 1998; Walsh 2000). Alternatively, it is possible that there are changes in the allocation of the precursor compound glucose-6-phosphate to sorbitol or sucrose. Aldose-6-phosphate reductase (A6PR; EC 1.1.1.200) is a key enzyme in sorbitol biosynthesis, whilst sucrose-phosphate synthase (SPS; EC 2.4.1.14) is involved in sucrose biosynthesis (Escobar-Gutiérrez *et al.* 1998). At elevated CO_2, coupled with drought, the A6PR enzyme rate could be reduced. It is possible then that the plant allocates more C into sucrose instead of sorbitol causing a shift in the activity of A6PR and SPS (Wang *et al.* 1996). A study of the activity of these enzymes in *P. maritima* grown at elevated CO_2 would answer this question.

Nitrogen-based compounds usually decrease in response to elevated CO_2 (Stitt & Krapp 1999). The decrease in the concentration of betaine and proline in *A. maritima* grown at a low N supply gives the impression that less N was being allocated to these compounds; however, the reverse was the actual situation as the percentage of N allocated to compatible solutes was increased (section 4.4). This was also shown in a similar study on N-based secondary compounds (cyanogenic glycosides) in *Eucalyptus cladocalyx* where an apparent decrease of cyanide and total N concentrations occurred in plants exposed to elevated CO_2. However, when calculated as a percentage of total tissue N, the plant had actually allocated more N to cyanide, not less as the basic data suggests (Gleadow *et al.* 1998). This highlights the importance of relating individual N-based compounds to the total N content of the plant and the importance of determining changes in individual metabolites.

As predicted, betaine and proline concentrations decreased in *A. maritima* grown in elevated CO_2 combined with drought (section 4.3.2); yet it is interesting that compatible solutes in both *A. maritima* and *P. maritima* showed similar responses to these conditions. As betaine, proline and sorbitol do not have the same biochemical pathways for biosynthesis, there has to be a more general factor responsible for this decrease. One possible explanation is that leaf transpiration (E) and stomatal conductance (g_s) are also reduced by elevated CO_2 (Bowes 1993; Wolfe *et al.* 1998; Wullschleger *et al.* 2002), which will be discussed below.

8.2 Plant transpiration, water-use-efficiency and water potential

There has been some discussion in the recent literature about the possibility that there would be less demand for compatible solute production in plants exposed to elevated CO_2 that also showed a reduction of E (Allen *et al.* 1998; Wall 2001; Wullschleger *et al.* 2002). A decrease in E and a related increased water-use-efficiency (*WUE*) is one of the most common plant responses to elevated CO_2. However, surprisingly few publications have investigated or even discussed how reductions in E at elevated CO_2 will affect the accumulation of known compatible solutes, or *vice versa*. In the current study, elevated CO_2 eventually decreased the rates of E and g_s in *P. maritima*, with E remaining higher for a longer period. In addition, rates of g_s and E in *A. maritima* were not affected by elevated CO_2 or drought (section 5.3). Therefore, the reductions of compatible solute concentrations in *P. maritima*, but not in *A. maritima*, could be due to reductions in leaf transpiration. Another possible consequence of a decrease in compatible solute accumulation caused by elevated CO_2, along with a possible reduction in g_s and E, is an increase (less negative) in plant water-potential (Ψ) (chapter 5). Studies such as Tognetti *et al.* 2000 b, Wall (2001), Wall *et al.* (2001) and Wullschleger *et al.* (2002) all detected higher Ψ in plants exposed to elevated CO_2. However, in *P. maritima* and *A. maritima*, Ψ was lowered in root and shoot tissue, respectively, in response to elevated CO_2. The lowering of plant tissue Ψ at elevated CO_2 could be due to an increase in cell expansion rates, which would cause 'cell-wall loosening' rather than a decrease in transpirational water loss or solute accumulation (Passioura 1982; Ferris & Taylor 1994). The risk of depleting soil water reserves in an already dry environment must be highlighted. If root Ψ is lowered by elevated CO_2, the Ψ gradient from the soil to the root tissue would be greater, allowing more water to be extracted from the soil and thereby depleting soil water at a quicker rate (Serraj & Sinclair 2002; Wullschleger *et al.* 2002). The detrimental effect of maintaining high rates of stomatal conductance, and hence soil water depletion, during drought and elevated CO_2 was highlighted in a study on beech trees (*Fagus sylvatica*; Heath & Kersteins 1997). The authors suggested that the cause of long-term damage in beech trees after periods of severe drought in the UK during the past few decades was a maintained E and quicker rates of soil water depletion during drought. This response was the same for plants grown at elevated CO_2. It is clear that our understanding of how elevated CO_2 affects Ψ is still poorly understood for many native, non-crop plant species. The fact that the plants did not show significant drought responses again

implies that either the level of drought was not sufficient to cause serious drought responses, or that as the plants are considered drought tolerant meant that they would not show the usual responses to drought until a much lower level of water availability when than many glycophytes. A glycophytic species, used as a control species, would have allowed the level of drought tolerance in these two species to be measured.

8.3 Growth and compatible solute partitioning

Jefferies *et al.* (1979) and Yeo (1983) proposed that the diversion of C and N to compatible solutes would have a detrimental effect on growth. Sheehy-Skeffington & Jeffrey (1988) have described how the biomass of *P. maritima* and *A. maritima* are lower under low N availability and salinity, especially in a combination of the two treatments. Compatible solutes increase in response to salinity in these species (Ahmed *et al.* 1979; Stewart *et al.* 1979) and although not measured in the study by Sheehy-Skeffington & Jeffrey (1988), it appears that the accumulation of such solutes did affect the biomass of these species, in particular, *A. maritima*. Although their study was on salt-marsh ecotypes, it provides a useful insight into the level of response to changes in resources that might be expected in cliff-top ecotypes of the species. Chapter 3, which investigated growth, and chapter 4, which investigated compatible solute accumulation, showed that *A. maritima* was more responsive than *P. maritima* in terms of growth and dry-matter partitioning to changes in C and N availability. The accumulation of compatible solutes was associated with decreased biomass of *A. maritima* but less so in *P. maritima*, particularly at ambient CO_2.

The biomass of *A. maritima* was not greatly affected by elevated CO_2 in the short-term (section 3.3), although decreases in specific leaf area (*SLA*) and leaf area ratio (*LAR*) indicated that the allocation of dry matter within the plant was affected. However, under well-watered conditions, after a period of one year, the biomasses of *A. maritima* and *P. maritima* were reduced and increased respectively in response to elevated CO_2 (section 6.3.1). As the two species in this study showed different responses to elevated CO_2 in terms of dry matter allocation and biomass production, this could influence interspecific competition in a possible future climate-change scenario (Leadley *et al.* 1999). This may be particularly important ecologically in coastal cliff-top habitats, where there is low species diversity (Goldsmith 1973 a & b; Rodwell 2000). Alterations in the growth response to elevated CO_2 may alter the species composition of such habitats (Davey *et al.* 1999). However, such a hypothesis should be taken with

caution as the study by Gray and Mogg (2001) showed that two coastal plants (salt-marsh *Puccinella maritima* and *Spartina anglica*) had different biomass responses to elevated CO_2 when grown together in pots, compared to grown alone in pots.

8.4 Models of resource allocation

The response to elevated CO_2 and reduced N availability to some extent fits literature models for predicting the allocation of C and N-based compounds (Bryant *et al.* 1983; Herms & Mattson 1992; Peñuelas & Estiarte 1998). The general basis of these models is that plants subjected to combined increased atmospheric CO_2 and low soil N availability accumulate C-based compounds and reduce concentrations of N-based compounds and growth as a whole. *Armeria maritima* actually increased its shoot biomass, and *SLA* and *LAR* were reduced in plants exposed to a combination of elevated CO_2 and low N availability (section 3.3). However, in *P. maritima*, when elevated CO_2 was coupled with low N supply the root biomass was reduced, compared to plants exposed to ambient CO_2. The allocation of C compounds is not compliant with predictions made within these models as in *P. maritima*, the sorbitol concentrations were not increased at elevated CO_2, even under low N availability, although starch concentrations did increase. Nitrogen-based compounds are also predicted to decrease in these models as N is used for other functions such as growth (Cotrufo *et al.* 1998; Gleadow *et al.* 1998; Peñuelas & Estiarte 1998). However, the reverse occurred in *A. maritima* because betaine and proline concentrations were generally higher in plants exposed to elevated CO_2 when well-watered but were lower when droughted. The non-structural carbohydrate concentrations were also lower in plants exposed to a combination of elevated CO_2 and low N availability, conflicting with the model predictions. The results of this study show that the blanket hypothesis that all C-based and N-based compounds are increased or decreased, respectively, in plants grown at elevated CO_2 should be approached with extreme caution. It is shown here that it is important to study individual compounds rather than assuming that all C and N compounds respond in a similar fashion (Stitt and Krapp 1999).

There has also been much discussion in the recent literature that these models such as the carbon-nutrient balance (CNB) (Bryant *et al.* 1983) and growth-differential balance (GDB) (Herms & Mattson 1992) modes are over-simplified. Although such models have provided a sound hypothesis for research, there is a need for them to be updated and to incorporate recent findings (Hamilton *et al.* 2001; Koricheva 2002; Lerdau 2002;

Nitao *et al.* 2002). Chapter 6 presented results using an updated model to assess the allocation of C and N compounds. The protein competition model of phenolic allocation (PCM, Jones & Hartley 1999) incorporates how plants allocate C and N to protein and phenolics, which is another major component of secondary metabolism. This enabled a clearer understanding of how the two species allocated C and N to these major C and N pools when exposed to ambient and elevated CO_2 and provided further evidence for a species-specific response to resource availability. In *P. maritima*, root phenolic concentrations increased and root and shoot soluble protein concentrations decreased at elevated CO_2 (section 6.3.2). However, in *A. maritima*, shoot phenolic concentrations were decreased and soluble protein concentrations increased, particularly in the roots, at elevated CO_2. Such an increase in total N, which implies increased protein concentration, was also observed in *A. maritima* exposed to elevated CO_2 in the 10-week study (section 3.4). It is also highlighted in chapter 6 that recent findings on the regulation of C and N by sensing and signalling mechanisms, such as hexokinase and nitrates, would have more of an effect on C and N portioning than any single point factor (ie. in the case of the PCM this would be the PAL enzyme).

Although the PCM model worked well in predicting the allocation of resources to total phenolic and protein compounds in *P. maritima*, the model does not indicate whether the increase in phenolic concentrations is due to an accumulation of all of the phenolic compounds or whether it is due to increases in only a few compounds. This study found that under elevated, as compared to ambient CO_2, there were increases in the concentrations of ferulic acid and verbascoside in the shoots after five weeks growth and caffeic acid in the shoot and *p*-coumaric acid in the roots after exposure for one year (section 7.6). There was a selective increase in the identified phenolic compounds in response to low N availability and drought, which is in agreement with results presented in Chapter 6 and with the CNB and PCM models that increased C availability increases the concentration of C compounds. This confirms recent hypotheses that models predicting the allocation of resources within plants at elevated CO_2 need to consider changes in the individual fluctuations of such metabolites as found in this study (Stitt & Krapp 1999; Hamilton *et al.* 2001).

One effect of modifying the accumulation of soluble phenolics would be on the associated activity of these compounds in protecting the plant from biotic stress (Levin 1976). Insect species, mainly from families Aphididae (eg. *Aphis longirostra*),

Noctuidae (eg. *Acronicta euphorbia*, Sweet Gale moth; *Eumichtis lichenea*, Feathered Ranunculus and *Polymixis xanthomista*), Thripidae (eg. *Thrips nigropilosus*) and Tortricidae (eg. *Eana penziana*) all feed on *Plantago maritima* and *Armeria maritima*, some exclusively (Ward & Spalding 1993). As insect herbivores are limited by dietary protein, a plant that has an increased soluble protein content and a reduction in soluble phenolics, as in the case of *A. maritima*, may well benefit feeding-insects by increasing their nutritional intake and reducing their intake of potentially harmful phenolics. This may cause the plant to be more susceptible to insect attack. However, in the studies reported here *P. maritima* had a lower soluble protein content and a higher soluble phenolic compound content in response to elevated CO_2. This is potentially harmful for the feeding insect due to a lower nutritional intake and in increased intake of phenolic compounds (Coviella & Trumble 1999). Ultimately, this may benefit *P. maritima* by reducing the plants susceptibility for insect attack (Roth & Lindroth 1995; Whittaker 2001) and more seriously may well affect the development, reproduction and mortality rate of herbivores feeding on the plant tissue (Lawler *et al.* 1997; Coviella *et al.* 2002). However, this benefit to the plant could be offset by the possibility that insects raised on plants at elevated CO_2 have been shown to increase tissue consumption to compensate for the low protein content as observed in Aspen (*Populus tremuloides*) (Lindroth *et al.* 1993; Coviella & Trumble 1999).

8.5 Plant ultrastructure and histochemistry

It has also been suggested that alterations in the growth, photosynthesis and phenolic metabolism of *P. maritima* might affect other phenolic compounds (Chapter 7). The level of lignification was studied and it was shown that the lignified vessel wall thicknesses were reduced in plants exposed to elevated CO_2. However, there were increases in leaf vessel and vein diameters and the number of lignified root vessels and stele width, implying an increased surface area for lignification. Significantly, the increase in stele width and xylem vessels may enable *P. maritima* to withstand periods of drought for a longer period of time and to be able to absorb more water (Atkinson & Taylor 1996) which may have some physiological advantage in plants on a cliff-top habitat. Thinner lignified walls may increase the risk of vessel collapse (Boudet 2000). However, the observation that there were more lignified fibres present in the roots of plants exposed to elevated CO_2 (section 7.9) suggest that the strength of the root might remain intact under elevated CO_2 conditions.

8.6 Photosynthetic acclimation to elevated CO_2

Contrasting levels of photosynthetic acclimation were also found between species. Measuring the A/ c_i response revealed that *P. maritima* showed some acclimation (reduced $V_{c,max}$) to elevated CO_2 whilst *A. maritima* did not. Interspecific differences in A/ c_i response curves have been collated from various earlier studies (Wullschleger 1993; Tuba *et al.* 1998; Castells *et al.* 2002). Contrasting responses in terms of acclimation to elevated CO_2 have also been observed in neighbouring grassland species (Tuba *et al.* 1998). Sage *et al.* (1989), who measured the A/ci responses of five plant species, also concluded that because different species have different levels of acclimation to elevated CO_2, their abilities to compete against other plant species may differ. Plants with a less economical acclamatory response may be at a disadvantage. In a previous study, Flanagan & Jefferies (1989a,b) measured the A/c_i response in *Plantago maritima* subjected to various external salt concentrations. Their measurements of $V_{c,max}$ and J_{max} were slightly higher (about 10 – 20 %) than values measured in this study, possibly because their plants were taken from a salt-marsh and the plants in the current study were from a cliff-top habitat. Subtle genetic differences within a species could alter the acclimation response, and therefore the metabolic response, to elevated CO_2.

8.7 Implications of the findings for future research

The different responses in growth, photosynthetic acclimation and compound accumulation to elevated CO_2 between *P. maritima* and *A. maritima* could lead to eventual shifts within their plant populations in future climate change scenarios (Leadley *et al.* 1999). A logical progression of the findings of this thesis would be an investigation on how these plants will survive and compete when grown together in mixed populations. A range of studies should be undertaken to determine the combined effects of elevated CO_2 and competition, ranging on growth, photosynthesis and primary and secondary metabolism as conducted in this study. The fact that these two plant species can dominate habitats that are frequently subjected to periods of drought is important and should be taken into account. If one species has an enhanced growth and biochemical response to an increased atmospheric CO_2 supply then it is possible that competition effects will cause a shift in the dynamics of such a narrow niche (Leadley *et al.* 1999; Gray and Mogg 2001). A variety of coastal plants that accumulate either C or N metabolites in response to adverse environmental conditions (as listed in table 1.1)

should be used to test the hypothesis that the response to elevated CO_2 is highly dependent on whether the plant is a C or a N accumulator (Gleadow *et al.* 1998). Few studies have addressed this and many studies concentrate on C compounds rather than N compounds. Therefore, models of C allocation should be updated to include possible interactions of N accumulation, which the protein-competition model (PCM) attempts to some extent. If there are trends in the type of plant response a plant has which is dependent on the main type of compound it accumulates, i.e. N or C-based, then this information could be used to enhance the C and N partitioning models already in the literature (eg. CNB/GDB/PCM). The models of C allocation to phenolics should also incorporate C and N sensing and signalling processes. This study also showed that the basic knowledge of what might happen in terms of altered water-use-efficiency in elevated CO_2 is still poorly studied. The basic hypotheses and logical assumptions need more rigorous testing, as it is clear that many current hypotheses are in error, ie. Increased WUE will lead to increased plant water potential (Wullschleger *et al.* 2002). This study has also highlighted the importance of investigating the fluctuations in concentration of individual compounds as well as measuring changes in the total pools of compounds such as non-structural carbohydrates or total N. There is also a need to study fluctuations in more individual compounds (Stitt & Krapp 1999). Taking an 'environmental metabolomics' approach would allow fluctuations in C and N resource allocation and regulation and source-sink interactions to be studied at a much finer and encompassing scale (Fiehn *et al.* 2000).

8.8 Conclusions

Overall, this thesis has contributed to our understanding of the effects of elevated CO_2 and resource partitioning on *Plantago maritima* and *Armeria maritima*. In particular, it highlights the fact that responses to elevated CO_2 are species specific, particularly when combined with soil water-deficit. The accumulation of betaine and proline in *A. maritima* was shown to be associated with poor growth, even when N availability was low or if the plants were droughted. The growth of *P. maritima* was less affected by the accumulation of sorbitol, even if C availability increased. These functions are not as closely related to stomatal and water potential functions as originally hypothesised. However, the levels of water deficit used in the experiments were likely to have been too low as many of the typical drought responses were not observed in the species. Therefore, any single measurement, which is significantly different, should be approached with caution.

The allocation of resources to other major pools of C and N within the plants was predicted using a model based on the level of photosynthetic acclimation and growth to elevated CO_2. This also revealed an opposing response between the species: *P. maritima* allocates resources into phenolic over protein biosynthesis, whilst *A. maritima* does the reverse. The increase of phenolic compounds was studied in detail, indicating a selective up-regulation of phenolic compounds and changes in the ultrastructure where lignin deposition within the plant was shown to be increased. Therefore, it appears that photosynthetic acclimation and growth limitations offset the potential benefits of increased atmospheric CO_2. Whether acclimation will ultimately benefit the plants in terms of increased protection by increases in phenolics in *P. maritima* and the ability to accumulate protein in *A. maritima* remains unanswered. Understandably, caution should be given when extrapolating relatively short-term responses to elevated CO_2 to possible longer-term ecophysiological consequences of climate change. However, any phytochemical and physiological alterations in *P. maritima* exposed to elevated CO_2, especially during the first year of growth, may allow the species some ecological advantage in establishment over neighbouring plants.

Appendix 1. Results of statistical tests for chapter 3

Appendix 1.1. Effects of resource availability on growth

Table 1.1.1. Results from a oneway ANOVA analyses to test for significant effects of **elevated CO_2** on growth parameters of *Plantago maritima* after five and ten weeks. All values have degrees of freedom of 1 between groups and degrees of freedom within groups are 21 or 22 for five week data and 19 for well-watered plants and 16 for droughted plants for ten week data. Significance level is set at $P \leq 0.05$.

Five weeks		Total dry weight		Shoot dry weight		Root dry weight		Root:Shoot ratio	
Growth conditions		*F*-value	*P*	*F*-value	*P*	*F*-value	*P*	*F*-value	*P*
Well-watered	High N	0.007	0.934	0.124	0.728	0.053	0.821	0.588	0.451
	Low N	5.349	0.030	1.565	0.225	6.391	0.019	2.647	0.119LN
Drought	High N	0.311	0.583	0.569	0.459	0.857	0.365	2.617	0.121LN
	Low N	0.263	0.613	0.090	0.767	1.211	0.283	3.698	0.068

Ten weeks		Total dry weight		Shoot dry weight		Root dry weight		Root:Shoot ratio	
Growth conditions		*F*-value	*P*	*F*-value	*P*	*F*-value	*P*	*F*-value	*P*
Well-watered	High N	1.310	0.267	0.079	0.782	1.910	0.183	1.708	0.207
	Low N	3.085	0.095	1.651	0.214	2.951	0.102	0.074	0.789
Drought	High N	0.069	0.796	0.015	0.905	0.083	0.777	0.359	0.558
	Low N	0.813	0.381	0.128	0.725	1.456	0.245	0.031	0.863

LN = data were natural log transformed.

Table 1.1.2. Results from a Oneway ANOVA analyses to test for significant effects of **nitrogen** availability on growth parameters of *Plantago maritima* after five and ten weeks. All values have degrees of freedom of 1 between groups and degrees of freedom within groups are 21 or 22 for five week data; 16 for ambient CO_2 plants and droughted plants under elevated CO_2 and 22 for well-watered plants under elevated CO_2 for ten week data. Significance level is set at $P \leq 0.05$.

Five weeks		Total dry weight		Shoot dry weight		Root dry weight		Root:Shoot ratio	
Growth conditions		*F*-value	*P*	*F*-value	*P*	*F*-value	*P*	*F*-value	*P*
Ambient CO_2	Well-watered	0.000	0.986	1.338	0260	0.248	0.624	7.157	0.014LN
	Drought	1.883	0.184	6.126	0.022	0.245	0.626	10.055	0.005LN
Elevated CO_2	Well-watered	8.396	0.008	10.189	0.004	5.729	0.026	0.543	0.469
	Drought	7.152	0.014	2.323	0.142	5.148	0.034	1.205	0.285

Ten weeks		Total dry weight		Shoot dry weight		Root dry weight		Root:Shoot ratio	
Growth conditions		*F*-value	*P*	*F*- value	*P*	*F*-value	*P*	*F*- value	*P*
Ambient CO_2	Well-watered	5.839	0.028	20.789	0.000	1.284	0.274	25.361	0.000LN
	Drought	6.048	0.026	8.146	0.011	2.743	0.117	0.106	0.749
Elevated CO_2	Well-watered	18.526	0.000	80.183	0.000	4.394	0.048	15.218	0.001LN
	Drought	11.624	0.004	16.058	0.001	7.748	0.013	0.506	0.487

LN = data were natural log transformed.

Table 1.1.3. Results from a Oneway ANOVA analyses to test for significant effects of **drought** on growth parameters of *Plantago maritima* after five and ten weeks. All values have degrees of freedom of 1 between groups and degrees of freedom within groups are 21 or 22 for five week data; 16 for ambient CO_2 plants and 19 for elevated CO_2 plants for ten week data. Significance level is set at $P \leq 0.05$.

Five weeks		Total dry weight		Shoot dry weight		Root dry weight		Root:Shoot ratio	
Growth conditions		*F*-value	*P*	*F*-value	*P*	*F*-value	*P*	*F*-value	*P*
Ambient CO_2	High N	0.009	0.924	0.322	0.576	0.131	0.720	1.644	0.213
	Low N	1.450	0.241	0.115	0.738	1.925	0.179	1.409	0.248
Elevated CO_2	High N	0.126	0.726	0.010	0.922	0.082	0.777	0.271	0.608
	Low N	1.192	0.287	3.113	0.092	0.256	0.618	2.141	0.158

Ten weeks		Total dry weight		Shoot dry weight		Root dry weight		Root:Shoot ratio	
Growth conditions		*F*-value	*P*	*F*-value	*P*	*F*-value	*P*	*F*-value	*P*
Ambient CO_2	High N	0.348	0.563	0.904	0.356	0.080	0.781	0.821	0.378
	Low N	0.531	0.477	0.003	0.958	0.826	0.377	5.471	0.034
Elevated CO_2	High N	0.000	1.000	2.010	0.172	0.239	0.631	2.239	0.151
	Low N	0.048	0.828	4.773	0.042	0.218	0.646	2.524	0.129

LN = data were natural log transformed.

Table 1.1.4. Results from a oneway ANOVA analyses to test for significant effects of **elevated CO_2** on growth parameters of *Armeria maritima* after five and ten weeks. All values have degrees of freedom of 1 between groups and degrees of freedom within groups are 21 or 22 for five week data and 18 or 19 for well-watered plants and 16 for droughted plants for ten week data. Significance level is set at $P \leq 0.05$.

Five weeks		Total dry weight		Shoot dry weight		Root dry weight		Root:Shoot ratio	
Growth conditions		*F*-value	*P*	*F*-value	*P*	*F*-value	*P*	*F*-value	*P*
Well-watered	High N	0.002	0.963	3.149	0.090	0.734	0.401	3.916	0.060
	Low N	0.001	0.970	3.843	0.063	1.308	0.265	9.471	0.006
Drought	High N	0.193	0.665	1.927	0.179	0.007	0.934	1.090	0.308
	Low N	0.223	0.642	0.242	0.628	0.135	0.717	0.073	0.789

Ten weeks		Total dry weight		Shoot dry weight		Root dry weight		Root:Shoot ratio	
Growth conditions		*F*-value	*P*	*F*-value	*P*	*F*-value	*P*	*F*-value	*P*
Well-watered	High N	0.471	0.501	2.759	0.113	0.035	0.854LN	1.933	0.180
	Low N	0.551	0.467	4.586	0.045	0.134	0.719	1.234	0.280
Drought	High N	0.144	0.709	0.225	0.642	0.437	0.518	0.789	0.388
	Low N	0.881	0.362	0.052	0.822LN	0.989	0.335	1.889	0.189

LN = data were natural log transformed.

Table 1.1.5. Results from a Oneway ANOVA analyses to test for significant effects of **nitrogen** availability on growth parameters of *Armeria maritima* after five and ten weeks. All values have degrees of freedom of 1 between groups and degrees of freedom within groups are 20 - 22 for five week data; 16 for ambient CO_2 plants and droughted plants under elevated CO_2 and 21 or 22 for well-watered plants under elevated CO_2 for ten week data. Significance level is set at $P \leq 0.05$.

Five weeks		Total dry weight		Shoot dry weight		Root dry weight		Root:Shoot ratio	
Growth conditions		*F*-value	*P*	*F*-value	*P*	*F*-value	*P*	*F*-value	*P*
Ambient CO_2	Well-watered	2.421	0.134	20.883	0.000	0.133	0.719	5.792	0.025
	Drought	3.204	0.087	11.887	0.002	0.627	0.437	2.988	0.098
Elevated CO_2	Well-watered	1.648	0.213	2.885	0.104	0.682	0.418	3.347	0.081
	Drought	4.400	0.048	4.340	0.050	2.101	0.162	0.041	0.841

Ten weeks		Total dry weight		Shoot dry weight		Root dry weight		Root:Shoot ratio	
Growth conditions		*F*-value	*P*	*F*-value	*P*	*F*-value	*P*	*F*-value	*P*
Ambient CO_2	Well-watered	19.634	0.000	57.215	0.000	6.153	0.025	23.653	0.000LN
	Drought	15.844	0.001	46.536	0.000	3.536	0.078	13.906	0.002
Elevated CO_2	Well-watered	11.597	0.003	51.917	0.000	2.678	0.116	14.808	0.001LN
	Drought	15.076	0.001	46.089	0.000LN	7.782	0.013	2.169	0.161

LN = data were natural log transformed.

Table 1.1.6. Results from a Oneway ANOVA analyses to test for significant effects of **drought** on growth parameters of *Armeria maritima* after five and ten weeks. All values have degrees of freedom of 1 between groups and degrees of freedom within groups are 21 or 22 for five week data; 16 for ambient CO_2 plants and 18 or 19 for elevated CO_2 plants for ten week data. Significance level is set at $P \leq 0.05$.

Five weeks		Total dry weight		Shoot dry weight		Root dry weight		Root:Shoot ratio	
Growth conditions		*F*-value	*P*	*F*-value	*P*	*F*-value	*P*	*F*-value	*P*
Ambient CO_2	High N	0.012	0.914	6.024	0.022	0.355	0.557	2.630	0.119
	Low N	0.117	0.735	3.616	0.070	1.203	0.285	7.559	0.012
Elevated CO_2	High N	0.130	0.722	1.437	0.243	0.061	0.808	1.480	0.237
	Low N	0.543	0.469	0.849	0.367	0.236	0.632	0.454	0.508

Ten weeks		Total dry weight		Shoot dry weight		Root dry weight		Root:Shoot ratio	
Growth conditions		*F*-value	*P*	*F*-value	*P*	*F*-value	*P*	*F*-value	*P*
Ambient CO_2	High N	4.354	0.053	0.121	0.733	7.288	0.016	5.450	0.033
	Low N	2.750	0.117	0.045	0.835	3.718	0.072	3.570	0.077
Elevated CO_2	High N	1.930	0.181	2.715	0.116	1.220	0.283	0.001	0.978
	Low N	11.356	0.003	8.896	0.008	9.537	0.006	3.092	0.096

LN = data were natural log transformed.

Table 1.1.7. Results from a Oneway ANOVA analyses to test for significant effects of **elevated CO_2** on water relations of *Plantago maritima* after five and ten weeks. All values have degrees of freedom of 1 between groups and degrees of freedom within groups are 21 or 22 for five week data and 19 for well-watered plants and 16 for droughted plants for ten week data. Significance level is set at $P \leq 0.05$.

Five weeks		Total water content		Shoot water content		Root water content	
Growth conditions		*F*-value	*P*	*F*-value	*P*	*F*-value	*P*
Well-watered	High N	1.870	0.185	0.451	0.509	3.240	0.086
	Low N	**22.482**	**0.000**	**5.970**	**0.023**	2.647	0.119
Drought	High N	0.616	0.441	**12.388**	**0.002**	3.343	0.082
	Low N	0.055	0.817	**8.577**	**0.008**	0.882	0.358

Ten weeks		Total water content		Shoot water content		Root water content	
Growth conditions		*F*-value	*P*	*F*-value	*P*	*F*-value	*P*
Well-watered	High N	0.084	0.775LN	**14.334**	**0.001**	0.874	0.362
	Low N	**11.147**	**0.003**	**46.154**	**0.000**	**6.823**	**0.017**
Drought	High N	0.014	0.907	0.638	0.436	0.067	0.799
	Low N	0.472	0.502	**5.419**	**0.033**	1.399	0.254

LN = data were natural log transformed.

Table 1.1.8. Results from a Oneway ANOVA analyses to test for significant effects of **nitrogen** availability on water relations of *Plantago maritima* after five and ten weeks. All values have degrees of freedom of 1 between groups and degrees of freedom within groups are 21 or 22 for five week data; 16 for ambient CO_2 plants and droughted plants under elevated CO_2 and 22 for well-watered plants under elevated CO_2 for ten week data. Significance level is set at $P \leq 0.05$.

Five weeks		Total water content		Shoot water content		Root water content	
Growth conditions		*F*- value	*P*	*F*-value	*P*	*F*-value	*P*
Ambient CO_2	Well-watered	**7.612**	**0.011**	**20.570**	**0.000**	3.332	0.082
	Drought	**12.953**	**0.002**	**31.236**	**0.000**	3.156	0.089
Elevated CO_2	Well-watered	0.133	0.718	3.858	0.063	0.002	0.967
	Drought	**8.986**	**0.007**	**18.117**	**0.000**	3.902	0.062

Ten weeks		Total water content		Shoot water content		Root water content	
Growth conditions		*F*-value	*P*	*F*- value	*P*	*F*-value	*P*
Ambient CO_2	Well-watered	**12.856**	**0.002**	**111.023**	**0.000**	3.502	0.080
	Drought	**5.462**	**0.033**	**6.030**	**0.026**	1.153	0.299
Elevated CO_2	Well-watered	**7.888**	**0.010**	**22.855**	**0.000**	3.181	0.088
	Drought	0.823	0.378	**11.856**	**0.003LN**	0.119	0.735

LN = data were natural log transformed.

Table 1.1.9. Results from a Oneway ANOVA analyses to test for significant effects of **drought** on water relations of *Plantago maritima* after five and ten weeks. All values have degrees of freedom of 1 between groups and degrees of freedom within groups are 21 or 22 for five week data; 16 for ambient CO_2 plants and 19 for elevated CO_2 plants for ten week data. Significance level is set at $P \leq 0.05$.

Five weeks		Total water content		Shoot water content		Root water content	
Growth conditions		*F*-value	*P*	*F*-value	*P*	*F*-value	*P*
Ambient CO_2	High N	0.110	0.743	1.757	0.199	0.233	0.634
	Low N	0.379	0.554	**5.122**	**0.034**	0.015	0.904
Elevated CO_2	High N	0.016	0.900	0.741	0.399	0.150	0.703
	Low N	**16.476**	**0.001**	**9.415**	**0.006**	**8.250**	**0.009**

Ten weeks		Total water content		Shoot water content		Root water content	
Growth conditions		*F*-value	*P*	*F*-value	*P*	*F*-value	*P*
Ambient CO_2	High N	1.977	0.179	1.704	0.210	1.462	0.244
	Low N	0.752	0.399	**7.980**	**0.012**	0.094	0.764
Elevated CO_2	High N	**4.756**	**0.042**	0.188	0.669	**6.153**	**0.023**
	Low N	1.350	0.260	**42.024**	**0.000**	0.200	0.660

LN = data were natural log transformed.

Table 1.1.10. Results from a Oneway ANOVA analyses to test for significant effects of **elevated CO_2** on water relations of *Armeria maritima* after five and ten weeks. All values have degrees of freedom of 1 between groups and degrees of freedom within groups are 21 or 22 for five week data and 18 or 19 for well-watered plants and 16 for droughted plants for ten week data. Significance level is set at $P \leq 0.05$.

Five weeks		Total water content		Shoot water content		Root water content	
Growth conditions		*F*-value	*P*	*F*-value	*P*	*F*-value	*P*
Well-watered	High N	3.632	0.070	2.097	0.162	**5.036**	**0.035**
	Low N	**6.846**	**0.016**	**69.916**	**0.000**	0.002	0.967
Drought	High N	1.954	0.176	0.116	0.736	1.374	0.254
	Low N	0.478	0.497LN	0.281	0.602	0.003	0.960

Ten weeks		Total water content		Shoot water content		Root water content	
Growth conditions		*F*-value	*P*	*F*- value	*P*	*F*-value	*P*
Well-watered	High N	0.914	0.351	0.450	0.511	1.424	0.247
	Low N	**10.555**	**0.004LN**	**165.488**	**0.000**	2.953	0.102
Drought	High N	**7.536**	**0.014**	3.014	0.102	**4.635**	**0.047**
	Low N	**5.261**	**0.036**	0.156	0.698	**7.251**	**0.016**

LN = data were natural log transformed.

Table 1.1.11. Results from a Oneway ANOVA analyses to test for significant effects of **nitrogen** availability on water relations of *Armeria maritima* after five and ten weeks. All values have degrees of freedom of 1 between groups and degrees of freedom within groups are 20 - 22 for five week data; 16 for ambient CO_2 plants and droughted plants under elevated CO_2 and 21 or 22 for well-watered plants under elevated CO_2 for ten week data. Significance level is set at $P \leq 0.05$.

Five weeks		Total water content		Shoot water content		Root water content	
Growth conditions		*F*-value	*P*	*F*- value	*P*	*F*-value	*P*
Ambient CO_2	Well-watered	**7.407**	**0.012**	**18.021**	**0.000**	3.934	0.060
	Drought	**11.998**	**0.002**	**13.174**	**0.001**	2.829	0.107
Elevated CO_2	Well-watered	**86.114**	**0.000**	**145.876**	**0.000**	**45.103**	**0.000**
	Drought	2.602	0.122	**29.450**	**0.000**	0.478	0.497

Ten weeks		Total water content		Shoot water content		Root water content	
Growth conditions		*F*-value	*P*	*F*- value	*P*	*F*- value	*P*
Ambient CO_2	Well-watered	**22.539**	**0.000**	**69.443**	**0.000**	**10.445**	**0.005**
	Drought	**37.069**	**0.000**	**22.585**	**0.000**	**13.100**	**0.002**
Elevated CO_2	Well-watered	**179.207**	**0.000LN**	**163.614**	**0.000LN**	**72.036**	**0.000**
	Drought	0.643	0.435	**62.859**	**0.000**	0.695	0.417

LN = data were natural log transformed.

Table 1.1.12. Results from a Oneway ANOVA analyses to test for significant effects of **drought** on water relations of *Armeria maritima* after five and ten weeks. All values have degrees of freedom of 1 between groups and degrees of freedom within groups are 21 or 22 for five week data; 16 for ambient CO_2 plants and 18 or 19 for elevated CO_2 plants for ten week data. Significance level is set at $P \leq 0.05$.

Five weeks		Total water content		Shoot water content		Root water content	
Growth conditions		*F*-value	*P*	*F*- value	*P*	*F*-value	*P*
Ambient CO_2	High N	0.067	0.798	**7.873**	**0.010**	0.940	0.343
	Low N	0.000	1.000	**15.928**	**0.001**	2.842	0.106
Elevated CO_2	High N	**21.680**	**0.000**	**16.867**	**0.000**	**13.169**	**0.001**
	Low N	3.210	0.088	**6.441**	**0.020**	0.237	0.631LN

Ten weeks		Total water content		Shoot water content		Root water content	
Growth conditions		*F*-value	*P*	*F*-value	*P*	*F*- value	*P*
Ambient CO_2	High N	0.061	0.808	**11.728**	**0.003**	0.226	0.641
	Low N	0.441	0.516	**7.832**	**0.013**	**5.904**	**0.027LN**
Elevated CO_2	High N	**19.075**	**0.000**	**4.755**	**0.042**	**14.049**	**0.001**
	Low N	**31.391**	**0.000LN**	**29.694**	**0.000**	**21.469**	**0.000**

LN = data were natural log transformed.

Table 1.1.13. Results from a Oneway ANOVA analyses to test for significant effects of **elevated CO_2** on leaf growth parameters of *Plantago maritima* after five and ten weeks. All values have degrees of freedom of 1 between groups and degrees of freedom within groups are 21 or 22 for five week data and 19 for well-watered plants and 16 for droughted plants for ten week data. Significance level is set at $P \leq 0.05$.

Five weeks		Leaf Area		*LAR*		*SLA*		*LWR*	
Growth conditions		*F*-value	*P*	*F*-value	*P*	*F*-value	*P*	*F*-value	*P*
Well-watered	High N	**4.334**	**0.049**	1.240	0.278	2.987	0.098LN	0.608	0.444
	Low N	3.347	0.081	0.716	0.407	0.695	0.414	2.389	0.137
Drought	High N	0.069	0.795	0.113	0.740	2.853	0.105	2.198	0.153
	Low N	0.134	0.718	0.233	0.634	4.059	0.057	4.244	0.052

Ten weeks		Leaf Area		*LAR*		*SLA*		*LWR*	
Growth conditions		*F*-value	*P*	*F*-value	*P*	*F*-value	*P*	*F*-value	*P*
Well-watered	High N	0.003	0.955	1.936	0.180	0.198	0.662	1.927	0.181
	Low N	0.179	0.677	3.688	0.070	**13.849**	**0.001**	0.670	0.423
Drought	High N	0.453	0.510	0.180	0.677LN	1.950	0.182	0.197	0.663
	Low N	0.232	0.637	1.393	0.255	0.105	0.750	1.143	0.301

LN = data were natural log transformed.

Table 1.1.14. Results from a Oneway ANOVA analyses to test for significant effects of **nitrogen** availability on leaf growth parameters of *Plantago maritima* after five and ten weeks. All values have degrees of freedom of 1 between groups and degrees of freedom within groups are 21 or 22 for five week data; 16 for ambient CO_2 plants and droughted plants under elevated CO_2 and 22 for well-watered plants under elevated CO_2 for ten week data. Significance level is set at $P \leq 0.05$.

Five weeks		Leaf Area		*LAR*		*SLA*		*LWR*	
Growth conditions		*F*-value	*P*	*F*-value	*P*	*F*-value	*P*	*F*-value	*P*
Ambient CO_2	Well-watered	**10.173**	**0.004**	**8.755**	**0.007**	2.94	0.593	**7.057**	**0.014**
	Drought	2.800	0.108	1.413	0.247	1.378	0.254	**10.095**	**0.005**
Elevated CO_2	Well-watered	**22.041**	**0.000**	1.628	0.215	1.124	0.301	0.787	0.385
	Drought	**18.177**	**0.000**	0.313	0.582	4.186	0.053LN	0.646	0.431

Ten weeks		Leaf Area		*LAR*		*SLA*		*LWR*	
Growth conditions		*F*- value	*P*	*F*-value	*P*	*F*- value	*P*	*F*-value	*P*
Ambient CO_2	Well-watered	**42.767**	**0.000**	**43.526**	**0.000**	**20.310**	**0.000LN**	**26.963**	**0.000**
	Drought	**18.231**	**0.001**	**6.289**	**0.023**	**6.188**	**0.024**	1.382	0.257
Elevated CO_2	Well-watered	**156.667**	**0.000LN**	**23.609**	**0.000**	**8.389**	**0.008**	**15.431**	**0.001**
	Drought	**20.102**	**0.000**	1.730	0.207	1.691	0.212	0.314	0.583

LN = data were natural log transformed.

Table 1.1.15. Results from a Oneway ANOVA analyses to test for significant effects of **drought** on leaf growth parameters of *Plantago maritima* after five and ten weeks. All values have degrees of freedom of 1 between groups and degrees of freedom within groups are 21 or 22 for five week data; 16 for ambient CO_2 plants and 19 for elevated CO_2 plants for ten week data. Significance level is set at $P \leq 0.05$.

Five weeks		Leaf Area		*LAR*		*SLA*		*LWR*	
Growth conditions		*F*-value	*P*	*F*-value	*P*	*F*-value	*P*	*F*-value	*P*
Ambient CO_2	High N	2.712	0.114	2.206	0.152	**7.651**	**0.011**	0.949	0.341
	Low N	0.398	0.534	0.120	0.732	0.644	0.431	1.015	0.325
Elevated CO_2	High N	0.003	0.954	0.572	0.458	0.215	0.647	0.001	0.975
	Low N	1.402	0.249	0.000	0.988	**4.828**	**0.039**	2.595	0.122LN

Ten weeks		Leaf Area		*LAR*		*SLA*		*LWR*	
Growth conditions		*F*-value	*P*	*F*-value	*P*	*F*-value	*P*	*F*-value	*P*
Ambient CO_2	High N	0.542	0.472	0.003	0.960	0.778	0.391	0.437	0.518
	Low N	0.320	0.579	2.929	0.106	**6.616**	**0.020**	1.116	0.307
Elevated CO_2	High N	**4.429**	**0.049**	**5.054**	**0.037**	1.696	0.208	1.941	0.180
	Low N	4.283	0.052	2.058	0.168	0.589	0.452	3.021	0.098

LN = data were natural log transformed.

Table 1.1.16. Results from a Oneway ANOVA analyses to test for significant effects of **elevated CO_2** on leaf growth parameters of *Armeria maritima* after five and ten weeks. All values have degrees of freedom of 1 between groups and degrees of freedom within groups are 21 or 22 for five week data and 18 or 19 for well-watered plants and 16 for droughted plants for ten week data. Significance level is set at $P \leq 0.05$.

Five weeks		Leaf Area		*LAR*		*SLA*		*LWR*	
Growth conditions		*F*-value	*P*	*F*-value	*P*	*F*-value	*P*	*F*-value	*P*
Well-watered	High N	0.596	0.448	0.340	0.566LN	0.062	0.806LN	3.079	0.093
	Low N	2.121	0.159	2.411	0.135LN	**32.670**	**0.000LN**	**15.328**	**0.001**
Drought	High N	**23.464**	**0.000**	**12.029**	**0.002**	**9.786**	**0.005**	0.340	0.566LN
	Low N	**13.221**	**0.002**	**7.971**	**0.010**	**8.118**	**0.010**	0.003	0.955

Ten weeks		Leaf Area		*LAR*		*SLA*		*LWR*	
Growth conditions		*F*-value	*P*	*F*-value	*P*	*F*-value	*P*	*F*- value	*P*
Well-watered	High N	0.052	0.822	0.107	0.748	**7.031**	**0.016**	2.612	0.123
	Low N	0.502	0.487	1.903	0.184	**53.527**	**0.000**	2.043	0.169
Drought	High N	1.199	0.290	1.164	0.297	0.673	0.424	0.629	0.439
	Low N	0.030	0.865	2.478	0.136	0.1582	0.228	0.1335	0.266

LN = data were natural log transformed.

Table 1.1.17. Results from a Oneway ANOVA analyses to test for significant effects of **nitrogen** availability on leaf growth parameters of *Armeria maritima* after five and ten weeks. All values have degrees of freedom of 1 between groups and degrees of freedom within groups are 20 - 22 for five week data; 16 for ambient CO_2 plants and droughted plants under elevated CO_2 and 21 or 22 for well-watered plants under elevated CO_2 for ten week data. Significance level is set at $P \leq 0.05$.

Five weeks		Leaf Area		*LAR*		*SLA*		*LWR*	
Growth conditions		*F*-value	*P*	*F*-value	*P*	*F*-value	*P*	*F*-value	*P*
Ambient CO_2	Well-watered	**8.668**	**0.008**	**4.331**	**0.049**	0.110	0.743	**7.778**	**0.011**
	Drought	**51.568**	**0.000**	**15.083**	**0.001**	4.028	0.057	3.339	0.081
Elevated CO_2	Well-watered	**24.904**	**0.000**	**51.347**	**0.000LN**	**47.712**	**0.000**	**4.651**	**0.042**
	Drought	**31.730**	**0.000**	**8.287**	**0.009**	**4.409**	**0.049**	1.157	0.295

Ten weeks		Leaf Area		*LAR*		*SLA*		*LWR*	
Growth conditions		*F*- value	*P*	*F*-value	*P*	*F*- value	*P*	*F*-value	*P*
Ambient CO_2	Well-watered	**128.255**	**0.000**	**52.487**	**0.000**	**15.466**	**0.001**	**24.497**	**0.000**
	Drought	**92.471**	**0.000**	**23.785**	**0.000**	**14.615**	**0.001**	**11.989**	**0.003**
Elevated CO_2	Well-watered	**88.345**	**0.000**	**30.775**	**0.000**	**35.610**	**0.000LN**	**15.150**	**0.001**
	Drought	**73.185**	**0.000LN**	3.767	0.071	2.099	0.168	1.899	0.188

LN = data were natural log transformed.

Table 1.1.18. Results from a Oneway ANOVA analyses to test for significant effects of **drought** on leaf growth parameters of *Armeria maritima* after five and ten weeks. All values have degrees of freedom of 1 between groups and degrees of freedom within groups are 21 or 22 for five week data; 16 for ambient CO_2 plants and 18 or 19 for elevated CO_2 plants for ten week data. Significance level is set at $P \leq 0.05$.

Five weeks		Leaf Area		*LAR*		*SLA*		*LWR*	
Growth conditions		*F*-value	*P*	*F*-value	*P*	*F*-value	*P*	*F*-value	*P*
Ambient CO_2	High N	2.630	0.119	1.085	0.309	0.682	0.418LN	2.611	0.120
	Low N	**0.973**	**0.012**	1.264	0.273	1.193	0.287	**8.912**	**0.007**
Elevated CO_2	High N	1.398	0.250	0.335	0.569	0.046	0.832LN	0.548	0.467
	Low N	0.037	0.849	1.371	0.255	4.276	0.051	0.030	0.864

Ten weeks		Leaf Area		*LAR*		*SLA*		*LWR*	
Growth conditions		*F*-value	*P*	*F*-value	*P*	*F*- value	*P*	*F*-value	*P*
Ambient CO_2	High N	2.222	0.156	0.822	0.378	**6.828**	**0.019**	**5.799**	**0.028**
	Low N	1.950	0.182	0.235	0.634	**20.544**	**0.000**	3.510	0.079
Elevated CO_2	High N	**4.633**	**0.045**	0.017	0.899	0.460	0.506	0.025	0.877
	Low N	1.688	0.209	**11.912**	**0.003**	**14.307**	**0.001**	2.890	0.106

LN = data were natural log transformed.

Appendix 1.2. Effects of resource availability on plant N concentrations

Table 1.2.1. Results from a Oneway ANOVA analyses to test for significant effects of **elevated CO_2** on N concentrations in *Plantago maritima* after five and ten weeks. All values have degrees of freedom of 1 between groups and degrees of freedom within groups are 9 or 10 for five week data and 10 for ten week data. Significance level is set at $P \leq 0.05$.

Five weeks		Total N		Shoot N		Root N	
Growth conditions		*F*-value	*P*	*F*-value	*P*	*F*-value	*P*
Well-watered	High N	0.935	0.359	0.016	0.901	4.055	0.075
	Low N	1.133	0.315	0.067	0.801	2.137	0.178
Drought	High N	**5.496**	**0.041**	0.300	0.596	**9.334**	**0.012**
	Low N	1.270	0.286	0.247	0.630	1.172	0.304

Ten weeks		Total N		Shoot N		Root N	
Growth conditions		*F*-value	*P*	*F*-value	*P*	*F*-value	*P*
Well-watered	High N	0.305	0.593	0.025	0.878	0.766	0.402LN
	Low N	2.356	0.156LN	0.327	0.580	**5.297**	**0.044LN**
Drought	High N	1.002	0.340	0.035	0.855	1.496	0.249
	Low N	0.414	0.534	1.989	0.189	0.020	0.890

LN = data were natural log transformed.

Table 1.2.2. Results from a Oneway ANOVA analyses to test for significant effects of **nitrogen** availability on N concentrations in *Plantago maritima* after five and ten weeks. All values have degrees of freedom of 1 between groups and degrees of freedom within groups are 7, 9 or 10 for five week data and 10 for ten week data. Significance level is set at $P \leq 0.05$.

Five weeks		Total N		Shoot N		Root N	
Growth conditions		*F*-value	*P*	*F*-value	*P*	*F*-value	*P*
Ambient CO_2	Well-watered	**6.012**	**0.034**	**12.107**	**0.006LN**	**10.772**	**0.009**
	Drought	**13.047**	**0.005LN**	3.538	0.089	**6.990**	**0.025**
Elevated CO_2	Well-watered	**7.668**	**0.028**	**8.174**	**0.017LN**	**5.328**	**0.050**
	Drought	3.629	0.086	**9.251**	**0.012**	0.588	0.461

Ten weeks		Total N		Shoot N		Root N	
Growth conditions		*F*-value	*P*	*F*-value	*P*	*F*-value	*P*
Ambient CO_2	Well-watered	4.752	0.054	0.435	0.524	1.895	0.199
	Drought	0.617	0.451	**15.069**	**0.003**	0.257	0.623
Elevated CO_2	Well-watered	2.613	0.137	2.184	0.170	**6.455**	**0.029**
	Drought	0.652	0.438	3.381	0.096	0.055	0.820

LN = data were natural log transformed.

Table 1.2.3. Results from a Oneway ANOVA analyses to test for significant effects of **drought** on N concentrations in *Plantago maritima* after five and ten weeks. All values have degrees of freedom of 1 between groups and degrees of freedom within groups are 8, 9 or 10 for five week data and 10 for ten week data. Significance level is set at $P \leq 0.05$.

Five weeks		Total N		Shoot N		Root N	
Growth conditions		*F*-value	*P*	*F*-value	*P*	*F*-value	*P*
Ambient CO_2	High N	0.066	0.802	0.701	0.422	0.106	0.752
	Low N	0.287	0.604	1.235	0.293	0.012	0.913
Elevated CO_2	High N	2.296	0.168	0.858	0.376LN	2.718	0.134
	Low N	0.012	0.914	0.047	0.832	0.002	0.968

Ten weeks		Total N		Shoot N		Root N	
Growth conditions		*F*-value	*P*	*F*-value	*P*	*F*-value	*P*
Ambient CO_2	High N	0.014	0.908	0.025	0.878	0.003	0.958
	Low N	0.087	0.774	**5.766**	**0.037**	0.990	0.343
Elevated CO_2	High N	1.556	0.241	0.035	0.855	2.939	0.117
	Low N	1.651	0.228	0.048	0.830	1.665	0.226

LN = data were natural log transformed.

Table 1.2.4. Results from a Oneway ANOVA analyses to test for significant effects of **elevated CO_2** on N concentrations in *Armeria maritima* after five and ten weeks. All values have degrees of freedom of 1 between groups and degrees of freedom within groups are 8 - 10 for five and ten week data. Significance level is set at $P \leq 0.05$.

Five weeks		Total N		Shoot N		Root N	
Growth conditions		*F*-value	*P*	*F*-value	*P*	*F*-value	*P*
Well-watered	High N	2.825	0.127	2.407	0.152LN	0.006	0.941
	Low N	0.978	0.349	0.318	0.585	0.447	0.520
Drought	High N	0.593	0.459	1.041	0.332	0.358	0.563
	Low N	0.757	0.405	1.110	0.317	0.513	0.490

Ten weeks		Total N		Shoot N		Root N	
Growth conditions		*F*-value	*P*	*F*-value	*P*	*F*-value	*P*
Well-watered	High N	0.035	0.857	2.112	0.177	0.930	0.360
	Low N	0.237	0.637	2.641	0.135	1.245	0.245
Drought	High N	1.044	0.331	**16.518**	**0.002LN**	**8.477**	**0.016**
	Low N	1.690	0.223	0.546	0.477	0.997	0.344

LN = data were natural log transformed.

Table 1.2.5. Results from a Oneway ANOVA analyses to test for significant effects of **nitrogen** availability on N concentrations in *Armeria maritima* after five and ten weeks. All values have degrees of freedom of 1 between groups and degrees of freedom within groups are 8 - 10 for five week data and 7 - 10 for ten week data. Significance level is set at $P \leq 0.05$.

Five weeks		Total N		Shoot N		Root N	
Growth conditions		*F*-value	*P*	*F*-value	*P*	*F*-value	*P*
Ambient CO_2	Well-watered	**14.260**	**0.004**	**28.537**	**0.000**	**6.147**	**0.033**
	Drought	**10.708**	**0.008**	**22.712**	**0.001**	3.589	0.087
Elevated CO_2	Well-watered	**7.183**	**0.028**	**17.209**	**0.002LN**	0.388	0.549
	Drought	**8.479**	**0.016**	**7.099**	**0.024**	4.046	0.072

Ten weeks		Total N		Shoot N		Root N	
Growth conditions		*F*-value	*P*	*F*-value	*P*	*F*- value	*P*
Ambient CO_2	Well-watered	4.120	0.070	**6.473**	**0.029**	0.988	0.344
	Drought	**13.803**	**0.004**	0.153	0.703	**24.114**	**0.001LN**
Elevated CO_2	Well-watered	**8.201**	**0.021**	0.235	0.638	3.361	0.100
	Drought	**8.876**	**0.014**	**10.849**	**0.008**	2.829	0.127

LN = data were natural log transformed.

Table 1.2.6. Results from a Oneway ANOVA analyses to test for significant effects of **drought** on N concentrations of *Armeria maritima* after five and ten weeks. All values have degrees of freedom of 1 between groups and degrees of freedom within groups are 8 - 10 for five week data and 7 - 10 for ten week data. Significance level is set at $P \leq 0.05$.

Five weeks		Total N		Shoot N		Root N	
Growth conditions		*F*-value	*P*	*F*-value	*P*	*F*-value	*P*
Ambient CO_2	High N	0.234	0.639	0.012	0.913	0.027	0.872
	Low N	0.109	0.748	0.001	0.971	0.230	0.642
Elevated CO_2	High N	0.024	0.880	0.593	0.459LN	0.574	0.466
	Low N	0.147	0.711	0.251	0.627	0.199	0.666

Ten weeks		Total N		Shoot N		Root N	
Growth conditions		*F*-value	*P*	*F*-value	*P*	*F*-value	*P*
Ambient CO_2	High N	**7.981**	**0.018**	0.353	0.566	**12.403**	**0.006LN**
	Low N	2.303	0.160	3.796	0.080LN	0.003	0.960
Elevated CO_2	High N	3.261	0.109LN	**5.717**	**0.038**	1.328	0.279
	Low N	0.250	0.628	0.549	0.476	0.027	0.872

LN = data were natural log transformed.

Appendix 1.3. Effects of resource availability on photosynthetic pigments and capacity

Table 1.3.1. Results from a Oneway ANOVA analyses to test for significant effects of **elevated CO_2** on photosynthetic pigments and capacity in *Plantago maritima* after five and ten weeks. All values have degrees of freedom of 1 between groups and degrees of freedom within groups are 8, 9 or 10 for five week data and 7-10 for ten week data. Significance level is set at $P \leq 0.05$.

Five weeks		Carotenoid		Total chlorophyll		Photosynthetic capacity	
Growth conditions		*F*-value	*P*	*F*-value	*P*	*F*- value	*P*
Well-watered	High N	**6.347**	**0.030**	**8.293**	**0.016**	**7.938**	**0.018LN**
	Low N	0.209	0.658	1.950	0.193LN	2.252	0.168
Drought	High N	1.434	0.262	1.001	0.343LN	**10.460**	**0.012LN**
	Low N	**25.787**	**0.000LN**	1.241	0.291	0.009	0.925

Ten weeks		Carotenoid		Total chlorophyll		Photosynthetic capacity	
Growth conditions		*F*-value	*P*	*F*-value	*P*	*F*- value	*P*
Well-watered	High N	3.235	0.106	1.249	0.290	0.108	0.749
	Low N	0.241	0.637	1.949	0.193	0.013	0.913
Drought	High N	1.353	0.275	1.624	0.231	1.103	0.321
	Low N	0.321	0.589	0.246	0.631	3.446	0.096

LN = data were natural log transformed.

Table 1.3.2. Results from a Oneway ANOVA analyses to test for significant effects of **nitrogen** availability on photosynthetic pigments and capacity in *Plantago maritima* after five and ten weeks. All values have degrees of freedom of 1 between groups and degrees of freedom within groups are 8, 9 or 10 for five week data and 7-10 for ten week data. Significance level is set at $P \leq 0.05$.

Five weeks		Carotenoid		Total chlorophyll		Photosynthetic capacity	
Growth conditions		*F*-value	*P*	*F*-value	*P*	*F*- value	*P*
Ambient CO_2	Well-watered	**30.009**	**0.000**	**7.526**	**0.021**	2.153	0.176LN
	Drought	**5.027**	**0.049**	2.202	0.169LN	2.666	0.137LN
Elevated CO_2	Well-watered	**5.322**	**0.044**	2.413	0.151	1.052	0.329
	Drought	3.851	0.081	4.658	0.059	1.101	0.325

Ten weeks		Carotenoid		Total chlorophyll		Photosynthetic capacity	
Growth conditions		*F*-value	*P*	*F*-value	*P*	*F*- value	*P*
Ambient CO_2	Well-watered	4.488	0.072	3.189	0.104LN	0.111	0.746
	Drought	2.981	0.123	0.057	0.816	**5.250**	**0.045**
Elevated CO_2	Well-watered	0.032	0.862	2.776	0.127	0.007	0.934
	Drought	0.037	0.853	0.000	0.992	0.720	0.421

LN = data were natural log transformed.

Table 1.3.3. Results from a Oneway ANOVA analyses to test for significant effects of **drought** on photosynthetic pigments ad capacity in *Plantago maritima* after five and ten weeks. All values have degrees of freedom of 1 between groups and degrees of freedom within groups are 8, 9 or 10 for five week data and 7-10 for ten week data. Significance level is set at $P \leq 0.05$.

Five weeks		Carotenoid		Total chlorophyll		Photosynthetic capacity	
Growth conditions		*F*-value	*P*	*F*-value	*P*	*F*- value	*P*
Ambient CO_2	High N	0.002	0.968	0.142	0.714LN	0.054	0.821LN
	Low N	**90.695**	**0.000**	0.004	0.949	0.086	0.775
Elevated CO_2	High N	0.048	0.830	1.354	0.272	0.004	0.948
	Low N	0.095	0.764	0.08	0.591	2.418	0.154

Ten weeks		Carotenoid		Total chlorophyll		Photosynthetic capacity	
Growth conditions		*F*-value	*P*	*F*-value	*P*	*F*- value	*P*
Ambient CO_2	High N	0.015	0.906	1.967	0.191	1.089	0.321
	Low N	2.414	0.164	2.525	0.143	0.009	0.927
Elevated CO_2	High N	0.103	0.754	0.962	0.350	0.038	0.851
	Low N	0.005	0.945	0.489	0.500	0.945	0.359

LN = data were natural log transformed.

Table 1.3.4. Results from a Oneway ANOVA analyses to test for significant effects of **elevated CO_2** on photosynthetic pigments and capacity in *Armeria maritima* after five and ten weeks. All values have degrees of freedom of 1 between groups and degrees of freedom within groups are 7, 9 or 10 for five week data and 5 - 7 for ten week data. Significance level is set at $P \leq 0.05$.

Five weeks		Carotenoid		Total chlorophyll		Photosynthetic capacity	
Growth conditions		*F*-value	*P*	*F*-value	*P*	*F*- value	*P*
Well-watered	High N	0.356	0.564	**12.365**	**0.006**	**16.368**	**0.002**
	Low N	0.635	0.446	**81.465**	**0.000**	0.630	0.446
Drought	High N	0.325	0.582	2.768	0.131LN	0.046	0.834
	Low N	0.327	0.585	0.022	0.886LN	2.843	0.126LN

Ten weeks		Carotenoid		Total chlorophyll		Photosynthetic capacity	
Growth conditions		*F*-value	*P*	*F*-value	*P*	*F*- value	*P*
Well-watered	High N	**7.037**	**0.033**	0.262	0.625	**13.959**	**0.007**
	Low N	3.157	0.119LN	**12.683**	**0.009LN**	2.175	0.184
Drought	High N	1.323	0.294	1.549	0.253	0.000	0.991
	Low N	**6.680**	**0.049**	0.016	0.902	**21.291**	**0.002LN**

LN = data were natural log transformed.

Table 1.3.5. Results from a Oneway ANOVA analyses to test for significant effects of **nitrogen** availability on photosynthetic pigments and capacity in *Armeria maritima* after five and ten weeks. All values have degrees of freedom of 1 between groups and degrees of freedom within groups are 8 - 10 for five week data and 3, 4, 9, 10 or 11 for ten week data. Significance level is set at $P \leq 0.05$.

Five weeks		Carotenoid		Total chlorophyll		Photosynthetic capacity	
Growth conditions		*F*-value	*P*	*F*- value	*P*	*F*- value	*P*
Ambient CO_2	Well-watered	0.555	0.474	**15.653**	**0.003**	**9.854**	**0.011**
	Drought	**16.830**	**0.003**	**12.105**	**0.007**	**6.471**	**0.032**
Elevated CO_2	Well-watered	**12.659**	**0.006LN**	**207.149**	**0.000**	0.492	0.499
	Drought	2.511	0.152	4.290	0.068	0.266	0.618

Ten weeks		Carotenoid		Total chlorophyll		Photosynthetic capacity	
Growth conditions		*F*-value	*P*	*F*-value	*P*	*F*- value	*P*
Ambient CO_2	Well-watered	0.082	0.789	0.568	0.493	1.420	0.299
	Drought	0.022	0.891	3.484	0.135	1.088	0.374
Elevated CO_2	Well-watered	**5.837**	**0.036**	**13.013**	**0.005LN**	0.199	0.665
	Drought	0.007	0.937LN	0.129	0.727	**6.990**	**0.027LN**

LN = data were natural log transformed.

Table 1.3.6. Results from a Oneway ANOVA analyses to test for significant effects of **drought** on photosynthetic pigments and capacity in *Armeria maritima* after five and ten weeks. All values have degrees of freedom of 1 between groups and degrees of freedom within groups are 7, 9 or 10 for five week data and 3, 4, 8, 9or 10 for ten week data. Significance level is set at $P \leq 0.05$.

Five weeks		Carotenoid		Total chlorophyll		Photosynthetic capacity	
Growth conditions		*F*-value	*P*	*F*-value	*P*	*F*- value	*P*
Ambient CO_2	High N	0.089	0.772	**8.805**	**0.016**	**11.345**	**0.008**
	Low N	1.964	0.195	1.940	0.194	**5.744**	**0.038LN**
Elevated CO_2	High N	0.900	0.365	**5.677**	**0.038LN**	0.162	0.696
	Low N	0.334	0.581LN	**13.344**	**0.005LN**	0.162	0.696

Ten weeks		Carotenoid		Total chlorophyll		Photosynthetic capacity	
Growth conditions		*F*-value	*P*	*F*-value	*P*	*F*- value	*P*
Ambient CO_2	High N	6.455	0.085	1.231	0.329	7.213	0.075
	Low N	1.564	0.279	1.514	0.286	0.625	0.474
Elevated CO_2	High N	0.203	0.662	0.749	0.407	0.537	0.482
	Low N	5.139	0.053	1.270	0.286LN	4.044	0.072LN

LN = data were natural log transformed.

Appendix 2. Results of statistical tests for chapter 4

Appendix 2.1 Effects of resource availability on metabolite concentrations

Table 2.1.1. Results from a oneway ANOVA analyses to test for significant effects of **elevated CO_2** on the metabolites in the shoots of *Plantago maritima* after five and ten weeks. All values have degrees of freedom of 1 between groups and degrees of freedom within groups are either 4, 5 or 6. Significance level is set at $P \leq 0.05$.

Five weeks		Sorbitol		Proline		Soluble carbohydrate		Starch	
Growth conditions		*F*-value	*P*	*F*-value	*P*	*F*-value	*P*	*F*-value	*P*
Well-watered	High N	0.274	0.623	0.794	0.423	0.003	0.958	3.308	0.129
	Low N	0.362	0.569	1.203	0.315	0.751	0.419	4.369	0.082
Drought	High N	4.935	0.068LN	4.735	0.095	0.926	0.380	**97.590**	**0.010**
	Low N	1.163	0.332	0.169	0.708	0.510	0.502	1.887	0.228

Ten weeks		Sorbitol		Proline		Soluble carbohydrate		Starch	
Growth conditions		*F*-value	*P*	*F*-value	*P*	*F*-value	*P*	*F*-value	*P*
Well-watered	High N	0.029	0.871	5.410	0.081	0.697	0.436	0.138	0.723
	Low N	0.018	0.898	3.860	0.097	1.328	0.293	**31.961**	**0.002**
Drought	High N	0.413	0.544	0.535	0.497	0.001	0.977	5.047	0.066
	Low N	**16.226**	**0.007**	0.318	0.597	2.024	0.205	0.929	0.372

LN = data were natural log transformed.

Table 2.1.2. Results from a Oneway ANOVA analyses to test for significant effects of **nitrogen** availability on the metabolites in the shoots of *Plantago maritima* after five and ten weeks. All values have degrees of freedom of 1 between groups and degrees of freedom within groups are either 4, 5 or 6. Significance level is set at $P \leq 0.05$.

Five weeks		Sorbitol		Proline		Soluble carbohydrate		Starch	
Growth conditions		*F*-value	*P*	*F*-value	*P*	*F*-value	*P*	*F*-value	*P*
Ambient CO_2	Well-watered	0.004	0.952	1.299	0.298	**14.044**	**0.010**	1.360	0.288
	Drought	0.022	0.888	0.547	0.500	0.314	0.595	1.788	0.274
Elevated CO_2	Well-watered	0.007	0.936	3.983	0.117	**15.786**	**0.011**	2.469	0.177
	Drought	3.168	0.125	2.502	0.212	0.975	0.369	2.205	0.212

Ten weeks		Sorbitol		Proline		Soluble carbohydrate		Starch	
Growth conditions		*F*-value	*P*	*F*- value	*P*	*F*- value	*P*	*F*-value	*P*
Ambient CO_2	Well-watered	0.170	0.694	0.202	0.672	0.123	0.738	0.102	0.760
	Drought	1.343	0.291	0.041	0.847	2.206	0.188	**6.327**	**0.046**
Elevated CO_2	Well-watered	0.437	0.533	3.536	0.119	2.720	0.150	**14.320**	**0.013**
	Drought	0.859	0.390	0.295	0.610	0.118	0.743	1.768	0.232

LN = data were natural log transformed.

Table 2.1.3. Results from a Oneway ANOVA analyses to test for significant effects of **drought** on the metabolites in the shoots of *Plantago maritima* after five and ten weeks. All values have degrees of freedom of 1 between groups and degrees of freedom within groups are either 4, 5 or 6. Significance level is set at $P \leq 0.05$.

Five weeks		Sorbitol		Proline		Soluble carbohydrate		Starch	
Growth conditions		*F*-value	*P*	*F*-value	*P*	*F*-value	*P*	*F*-value	*P*
Ambient CO_2	High N	**7.617**	**0.033**	1.202	0.323	2.368	0.175	**12.632**	**0.024**
	Low N	3.203	0.124	1.975	0.219	0.001	0.972	0.303	0.605
Elevated CO_2	High N	0.503	0.510	3.461	0.160	0.491	0.522	3.333	0.487
	Low N	0.141	0.721	2.362	0.199	0.225	0.652	0.005	0.948

Ten weeks		Sorbitol		Proline		Soluble carbohydrate		Starch	
Growth conditions		*F*-value	*P*	*F*-value	*P*	*F*-value	*P*	*F*-value	*P*
Ambient CO_2	High N	0.001	0.971	0.050	0.834	0.764	0.416	1.814	0.227
	Low N	2.601	0.158	0.374	0.563	1.092	0.336	2.850	0.142
Elevated CO_2	High N	0.448	0.528	1.583	0.264	0.164	0.700	2.978	0.135
	Low N	1.953	0.212	0.261	0.631	2.427	0.170	3.526	0.119

LN = data were natural log transformed.

Table 2.1.4. Results from a oneway ANOVA analyses to test for significant effects of **elevated CO_2** on the metabolites in the roots of *Plantago maritima* after five and ten weeks. All values have degrees of freedom of 1 between groups and degrees of freedom within groups are either 4, 5 or 6. Significance level is set at $P \leq 0.05$.

Five weeks		Sorbitol		Proline		Soluble carbohydrate		Starch	
Growth conditions		*F*-value	*P*	*F*-value	*P*	*F*-value	*P*	*F*-value	*P*
Well-watered	High N	0.224	0.186LN	0.107	0.757	0.779	0.418	**3.360**	**0.045**
	Low N	0.229	0.650LN	3.553	0.108	**7.275**	**0.036**	4.656	0.074
Drought	High N	0.000	0.996	1.970	0.219	0.187	0.680	6.474	0.052
	Low N	5.551	0.057	**13.818**	**0.014**	0.640	0.454	**13.066**	**0.011**

Ten weeks		Sorbitol		Proline		Soluble carbohydrate		Starch	
Growth conditions		*F*-value	*P*	*F*-value	*P*	*F*-value	*P*	*F*-value	*P*
Well-watered	High N	0.001	0.971	1.429	0.286	0.084	0.782	3.409	0.124
	Low N	0.506	0.509	1.000	0.356	4.618	0.084	**8.613**	**0.026**
Drought	High N	0.465	0.521	4.910	0.078	0.064	0.809	0.576	0.477
	Low N	4.247	0.094	2.870	0.151	1.240	0.308	0.220	0.656

LN = data were natural log transformed.

Table 2.1.5. Results from a Oneway ANOVA analyses to test for significant effects of **nitrogen** availability on the metabolites in the roots of *Plantago maritima* after five and ten weeks. All values have degrees of freedom of 1 between groups and degrees of freedom within groups are either 4, 5 or 6. Significance level is set at $P \leq 0.05$. (/ = analysis not performed).

Five weeks		Sorbitol		Proline		Soluble carbohydrate		Starch	
Growth conditions		*F*-value	*P*	*F*-value	*P*	*F*-value	*P*	*F*-value	*P*
Ambient CO_2	Well-watered	**17.735**	**0.06LN**	2.637	0.156	0.287	0.611	4.904	0.069
	Drought	0.389	0.556	4.808	0.080	1.277	0.302	0.656	0.449
Elevated CO_2	Well-watered	4.568	0.076	**11.398**	**0.020**	**25.792**	**0.004**	0.022	0.886
	Drought	4.247	0.085	0.494	0.513	1.174	0.320	0.016	0.906

Ten weeks		Sorbitol		Proline		Soluble carbohydrate		Starch	
Growth conditions		*F*-value	*P*	*F*- value	*P*	*F*-value	*P*	*F*-value	*P*
Ambient CO_2	Well-watered	0.389	0.556	/	/	0.146	0.716	0.002	0.968
	Drought	0.162	0.704	1.389	0.292	3.730	0.102	1.075	0.340
Elevated CO_2	Well-watered	0.640	0.460	0.000	0.983	**12.861**	**0.016**	0.142	0.719
	Drought	5.429	0.059	0.010	0.925	0.203	0.668	0.074	0.795

LN = data were natural log transformed.

Table 2.1.6. Results from a Oneway ANOVA analyses to test for significant effects of **drought** on the metabolites in the roots of *Plantago maritima* after five and ten weeks. All values have degrees of freedom of 1 between groups and degrees of freedom within groups are either 4, 5 or 6. Significance level is set at $P \leq 0.05$.

Five weeks		Sorbitol		Proline		Soluble carbohydrate		Starch	
Growth conditions		*F*-value	*P*	*F*-value	*P*	*F*-value	*P*	*F*-value	*P*
Ambient CO_2	High N	4.599	0.076	5.312	0.069	2.368	0.175	**6.396**	**0.045**
	Low N	0.072	0.798	2.782	0.146	0.022	0.888	5.583	0.056LN
Elevated CO_2	High N	1.427	0.277LN	0.616	0.468	0.563	0.487	5.085	0.074
	Low N	**16.554**	**0.007LN**	1.316	0.303	0.749	0.420	**12.937**	**0.011**

Ten weeks		Sorbitol		Proline		Soluble carbohydrate		Starch	
Growth conditions		*F*-value	*P*	*F*-value	*P*	*F*-value	*P*	*F*-value	*P*
Ambient CO_2	High N	1.472	0.271LN	**14.730**	**0.012**	3.117	0.128	1.946	0.222
	Low N	**16.661**	**0.010**	**9.282**	**0.023**	0.019	0.896	2.236	0.185
Elevated CO_2	High N	2.848	0.142	0.158	0.708	0.623	0.460	1.0431	0.277
	Low N	1.450	0.282	0.254	0.636	**16.381**	**0.010**	3.817	0.099

LN = data were natural log transformed.

Table 2.1.7. Results from a oneway ANOVA analyses to test for significant effects of **elevated CO_2** on the metabolites in the shoots of *Armeria maritima* after five and ten weeks. All values have degrees of freedom of 1 between groups and degrees of freedom within groups are either 4, 5 or 6. Significance level is set at $P \leq 0.05$.

Five weeks		Betaine		Proline		Soluble carbohydrate		Starch	
Growth conditions		*F*-value	*P*	*F*-value	*P*	*F*-value	*P*	*F*-value	*P*
Well-watered	High N	6.717	0.049	6.427	0.044 LN	7.673	0.039	56.646	0.001
	Low N	0.479	0.515	0.380	0.560	0..360	0.857	0.188	0.687
Drought	High N	10.726	0.022	0.840	0.401	0.871	0.394	0.452	0.526
	Low N	4.243	0.094	0.489	0.516	0.548	0.492	0.044	0.842

Ten weeks		Betaine		Proline		Soluble carbohydrate		Starch	
Growth conditions		*F*-value	*P*	*F*-value	*P*	*F*-value	*P*	*F*-value	*P*
Well-watered	High N	6.713	0.049	0.006	0.940	0.033	0.861	0.422	0.540
	Low N	1.193	0.317	3.905	0.096	0.287	0.611	6.471	0.044
Drought	High N	0.227	0.659	0.675	0.449	4.409	0.081	0.272	0.621
	Low N	3.145	0.136	1.027	0.350	2.430	0.170	1.838	0.247

LN = data were natural log transformed.

Table 2.1.8. Results from a Oneway ANOVA analyses to test for significant effects of **nitrogen** availability on the metabolites in the shoots of *Armeria maritima* after five and ten weeks. All values have degrees of freedom of 1 between groups and degrees of freedom within groups are either 4, 5 or 6. Significance level is set at $P \leq 0.05$.

Five weeks		Betaine		Proline		Soluble carbohydrate		Starch	
Growth conditions		*F*-value	*P*	*F*-value	*P*	*F*-value	*P*	*F*-value	*P*
Ambient CO_2	Well-watered	0.087	0.778	7.010	0.038	0.153	0.709	6.796	0.060
	Drought	8.956	0.030	8.384	0.034	6.404	0.052 LN	2.666	0.154
Elevated CO_2	Well-watered	0.001	0.975	12.571	0.012	12.774	0.016	4.024	0.101 LN
	Drought	1.8950	0.232	3.779	0.109	0.009	0.930	0.109	0.753

Ten weeks		Betaine		Proline		Soluble carbohydrate		Starch	
Growth conditions		*F*-value	*P*	*F*- value	*P*	*F*- value	*P*	*F*-value	*P*
Ambient CO_2	Well-watered	16.342	0.007	0.287	0.611	0.158	0.704	14.657	0.009
	Drought	0.181	0.688	0.067	0.806	0.179	0.687	1.562	0.267
Elevated CO_2	Well-watered	0.180	0.689	3.759	0.110	0.860	0.390	0.667	0.445
	Drought	1.584	0.277	0.286	0.612	8.938	0.024	0.707	0.439

LN = data were natural log transformed.

Table 2.1.9. Results from a Oneway ANOVA analyses to test for significant effects of **drought** on the metabolites in the shoots of *Armeria maritima* after five and ten weeks. All values have degrees of freedom of 1 between groups and degrees of freedom within groups are either 4, 5 or 6. Significance level is set at $P \leq 0.05$.

Five weeks		Betaine		Proline		Soluble carbohydrate		Starch	
Growth conditions		*F*-value	*P*	*F*-value	*P*	*F*-value	*P*	*F*-value	*P*
Ambient CO_2	High N	18.060	0.008	1.995	0.217	0.743	0.422	106.145	0.000
	Low N	0.012	0.915	1.223	0.311	0.476	0.521	76.145	0.000
Elevated CO_2	High N	3.466	0.122	0.083	0.782	9.174	0.039	0.806	0.404
	Low N	2.860	0.152 LN	0.006	0.943	0.101	0.762	5.353	0.069 LN

Ten weeks		Betaine		Proline		Soluble carbohydrate		Starch	
Growth conditions		*F*-value	*P*	*F*-value	*P*	*F*-value	*P*	*F*-value	*P*
Ambient CO_2	High N	6.935	0.046	0.540	0.495	0.438	0.533	0.010	0.925
	Low N	3.699	0.103	0.003	0.957	0.985	0.359	5.393	0.068
Elevated CO_2	High N	0.404	0.559	1.371	0.294	2.513	0.164	0.028	0.874
	Low N	1.004	0.362	11.289	0.015	1.223	0.311 LN	5.044	0.075

LN = data were natural log transformed.

Table 2.1.10. Results from a oneway ANOVA analyses to test for significant effects of **elevated CO_2** on the metabolites in the roots of *Armeria maritima* after five and ten weeks. All values have degrees of freedom of 1 between groups and degrees of freedom within groups are either 4, 5 or 6. Significance level is set at $P \leq 0.05$.

Five weeks		Betaine		Proline		Soluble carbohydrate		Starch	
Growth conditions		*F*-value	*P*	*F*-value	*P*	*F*-value	*P*	*F*-value	*P*
Well-watered	High N	1.482	0.269	0.414	0.544	0.752	0.435	**20.632**	**0.004**
	Low N	**7.238**	**0.043**	1.795	0.238	2.085	0.222	**32.715**	**0.001**
Drought	High N	**12.590**	**0.016**	**27.482**	**0.003**	0.091	0.775	1.211	0.313
	Low N	0.090	0.775	0.222	0.654	0.002	0.969	1.049	0.345

Ten weeks		Betaine		Proline		Soluble carbohydrate		Starch	
Growth conditions		*F*-value	*P*	*F*-value	*P*	*F*-value	*P*	*F*-value	*P*
Well-watered	High N	0.555	0.484	**9.067**	**0.024**	2.129	0.195	0.375	0.563
	Low N	**16.770**	**0.009**	1.726	0.246	0.348	0.581	2.406	0.182
Drought	High N	3.907	0.105	0.476	0.521	0.220	0.656	0.112	0.755
	Low N	0.080	0.786	0.138	0.723	0.587	0.473	5.047	0.075

LN = data were natural log transformed.

Table 2.1.11. Results from a Oneway ANOVA analyses to test for significant effects of **nitrogen** availability on the metabolites in the roots of *Armeria maritima* after five and ten weeks. All values have degrees of freedom of 1 between groups and degrees of freedom within groups are either 4, 5 or 6. Significance level is set at $P \leq 0.05$.

Five weeks		Betaine		Proline		Soluble carbohydrate		Starch	
Growth conditions		*F*-value	*P*	*F*-value	*P*	*F*-value	*P*	*F*-value	*P*
Ambient CO_2	Well-watered	**10.763**	**0.022**	2.815	0.154	2.142	0.217	0.112	0.749
	Drought	**18.266**	**0.005**	**21.268**	**0.004**	0.049	0.832 LN	2.920	0.138
Elevated CO_2	Well-watered	0.000	0.993	**7.030**	**0.038**	0.792	0.424	0.364	0.568
	Drought	0.291	0.613	0.592	0.476	0.026	0.878	2.975	0.135

Ten weeks		Betaine		Proline		Soluble carbohydrate		Starch	
Growth conditions		*F*-value	*P*	*F*- value	*P*	*F*-value	*P*	*F*-value	*P*
Ambient CO_2	Well-watered	1.160	0.331	0.202	0.672	1.845	0.232	3.091	0.139
	Drought	0.659	0.448	0.464	0.521	0.004	0.954	0.709	0.447
Elevated CO_2	Well-watered	**7.942**	**0.030**	0.589	0.472	0.345	0.579	0.453	0.526
	Drought	2.732	0.159	0.167	0.700	0.051	0.828	0.570	0.484

LN = data were natural log transformed.

Table 2.1.12. Results from a Oneway ANOVA analyses to test for significant effects of **drought** on the metabolites in the roots of *Armeria maritima* after five and ten weeks. All values have degrees of freedom of 1 between groups and degrees of freedom within groups are either 4, 5 or 6. Significance level is set at $P \leq 0.05$.

Five weeks		Betaine		Proline		Soluble carbohydrate		Starch	
Growth conditions		*F*-value	*P*	*F*-value	*P*	*F*-value	*P*	*F*-value	*P*
Ambient CO_2	High N	**11.214**	**0.015**	**55.940**	**0.000**	0.001	0.982	**18.922**	**0.005**
	Low N	**20.505**	**0.006**	**92.669**	**0.000**	3.139	0.137	**25.776**	**0.002**
Elevated CO_2	High N	0.412	0.549	4.082	0.099	0.000	0.984	2.907	0.139
	Low N	1.092	0.336	6.043	0.049	0.073	0.798	3.559	0.108

Ten weeks		Betaine		Proline		Soluble carbohydrate		Starch	
Growth conditions		*F*-value	*P*	*F*-value	*P*	*F*-value	*P*	*F*-value	*P*
Ambient CO_2	High N	0.106	0.756	0.182	0.684	1.072	0.340	5.003	0.076
	Low N	5.706	0.062	0.339	0.586 LN	0.212	0.664	**15.292**	**0.017**
Elevated CO_2	High N	0.327	0.592	0.637	0.461	0.001	0.976	1.721	0.247
	Low N	0.001	0.970	0.365	0.568	0.907	0.378	0.025	0.880

LN = data were natural log transformed.

Appendix 2.2 Effects of resource availability on the % N and % C allocated to compatible solutes

Table 2.2.1. Results from a oneway ANOVA analyses to test for significant effects of **elevated CO_2** on the % N allocated to compatible solutes in *Armeria maritima* and *Plantago maritima* after five and ten weeks. All values have degrees of freedom of 1 between groups and degrees of freedom within groups are either 4, 5 or 6. Significance level is set at $P \leq 0.05$.

Five weeks		*Armeria maritima* shoot		*Armeria maritima* root		*Plantago maritima* shoot		*Plantago maritima* root	
Growth conditions		*F*-value	*P*	*F*-value	*P*	*F*-value	*P*	*F*-value	*P*
Well-watered	High N	0.184	0.686	0.014	0.908	0.407	0.558	1.690	0.241
	Low N	0.003	0.958	3.468	0.122	1.341	0.291	**16.537**	**0.007**
Drought	High N	**56.588**	**0.001**	2.960	0.146	1.731	0.245	2.413	0.181
	Low N	**9.837**	**0.026**	1.610	0.260	0.854	0.398	5.940	0.051

Ten weeks		*Armeria maritima* shoot		*Armeria maritima* root		*Plantago maritima* shoot		*Plantago maritima* root	
Growth conditions		*F*-value	*P*	*F*-value	*P*	*F*-value	*P*	*F*-value	*P*
Well-watered	High N	5.663	0.055	0.200	0.670	2.344	0.200	1.429	0.286
	Low N	0.146	0.715	**22.077**	**0.003**	2.163	0.192	1.000	0.356
Drought	High N	0.433	0.540	0.020	0.893	3.216	0.133	**9.917**	**0.020**
	Low N	0.622	0.466	0.646	0.452	0.395	0.553	0.104	0.760

LN = data were natural log transformed.

Table 2.2.2. Results from a Oneway ANOVA analyses to test for significant effects of **nitrogen** availability on the % N allocated to compatible solutes in *Armeria maritima* and *Plantago maritima* after five and ten weeks. All values have degrees of freedom of 1 between groups and degrees of freedom within groups are either 4, 5 or 6. Significance level is set at $P \leq 0.05$. / = analysis not carried out.

Five weeks		*Armeria maritima* shoot		*Armeria maritima* root		*Plantago maritima* shoot		*Plantago maritima* root	
Growth conditions		*F*-value	*P*	*F*-value	*P*	*F*-value	*P*	*F*-value	*P*
Ambient CO_2	Well-watered	4.834	0.070	3.020	0.143	4.307	0.083	**6.628**	**0.042**
	Drought	5.238	0.071	1.133	0.328	1.384	0.305	4.460	0.088
Elevated CO_2	Well-watered	2.532	0.172	2.478	0.167	3.715	0.126	**20.873**	**0.004**
	Drought	0.920	0.382	2.380	0.198	0.473	0.517	0.772	0.414

Ten weeks		*Armeria maritima* shoot		*Armeria maritima* root		*Plantago maritima* shoot		*Plantago maritima* root	
Growth conditions		*F*-value	*P*	*F*- value	*P*	*F*- value	*P*	*F*-value	*P*
Ambient CO_2	Well-watered	1.145	0.326	2.160	0.192	**9.553**	**0.021**	/	/
	Drought	1.520	0.272	3.601	0.107	3.220	0.123	**42.856**	**0.001**
Elevated CO_2	Well-watered	2.036	0.203	**8.119**	**0.029**	3.955	0.118	0.592	0.477
	Drought	3.912	0.105	3.072	0.130	2.198	0.198	0.022	0.887

LN = data were natural log transformed.

Table 2.2.3. Results from a Oneway ANOVA analyses to test for significant effects of **drought** on the % N allocated to compatible solutes in *Armeria maritima* and *Plantago maritima* after five and ten weeks. All values have degrees of freedom of 1 between groups and degrees of freedom within groups are either 4, 5 or 6. Significance level is set at $P \leq 0.05$.

Five weeks		*Armeria maritima* shoot		*Armeria maritima* root		*Plantago maritima* shoot		*Plantago maritima* root	
Growth conditions		*F*-value	*P*	*F*-value	*P*	*F*-value	*P*	*F*-value	*P*
Ambient CO_2	High N	**11.038**	**0.021**	3.174	0.125	0.820	0.407	5.118	0.073
	Low N	0.513	0.501	5.635	0.064	0.560	0.488	3.123	0.128
Elevated CO_2	High N	1.195	0.324	0.375	0.567	0.965	0.382	0.879	0.385
	Low N	3.291	0.129	0.310	0.602	2.601	0.158	0.104	0.158

Ten weeks		*Armeria maritima* shoot		*Armeria maritima* root		*Plantago maritima* shoot		*Plantago maritima* root	
Growth conditions		*F*-value	*P*	*F*-value	*P*	*F*-value	*P*	*F*-value	*P*
Ambient CO_2	High N	2.804	0.155	0.410	0.545	0.680	0.441	**519.49**	**0.000**
	Low N	0.301	0.603	5.261	0.062	0.834	0.396	4.130	0.098
Elevated CO_2	High N	0.695	0.436	2.244	0.185	1.490	0.309	0.300	0.608
	Low N	0.367	0.571	0.265	0.625	0.918	0.375	3.891	0.096

LN = data were natural log transformed.

Table 2.2.4. Results from a oneway ANOVA analyses to test for significant effects of **elevated CO_2** on the % allocation of carbon to sorbitol in *Plantago maritima* after five and ten weeks. All values have degrees of freedom of 1 between groups and degrees of freedom within groups are either 4, 5 or 6. Significance level is set at $P \leq 0.05$.

Five weeks		*Plantago maritima* shoot		*Plantago maritima* root	
Growth conditions		*F*-value	*P*	*F*-value	*P*
Well-watered	High N	2.967	0.146	5.644	0.055
	Low N	0.011	0.919	1.956	0.211
Drought	High N	2.133	0.204	0.164	0.699
	Low N	4.399	0.081	0.046	0.838

Ten weeks		*Plantago maritima* shoot		*Plantago maritima* root	
Growth conditions		*F*-value	*P*	*F*-value	*P*
Well-watered	High N	0.432	0.540	0.314	0.595
	Low N	0.398	0.552	4.649	0.084
Drought	High N	4.467	0.088	0.393	0.554
	Low N	1.922	0.215	5.607	0.064

LN = data were natural log transformed.

Table 2.2.5. Results from a Oneway ANOVA analyses to test for significant effects of **nitrogen** availability on the % allocation of carbon to sorbitol in *Plantago maritima* after five and ten weeks. All values have degrees of freedom of 1 between groups and degrees of freedom within groups are either 4, 5 or 6. Significance level is set at $P \leq 0.05$.

Five weeks		*Plantago maritima* shoot		*Plantago maritima* root	
Growth conditions		*F*-value	*P*	*F*-value	*P*
Ambient CO_2	Well-watered	1.153	0.324	**7.274**	**0.036**
	Drought	0.082	0.784	0.070	0.800
Elevated CO_2	Well-watered	0.236	0.648	2.463	0.168
	Drought	0.038	0.854	0.736	0.424

Ten weeks		*Plantago maritima* shoot		*Plantago maritima* root	
Growth conditions		*F*- value	*P*	*F*-value	*P*
Ambient CO_2	Well-watered	0.121	0.740	0.569	0.479
	Drought	0.723	0.428	0.564	0.487
Elevated CO_2	Well-watered	2.486	0.176	1.777	0.240
	Drought	0.216	0.662	4.019	0.092

LN = data were natural log transformed.

Table 2.2.6. Results from a Oneway ANOVA analyses to test for significant effects of **drought** on the % allocation of carbon to sorbitol in *Plantago maritima* after five and ten weeks. All values have degrees of freedom of 1 between groups and degrees of freedom within groups are either 4, 5 or 6. Significance level is set at $P \leq 0.05$.

Five weeks		*Plantago maritima* shoot		*Plantago maritima* root	
Growth conditions		*F*-value	*P*	*F*-value	*P*
Ambient CO_2	High N	1.208	0.314	**9.533**	**0.021**
	Low N	3.989	0.093	0.132	0.729
Elevated CO_2	High N	1.236	0.329	0.698	0.435
	Low N	0.440	0.532	**11.557**	**0.015**

Ten weeks		*Plantago maritima* shoot		*Plantago maritima* root	
Growth conditions		*F*-value	*P*	*F*-value	*P*
Ambient CO_2	High N	0.561	0.482	0.680	0.441
	Low N	0.098	0.765	3.393	0.125
Elevated CO_2	High N	4.227	0.109	0.885	0.383
	Low N	0.087	0.779	**6.761**	**0.048**

LN = data were natural log transformed.

References

Ahmad I, Larher F, Stewart GR (1979) Sorbitol, a compatible osmotic solute in *Plantago maritima*. New Phytologist **82:** 671-678

Allen LH, Valle RR, Jones JW, Jones PH (1998) Soybean leaf water potential responses to carbon dioxide and drought. Agronomy Journal **90:** 375-383

Anterola AM, Lewis NG (2002) Trends in lignin modification: a comprehensive analysis of the effects of genetic manipulations/mutations on lignification and vascular integrity. Phytochemistry **61:** 221-294

Arp WJ (1991) Effects of source-sink relations on photosynthetic acclimation to elevated CO_2. Plant, cell and environment **14:** 869-875

Arp WJ, Van Mierlo JEM, Berense F, Snijders W (1998) Interactions between elevated CO_2 concentration, nitrogen and water: effects on growth and water use of six perennial plant species. Plant, Cell and Environment **21:** 1-11

Atkinson CJ, Taylor JM (1996) Effects of elevated CO_2 on stem growth, vessel area and hydraulic conductivity of oak and cherry seedlings. New Phytologist **133:** 617-626

Barkosky RR, Einhellig FA, Butler JL (2000) Caffeic acid-induced changes in plant-water relationships and photosynthesis in leafy spurge *Euphorbia esula*. Journal of Chemical Ecology **26:** 2095-2109

Batanouny KH, Ebeid MM (1981) Diurnal changes in proline content of desert plants. Oecologia **51:** 250-252

Baxter R, Ashenden TW, Farrar JF (1997) Effect of elevated CO_2 and nutrient status on growth, dry matter partitioning and nutrient content of *Poa alpina* var. *vivipara* L. Journal of experimental botany **48:** 1477-1486

Baxter R, Ashenden TW, Sparks TH, Farrar JF (1994) Effects of elevated carbon dioxide on three montane grass species I. Growth and dry matter partitioning. Journal of Experimental Botany **45:** 305-315

Baxter R, Bell SA, Sparks TH, Ashenden TW, Farrar JF (1995) Effects of elevated CO_2 concentrations on three montane grass species III. Source leaf metabolism and whole plant carbon partitioning. Journal of Experimental Botany **46:** 917-929

Baxter R, Gantley M, Ashenden TW, Farrar JF (1994) Effects of elevated carbon dioxide on three grass species from montane pasture II. Nutrient uptake, allocation and efficiency of use. Journal of Experimental Botany **45:** 1267-1278

Bazzaz FA (1990) The response of natural ecosystems to the rising global CO_2 levels. Annual review of ecology and systematics **21:** 167-196

Beadle CL (1993) Growth analysis. *In* DO Hall, JMO Scurlock, HR Bolhar-Nordenkampf, RC Lee, SP Long, eds, Photosynthesis and production in a changing environment: a field and laboratory manual. Chapman and Hall, London, pp 36-46

Bezemer TM, Jones TH, Newington JE (2000) Effects of carbon dioxide and nitrogen fertilization on phenolic content in *Poa annua* L. Biochemical Systematics and Ecology **28:** 839-846

Bohnert HJ, Nelson DE, Jensen RG (1995) Adaptations to environmental stress. The Plant Cell **7:** 1099-1111

Booker FL, Maier CA (2001) Atmospheric carbon dioxide, irrigation, and fertilization effects on phenolic and nitrogen concentrations in loblolly pine (*Pinus taeda*) needles. Tree Physiology **21:** 609-616

Boudet AM (2000) Lignins and lignification: Selected issues. Plant Physiology and Biochemistry **38:** 81-96

Bowes G (1991) Growth at elevated CO_2: photosynthetic responses mediated through Rubisco. Plant, Cell and Environment **14:** 795-806

Bowes G (1993) Facing the inevitable: plants and increasing atmospheric CO_2. Annual Review of Plant Physiology and Plant Molecular Biology **44:** 309-332

Bremner JM (1996) Nitrogen-Total. *In* JM Bigham, ed, Methods of Soil Analysis. Soil Science Society of America and American Society of Agronomy. Part 3 Chemical Methods, Madison, pp 1085-1121

Brown AD, Simpson JR (1972) Water relations of sugar-tolerant yeasts: the role of intracellular polyols. Journal of General Microbiology **72:** 589-591

Brown RW, Bartos DL (1982) A calibration model for screen-caged peltier thermocouple psychrometers. United States Department of Agriculture Forest Service Research Paper INT-293

Bryant JP, Chapin FS, Klein DR (1983) Carbon/nutrient balance of boreal plants in relation to vertebrate herbivory. Oikos **40:** 357-368

Buckland SM, Price AH, Hendry GAF (1991) The role of ascorbate in drought-treated *Cochlearia atlantica* Pobed and *Armeria maritima* (Mill) Willd. New Phytologist **119:** 155-160

Burghardt M, Riederer M (2003) Ecophysiological relevance of cuticular transpiration of deciduous and evergreen plants in relation to stomatal closure and leaf water potential. Journal of Experimental Botany **54:** 1941-1949

Chapin FS (1980) The mineral nutrition of wild plants. Annual Review of Ecology and Systematics **11:** 233-260

Cheng L, Fuchigami LH (2000) Rubisco activation state decreases with increasing nitrogen content in apple leaves. Journal of Experimental Botany **51:** 1687-1694

Cheng WH, Endo A, Zhou L, Penney J, Chen HC, Arroyo A, Leon P, Nambara E, Asami T, Seo M, Koshiba T, Sheen J (2002) A unique short-chain dehydrogenase/reductase in Arabidopsis glucose signaling and abscisic acid biosynthesis and functions. The Plant Cell **14:** 2723-2743

Clark H, Newton PCD, Barker DJ (1999) Physiological and morphological responses to elevated CO_2 and a soil moisture deficit of temperate pasture species growing in an established plant community. Journal of Experimental Botany **50:** 233-242

Cook AC, Tissue DT, Roberts SW, Oechel WC (1998) Effects of long-term elevated [CO_2] from natural CO_2 springs on *Nardus stricta*: photosynthesis, biochemistry, growth and phenology. Plant, Cell and Environment **21:** 417-425

Coruzzi G, Bush DR (2001) Nitrogen and carbon nutrient and metabolite signaling in plants. Plant Physiology **125:** 61-64

Coruzzi GM, Zhou L (2001) Carbon and nitrogen sensing and signaling in plants: emerging 'matrix effects'. Current opinion in plant biology **4:**247-253

Cotrufo MF, Ineson P, Scott A (1998) Elevated CO_2 reduces the nitrogen concentration of plant tissues. Global Change Biology **4:** 43-54

Coviella CE, Stipanovic RD, Trumble JT (2002) Plant allocation to defensive compounds: interactions between elevated CO_2 and nitrogen in transgenic cotton plants. Journal of Experimental Botany **53:** 323-331

Coviella CE, Trumble JT (1999) Effects of elevated atmospheric carbon dioxide on insect-plant interactions. Conservation Biology **13:** 700-712

Daayf F, Ongena M, Boulanger R, Hadrami IE, Bélanger RR (2000) Induction of phenolic compounds in two cultivars of cucumber by treatment of healthy and powdery mildew-infected plants with extracts of *Reynoutria sachalinensis*. Journal of Chemical Ecology **26:** 1579-1593

Davey PA, Parsons AJ, Atkinson L, Wadge K, Long SP (1999) Does photosynthetic acclimation to elevated CO_2 increase photosynthetic nitrogen-use efficiency? A study of three native UK grassland species in open top chambers. Functional Ecology **13:** 21-28

Dawson RMC, Elliot DC, Elliot WH, Jones KM (1989) Data for biochemical research, Ed 3rd. Oxford University Press, New York

De Pinheiro Henriques AR, Marcelis LFM (2000) Regulation of growth at steady-state nitrogen nutrition in lettuce (*Lactuca sativa* L.): Interactive effects of nitrogen and irradiance. Annals of botany **86:** 1073-1080

Delauney AJ, Verma DPS (1993) Proline biosynthesis and osmoregulation in plants. The Plant Journal **4:** 215-223

den Hertog J, Stulen I, Fonseca F, Delea P (1996) Modulation of carbon and nitrogen allocation in *Urtica dioica* and *Plantago major* by elevated CO_2: impact of accumulation of non-structural carbohydrates and ontogenetic drift. Physiologia Plantarum **98:** 77-88

den Hertog J, Stulen I, Lambers H (1993) Assimilation, respiration and allocation of carbon in *Plantago major* as affected by atmospheric CO_2 levels - a case-study. Vegetatio **104:** 369-378

den Hertog J, Stulen I, Posthumus F, Poorter H (1998) Interactive effects of growth-limiting N supply and elevated atmospheric CO_2 concentration on growth and carbon balance of *Plantago major*. Physiologia Plantarum **103:** 451-460

Dixon RA, Paiva NL (1995) Stress-Induced Phenylpropanoid Metabolism. The Plant Cell **7:** 1085-1097

Drake BG, Gonzàlez-Meler MA, Long SP (1997) More efficient plants: A consequence of rising atmospheric CO_2? Annual Review of Plant Physiology and Plant Molecular Biology **48:** 609-639

Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F (1956) Colorimetric method for determination of sugars and related substances. Analytical Chemistry **28:** 350-356

Eamus D (1991) The interaction of rising CO_2 and temperatures with water use efficiency. Plant, Cell and Environment **14:** 843-852

Edwards R, Kessmann H (1992) Isoflavonoid phytoalexins and their biosynthetic enzymes. *In* SJ Gurr, MJ McPherson, DJ Bowles, eds, Molecular Plant Pathology. Volume II A Practicle Approach. Oxford University Press, New York, pp 45-62

Escobar-Gutiérrez AJ, Gaudillère JP (1996) Distribution, métabolisme et rôle du sorbitol chez les plantes supérieures. Synthèse. Agronomie **16:** 281-298

Escobar-Gutierrez AJ, Gaudillere JP (1997) Carbon partitioning in source leaves of peach, a sorbitol-synthesizing species, is modified by photosynthetic rate. Physiologia Plantarum **100:** 353-360

Escobar-Gutierrez AJ, Zipperlin B, Carbonne F, Moing A, Gaudillere JP (1998) Photosynthesis, carbon partitioning and metabolic content during drought stress in peach seedlings. Australian Journal of Plant Physiology **25:** 197-205

Estiarte M, Peñuelas J, Kimball BA, Hendrix DL, Pinter PJ, Wall GW, LaMorte RL, Hunsaker DJ (1999) Free-air CO_2 enrichment of wheat: leaf flavonoid concentration throughout the growth cycle. Physiologia Plantarum **105:** 423-433

Evans JR, Poorter H (2001) Photosynthetic acclimation of plants to growth irradiance: the relative importance of specific leaf area and nitrogen partitioning in maximizing carbon gain. Plant, Cell and Environment **24:** 755-767

Fajer ED, Bowers MD, Bazzaz FA (1992) The effect of nutrients and enriched CO_2 environments on production of carbon-based allelochemicals in *Plantago*: a test of the carbon/nutrient balance hypothesis. The American Naturalist **140:** 707-723

FAO/IAEA (2000) Quantification of Tannins in Tree Foliage. *In*. Joint FAO/IAEA division of nuclear techniques in food and agriculture, Vienna
http://vcampus.uom.ac.mu/upload/public/2002930133557.pdf

Farquhar GD, von Caemmerer S (1982) Modelling of Photosynthetic Response to Environmental Conditions. *In* OL Lange, PS Nobel, CB Osmond, H Ziegler, eds, Physiological Plant Ecology II Water Relations and Carbon Assimilation, Vol 12 B. Springer-Verlag, Berlin, pp 549-587

Farrar J (1993) Carbon partitioning. *In* DO Hall, JMO Scurlock, HR Bolhar-Nordenkampf, RC Lee, SP Long, eds, Photosynthesis and production in a changing environment: a field and laboratory manual. Chapman and Hall, London, pp 232-246

Farrar J (1999) Acquisition, partitioning and loss of carbon. *In* MC Press, JD Scholes, MG Marker, eds, Physiological plant ecology. Blackwell Science Ltd., pp 25-43

Farrar JF, Williams ML (1991) The effects of increased atmospheric carbon dioxide and temperature on carbon partitioning, source-sink relations and respiration. Plant, Cell and Environment **14:** 819-830

Ferris R, Taylor G (1994) Increased root growth in elevated CO_2: a physical analysis of root cell elongation. Journal of Experimental Botany **45:** 1603-1612

Ferris R, Taylor G (1995) Contrasting effects of elevated CO_2 and water deficit on two native herbs. New Phytologist **131:** 491-501

Fiehn O, Kopka J, Trethewey RN, Willmitzer L (2000) Identification of uncommon plant metabolites based on calculation of elemental compositions using gas chromatography and quadruploe mass spectrometry. Analytical Chemistry **72:** 3573-3580

Flanagan LB, Jefferies RL (1989 a) Effect of increased salinity on CO_2 assimilation, O_2 evolution and the $\delta^{13}C$ values of leaves of *Plantago maritima* L. developed at low and high NaCl levels. Planta **178:** 377-384

Flanagan LB, Jefferies RL (1989 b) Photosynthetic and stomatal responses of the halophyte, *Plantago maritima* L. to flucuations in salinity. Plant, Cell and Environment **12:** 559-568

Flexas J, Gulías J, Jonasson S, Medbrano H, Mus M (2001) Seasonal patterns and control of gas exchange in local populations of the Mediterranean evergreen shrub *Pistacia lentiscus* L. Acta Oecologia **22:** 33-43

Fons F, Tousch D, Rapior S, Gueiffier A, Roussel JL, Gargadennec A, Andary C (1999) Phenolic profiles of untransformed and hairy root cultures of *Plantago lanceolata*. Plant Physiology and Biochemistry **37:** 291-296

Gates P (1993) Plant Anatomy, Histology and Taxonomy for Molecular Biologists. *In* RRD Croy, ed, Plant Molecular Biology Labfax. Bios Scientific Publishers, pp 1-20

Gleadow RM, Foley WJ, Woodrow IE (1998) Enhanced CO_2 alters the relationship between photosynthesis and defence in cyanogenic *Eucalyptus cladocalyx* F. Muell. Plant, Cell and Environment **21:** 12-22

Glenn EP, O'Leary JW (1984) Relationship between salt and accumulation and water content of dicotyledonous halophytes. Plant, Cell and Environment **7:** 253-261

Goldsmith FB (1967) Some aspects of the vegetation of sea cliffs. PhD. University of Wales, Bangor

Goldsmith FB (1973 a) The Vegetation of Exposed Sea Cliffs at South Stack, Anglesey. Journal of Ecology **61:** 787-818

Goldsmith FB (1973 b) The vegetation of Exposed Sea Cliffs at South Stack, Anglesey: II. Experimental Studies. Journal of Ecology **61:** 819-829

Gorham J (1984) Separation of plant betaines and their sulphur analogues by cation-exchange high-performance liquid chromatography. Journal of Chromatography **287:** 345-351

Gorham J, Hughes LI, Wyn Jones RG (1980) Chemical composition of salt marsh plants from Ynys Mon (Anglesey): the concept of physiotypes. Plant, Cell and Environment **3:** 309-318

Gray AJ, Mogg RJ (2001) Climate impacts on pioneer saltmarsh plants. Climate Research **18:** 105-112

Grayer RJ, de Kok RPJ (1998) Flavonoids and verbascoside as chemotaxonomic characters in the genera *Oxera* and *Faradaya* (Labiatae). Biochemical Systematics and Ecology **26:** 729-741

Gregor JW (1930) Experiments on the genetics of wild populations. I. *Plantago maritima*. Journal of Genetics **22:** 15-25

Gzik A (1996) Accumulation of proline and pattern of α-amino acids in sugar beet plants in response to osmotic, water and salt stress. Environmental and Experimental Botany **36:** 29-38

Hamilton JG, Zangeri AR, DeLucia EH, Berenbaum MR (2001) The carbon-nutrient hypothesis: its rise and fall. Ecology Letters **4:** 86-95

Hanson AD, Rathinasabapathi B, Rivoal J, Burnet M, Dillon MO, Gage A (1994) Osmoprotective compounds in the Plumbaginaceae: A natural experiment in metabolic engineering of stress tolerance. Proceedings of the National Academy of Science **91:** 306-310

Hanson AD, Rivoal J, Burnet M, Rathinasabapathi B (1995) Biosynthesis of quaternary ammonium and tertiary sulphonium compounds in response to water deficit. *In* N Smirnoff, ed, Environmental Plant Metabolism. Bios Scientific Publishers, London, pp 189-197

Harbourne JB (1997) Introduction to Ecological Biochemistry, Ed 4th. Academic Press Limited, London

Hare PD, Cress WA (1997) Metabolic implications of stress-induced proline accumulation in plants. Plant Growth Regulation **21:** 79-102

Hare PD, Cress WA, Van Standen J (1998) Dissecting the roles of osmolyte accumulation during stress. Plant, Cell and Environment **21:** 535-553

Harley PC, Thomas RB, Refnolds JF, Strain BR (1992) Modelling photosynthesis of cotton grown in elevated CO_2. Plant, Cell and Environment **15:** 271-282

Harmens H, Stirling CM, Marshall C, Farrar JF (1998) Elevated and CO_2 and N: interactive effects on photosynthesis of *Dactylis glomerata* L. (Cocksfoot). *In* LJ De Kok, I Stulen, eds, Responses of plant metabolism to air pollution and global change. Backhuys, Leiden, The Netherlands, pp 319-322

Harmens H, Stirling CM, Marshall C, Farrar JF (2000) Does down-regulation of photosynthetic capacity by elevated CO_2 depend on N supply in *Dactylis glomerata*? Physiologia Plantarum **108:** 43-50

Hartley SE, Jones CG, Couper GC, Hefin Jones T (2000) Biosynthesis of plant phenolic compounds in elevated atmospheric CO_2. Global Change Biology **6:** 497-506

Heath J, Kerstiens G (1997) Effects of elevated CO_2 on leaf gas exchange in beech and oak at two levels of nutrient supply: consequences for sensitivity to drought in beech. Plant, Cell and Environment **20:** 57-67

Hepburn I (1943) A Study of the Vegetation of Sea-Cliffs in North Cornwall. Journal of Ecology **31:** 30-39

Herms DA, Mattson WJ (1992) The dilemma of plants: to grow or defend. The Quarterly Review of Biology **67:** 283-335

Hewitt EJ (1966) Sand and Water culture methods used in the study of Plant Nutrition, Ed 2nd. The Eastern Press, London and Reading

Hillel D (1971) Soil and water. Physiological principles and processes. Academic press

Hind G (1993) Thylakoid components and processes. *In* RC Leegood, SP Long, eds, Photosynthesis and production in a changing environment: a field and laboratory manual. Chapman and Hall, London, pp 283-297

Hsieh MH, Lam HM, van de Loo FJ, Coruzzi G (1998) A PII-like protein in *Arabidopsis*: putative role in nitrogen sensing. Proceedings of the National Academy of Sciences USA **95:** 13965-13970

Humphreys JM, Chapple C (2002) Rewriting the lignin roadmap. Current Opinion in Plant Biology **5:** 224-229

Hunt R (1990) Basic growth analysis. Unwin Hyman, London

Idso KE, Hoober JK, Idso SB, Wall GW, Kimball BA (2001) Atmospheric CO_2 enrichment influences the synthesis and mobilization of putative vacuolar storage proteins in sour orange tree leaves. Environmental and Experimental Botany **48:** 199-211

IPCC (2001) Intergovernmental Panel on Climate Change. Third Assessment Report. Contribution of the working group I: Summary for policy makers. Cambridge University Press, Cambridge

Irigoyen JJ, Emerich DW, Sánchez-Díaz M (1992) Water stress induced changes in concentrations of proline and total soluble sugars in nodulated alfalfa (*Medicago sativa*) plants. Physiologia Plantarum **84:** 55-60

Jackson DL, McLeod CR (2002) Handbook on the UK status of EC Habitats Directive interest features: provisional data on the UK distribution and extent of Annex I habitats and the UK distribution and population size of Annex II species. Version 2. *In.* Joint Nature Conservation Committee (JNCC) Report, Peterborough, UK

Jang JC, Sheen J (1997) Sugar sensing in higher plants. Trends in Plant Science **2:** 208-214

Jefferies RL (1977) Growth responses of coastal halophytes to inorganic nitrogen. Journal of Ecology **65:** 847-865

Jefferies RL (1980) The role of organic solutes in osmoregulation in halophytic higher plants. *In* DW Rains, RC Valentine, A Hollaender, eds, Genetic engineering of osmoregulation. Impact on plant productivity for food, chemicals, and energy. Plenum Publishing Corporation, New York, pp 135-154

Jefferies RL, Davy AJ, Rudmik T (1979) The growth strategies of coastal halophytes. *In* RL Jefferies, AJ Davy, eds, Ecological processes in coastal environments. Blackwell scientific publications, Oxford, pp 243-268

Johnson JD, Tognetti R, Paris P (2002) Water relations and gas exchange in poplar and willow under water stress and elevated atmospheric CO_2. Physiologia Plantarum **115:** 93-100

Jones CG, Hartley SE (1999) A protein competition model of phenolic allocation. Oikos **86:** 27-44

Kang J, Turano FJ (2003) The putative glutamate receptor 1.1 (AtGLR1.1) functions as a regulator of carbon and nitrogen metabolism in *Arabidopsis thaliana*. Proceedings of the National Academy of Sciences USA **100:** 6872-6877

Kikuzaki H, Hisamoto M, Hirose K, Akiyama K, Taniguchi H (2002) Antioxidant properties of ferulic acid and its related compounds. Journal of Agricultural and Food Chemistry **50:** 2161-2168

King TS, Mann T (1959) Sorbitol metabolism in spermatozoa. Proceedings of the Royal Society **151:** 226-243

Klus DL, Kalisz S, Curtis PS, Teeri JA, Tonsor SL (2001) Varied responses of *Plantago lanceolata* to elevated CO_2. American Journal of Botany **88:** 1080-1087

Köhl K (1996) Population-specific Traits and their Implication for the Evolution of a Drought-adapted Ecotype in *Armeria maritima*. Botanica Acta **109:** 206-215

Köhl KI (1997 a) The effect of NaCl on growth, dry matter allocation and ion uptake insalt marsh and inland populations of *Armeria maritima*. New Phytologist **135:** 213-225

Köhl KI (1997 b) NaCl homoeostasis as a factor for the survival of the evergreen halophyte *Armeria maritima* (Mill.) Willd. under salt stress in winter. Plant, Cell and Environment **20:** 1253-1263

Koricheva J (2002) The Carbon-Nutrient Balance hypothesis is dead; long live the carbon-nutrient balance hypothesis? Oikos **98:** 537-539

Koricheva J, Larsson S, Haukioja E, Keinänen M (1998) Regulation of woody plant secondary metabolism by resource availability: hypothesis testing by means of meta-analysis. Oikos **83:** 212-226

Körner C, Menendez-Riedl SP (1989) The significance of developmental aspects in plant growth analysis. *In* H Lambers, ed, Causes and consequences of variation in growth rate and productivity of higher plants. SPB Academic Publishing, The Hague

Lam HM, Chiu J, Hsieh MH, Meisel L, Oliveira IC, Shin M, Coruzzi G (1998) Glutamate-receptor genes in plants. Nature **396:** 125-126

Lavola A, Julkunen-Tiitto R, de la Rosa TM, Lehto T, Aphalo PJ (2000) Allocation of carbon to growth and secondary metabolites in birch seedlings under UV-B radiation and CO_2 exposure. Physiologia Plantarum **109:** 260-267

Lawler IR, Foley WJ, Woodrow IE, Cork SJ (1997) The effects of elevated CO_2 atmospheres on the nutritional quality of Eucalyptus foliage and its interaction with soil nutrient and light availability. Oecologia **109:** 59-68

Leadley PW, Niklaus PA, Stocker R, Körner C (1999) A field study of the effects of elevated CO_2 on plant biomass and community structure in a calcareous grassland. Oecologia **118:** 39-49

Lenssen GM (1993) Responses of C_3 and C_4 species from Dutch salt marshes to atmospheric CO_2 enrichment. PhD. Vrije Universiteit Amsterdam, Amsterdam, The Netherlands

Lenssen GM, van Duin WE, Jak P, Rozema J (1995) The response of *Aster tripolium* and *Puccinella maritima* to atmospheric carbon dioxide enrichment and their interactions with flooding and salinity. Aquatic Botany **50:** 181-192

Lerdau M, Coley PD (2002) Benefits of the Carbon-Nutrient Balance Hypothesis. Oikos **98:** 534-536

Levin DA (1976) The chemical defenses of plants to pathogens and herbivores. Annual Review of Ecology and Systematics **7:** 121-159

Liang N, Maruyama K (1995) Interactive effects of CO_2 enrichment and drought stress on gas exchange and water-use efficiency in *Alnus firma*. Environmental and Experimental Botany **35:** 353-361

Lichtenthaler HK, Wellburn AR (1983) Determinations of total carotenoids and chlorophylls *a* and *b* of leaf extracts in different solvents. Biochemical Society Transactions **11:** 591-592

Lindroth RL, Kinney KK, Platz CL (1993) Responses of deciduous trees to elevated atmospheric CO_2: productivity, phytochemistry, and insect performance. Ecology **74:** 763-777

Lombardini L, Flore JA, Everard JD (1997) Study of growth analysis. water relations, gas exchange, and carbon partitioning in one plum species during water stress and relief. Acta Horticulturae **451:** 361-368

Long SP, Hällgren JE (1993) Measurement of CO_2 assimilation by plants in the field and the laboratory. *In* DO Hall, JMO Scurlock, HR Bolhar-Nordenkampf, RC Leegood, SP Long, eds, Photosynthesis and production in a changing environment: a field and laboratory manual. Chapman and Hall, London, pp 129-167

Luo Y, Reynolds J, Wang Y, Wolfe D (1999) A search for predictive understanding of plant responses to elevated [CO_2]. Global Change Biology **5:** 143-156

Mabry TJ, Markham KR, Thomas MB (1970) The Systematic Identification of Flavonoids. Springer-Verlag, Berlin

Magne C, Larher F (1992) High sugar content of extracts interferes with colorimetric determination of amino acids and free proline. Analytical Chemistry **200:** 115-118

Maher EA, Bate NJ, Ni W, Elkind Y, Dixon RA, Lamb CJ (1994) Increased disease susceptibility of transgenic tobacco plants with suppressed levels of preformed phenylpropanoid products. Proceedings of the National Academy of Science **91:** 7802-7806

Malloch AJC (1971) Vegetation of the Maritime Cliff-Tops of the Lizard and Land's End Peninsulas, West Cornwall. New Phytologist **70:** 1155-1197

Margna U (1977) Control at the level of substrate supply - an alternative in the regulation of phenylpropanoid accumulation in plant cells. Phytochemistry **16:** 419-426

Marigo G (1973) Sur une méthode de fractionnement et d'estimation des composés phénoliques chez les végétaux. Analusis **2:** 106-110

Matt P, Krapp A, Haake V, Mock HP, Stitt M (2002) Decreased Rubisco activity leads to dramatic changes of nitrate metabolism, amino acid metabolism and the levels of phenylpropanoids and nicotine in tobacco antisense *RBCS* transformants. The Plant Journal **30:** 663-677

McNeil SD, Nuccio ML, Hanson AD (1999) Betaines and Regulated Osmoprotectants. Targets for Metabolic Engineering of Stress Resistance. Plant Physiology **120:** 945-949

Moore DM, Williams CA, Yates B (1972) Studies on Bipolar Disjunct Species II. *Plantago maritima* L. Botaniska Notiser. **125:** 261-272

Munns R, Greenway H, Kirst GO (1983) Halotolerant Eukaryotes. *In* OL Lange, PS Nobel, CB Osmond, H Ziegler, eds, Osmoregulation. Physiological Plant Ecology III. Springer-Verlag, Berlin, pp 59-136

Nakano H, Makino A, Mae T (1997) The effect of elevated partial pressures of CO_2 on the relationship between photosynthetic capacity and N content in rice leaves. Plant Physiology **115:** 191-198

Nitao JK, Zangeri AR, Berenbaum MR (2002) CNB: requiescat in pace? Oikos **98:** 540-546

Olssen T, Leverenz JW (1994) Non-uniform stomatal closure and the apparent convexity of the photosynthetic photon flux density response curve. Plant, Cell and Environment **17:** 701-710

Osmond CB (1980) Integration of photosynthetic carbon metabolism during stress. *In* DW Rains, RC Valentine, A Hollaender, eds, Genetic engineering of osmoregulation. Impact on plant productivity for food, chemicals, and energy. Plenum Publishing Corporation, New York, pp 171-185

Paez A, Hellmers H, Strain BR (1984) Carbon-dioxide enrichment and water-stress interaction on growth of 2 tomato cultivars. Journal of Agricultural Science **102:** 687-693

Pan Q, Wang Z, Quebedeaux B (1998) Responses of the apple plant to CO_2 enrichment: changes in photosynthesis, sorbitol, other soluble sugars, and starch. Australian Journal of Plant Physiology **25:** 293-297

Passioura JB (1982) Water in the soil-plant-atmosphere continuum. *In* OL Lange, PS Nobel, CB Osmond, H Ziegler, eds, Water in tissues and cells. Physiological Plant Ecology. Springer-Verlag, Berlin, pp 5-34

Peltier JP, Marigo D, Marigo G (1997) Involvement of malate and mannitol in the diurnal regulation of the water status in members of Oleaceae. Trees **12:** 27-34

Peñuelas J, Castells E, Joffre R, Tognetti R (2002) Carbon-based secondary and structural compounds in Mediterranean shrubs growing near a natural CO_2 spring. Global Change Biology **8:** 281-288

Peñuelas J, Estiarte M (1998) Can elevated CO_2 affect secondary metabolism and ecosystem function? Trends in Ecology and Evolution TREE **13:** 20-24

Peñuelas J, Estiarte M, Llusià J (1997) Carbon-based secondary compounds at elevated CO_2. Photosynthetica **33:** 313-316

Peri PL, Moot DL, McNeil DL, Varella AC, Lucas RJ (2002) Modelling net photosynthetic rate of field-grown cocksfoot leaves under different nitrogen, water and temperature regimes. Grass and Forage Science **57:** 61-71

Petrusa LM, Winicov I (1997) Proline status in salt-tolerant and salt-sensitive alfalfa cell lines and plants in response to NaCl. Plant Physiology and Biochemistry **35:** 303-310

Poorter H, Pérez-Soba M (2001) The growth response of plants to elevated CO_2 under non-optimal environmental conditions. Oecologia **129:** 1-20

Poorter H, Van Berkel Y, Baxter R, Den Hertog J, Dijkstra P, Gifford RM, Griffin KL, Roumet C, Roy J, Wong SC (1997) The effect of elevated CO_2 on the chemical composition and construction costs of leaves of 27 C_3 species. Plant, Cell and Environment **20:** 472-482

Popp M, Smirnoff N (1995) Polyol accumulation and metabolism during water deficit. *In* N Smirnoff, ed, Environmental Plant Metabolism. Bios Scientific, London, pp 199-215

Pospíšilová J, Catský J (1999) Development of water stress under increased atmospheric CO_2 concentration. Biologia Plantarum **42:** 1-24

Preston CD, Pearman DA, Dines TD (2002) New Atlas of the British and Irish Flora: An atlas of the vascular plants of Britain, Ireland, the Isle of Man and the Channel Islands. Oxford University Press, Oxford

Pritchard SG, Rogers HH, Prior SA, Peterson CM (1999) Elevated CO_2 and plant structure: a review. Global Change Biology **5:** 807-837

Quick WP, Chaves MM, Wendler R, David M, Rodrigues ML, Passaharinho JA, Pereira JS, Adcock MD, Leegood RC, Stitt M (1992) The effect of water stress on photosynthetic carbon metabolism in four species grown under field conditions. Plant, Cell and Environment **15:** 25-35

Rafarel CR, Ashenden TW, Roberts TM (1995) An improved Solardome system for exposing plants to elevated CO_2 and temperature. New Phytologist **131:** 481-490

Rathinasabapathi B, Fouad, WM, Sigua C (2001) β-alanine betaine synthesis in the Plumbaginaceae. Purification and characterization of a trifunctional, S-adenosyl-L-methionine-dependent *N*-methyltransferase from *Limonium latifolium* leaves

Rathinasabapathi B, Sigua C, Ho J, Gage A (2000) Osmoprotectant β-alanine betaine synthesis in the Plumbaginaceae: *S*-adenosyl-L-methionine dependent *N*-methylation of β-alanine to its betaine is via *N*-methyl and *N,N*-dimethyl β-alanines. Physiologia Plantarum **109:** 225-231

Ravn H, Brimer L (1988) Structure and antibacterial activity of plantamajoside, a caffeic acid sugar ester from *Plantago major* subsp. *major*. Phytochemistry **27:** 3433-3437

Razal RA, Ellis S, Singh S, Lewis NG, Towers GHN (1996) Nitrogen recycling in phenylpropanoid metabolism. Phytochemistry **41:** 31-35

Rey A, Jarvis PG (1998) Long-term photosynthetic acclimation to increased atmospheric CO_2 concentration in young birch (*Betula pendula*) trees. Tree Physiology **18:** 441-450

Riipi M, Ossipov V, Lempa K, Haukioja E, Koricheva J, Ossipova S, Pihlaja K (2002) Seasonal changes in birch chemistry: are there trade-offs between leaf growth and accumulation of phenolics? Oecologia **130:** 380-390

Rodwell JS, ed (2000) British plant communities, Vol 5. Cambridge University Press, Cambridge

Rogers HH, Prior SA, Runion GB, Mitchell RJ (1996) Root to shoot ratio of crops as influenced by elevated CO_2. Plant and Soil **187:** 229-248

Rolland F, Moore, B, Sheen J (2002) Sugar sensing and signaling in plants. The Plant Cell. Supplement: S185-S205.

Rønsted N, Göbel E, Franzyk H, Jensen SR, Olsen CE (2000) Chemotaxonomy of *Plantago*. Iridoid glucosides and caffeoyl phenylethanoid glycosides. Phytochemistry **55:** 337-348

Rose F (1981) The Wild Flower Key - British Isles - N.W. Europe. Frederick Warne, London

Ross-Craig S (1964) Drawings of British Plants, Vol. XX. G. Bell and Sons Ltd., London

Ross-Craig S (1968) Drawings of British Plants, Vol. XXV. G. Bell and Sons Ltd., London

Roth SK, Lindroth RL (1995) Elevated atmospheric CO_2 effects on phytochemistry, insect performance and insect parasitoid interactions. Global Change Biology **1:** 173-182

Roumet C, Laurent G, Roy J (1999) Leaf structure and chemical composition as affected by elevated CO_2: genotypic responses of two perennial grasses. New Phytologist **143:** 73-81

Ryan D, Robards K, Prenzler P, Jardine D, Herlt T, Antolovich M (1999) Liquid chromatography with electrospray ionisation mass spectrometric detection of phenolic compounds from *Olea europaea*. Journal of Chromatography A **855:** 529-537

Ryttari T, Lahti T (1992) *Armeria maritima* subsp intermedia, a rare archeophyte in SE Finland - history, biology and conservation. Annales Botanici Fennici **29:** 107-117

Sage RF, Sharkey TD, Seemann JR (1989) Acclimation of photosynthesis to elevated CO_2 in five C_3 species. Plant Physiology **89:** 590-596

Saralabai VC, Vivekanandan M, Suresh Babu R (1997) Plant responses to high CO_2 concentration in the atmosphere. Photosynthetica **33:** 7-37

Schulze ED, Hall AE (1982) Stomatal responses, water loss and CO_2 assimilation rates of plants in contrasting environments. *In* OL Lange, PS Nobel, CB Osmond, H Ziegler, eds, Physiological Plant Ecology II. Springer-Verlag, Berlin, pp 181-230

Schwanz P, Polle A (2001) Differential stress responses of antioxidative systems to drought in pendunculate oak (*Quercus robur*) and maritime pine (*Pinus pinaster*) grown under high CO_2 concentrations. Journal of Experimental Botany **52:** 133-143

Serraj R, Sinclair TR (2002) Osmolyte accumulation: can it really help increase crop yield under drought conditions? Plant, Cell and Environment **25:** 333-341

Sheehy Skeffington MJ, Jeffrey DW (1985) Growth performance of an inland population of *Plantago maritima* in response to nitrogen and salinity. Vegetatio **61:** 265-272

Sheehy Skeffington MJ, Jeffrey DW (1988) Response of *Armeria maritima* (Mill.) Willd. and *Plantago maritima* L. from an Irish salt marsh to nitrogen and salinity. New Phytologist **110:** 399-408

Sicher RC, Kremer DF, Rodermel SR (1994) Photosynthetic acclimation to elevated CO_2 occurs in transformed tobacco with decreased ribulose-1,5-bisphosphate carboxylase/oxygenase content. Plant Physiology **104:** 409-415

SigmaDiagnostics (1995) Procedure sheet number 50-UV, Sorbitol Dehydrogenase. *In.* Sigma Chemical Company, St. Louis

Sims DA, Luo Y, Seemann JR (1998) Comparison of photosynthetic acclimation to elevated CO_2 and limited nitrogen supply in soybean. Plant, Cell and Environment **21:** 945-952

Singh TN, Paleg LG, Aspinall D (1973) Stress metabolism I. Nitrogen metabolism and growth in the barley plant during water stress. Australian Journal of Biological Science **26:** 45-56

Skalar (1995) The SAN^{plus} segmented flow analyser Soil & Plant analysis. Skalar Analytical B.V., De Breda, The Netherlands

Smirnoff N, Stewart GR (1985) Stress metabolites and their role in coastal plants. Vegetatio **62:** 273-278

Son S, Lewis BA (2002) Free radical scavenging and antioxidative activity of caffeic acid amide and ester analogues: structure-activity relationship. Journal of Agricultural and Food Chemistry **50:** 468-472

St Omer L, Horvath SM (1984) Developmental changes in anatomy, morphology and biochemistry of *Layia platyglossa* exposed to elevated carbon dioxide. American Journal of Botany **71:** 693-699

Stewart AJ, Chapman W, Jenkins GI, Graham I, Martin T, Crozier A (2001) The effect of nitrogen and phosphorus deficiency on flavonol accumulation in plant tissues. Plant, Cell and Environment **24:** 1189-1197

Stewart GR, Larher F (1980) Accumulation of Amino Acids and Related Compounds in Relation to Environmental Stress. *In* The Biochemistry of Plants, Vol 5. Academic Press, pp 609-635

Stewart GR, Larher F, Ahmed I, Lee JA (1979) Nitrogen Metabolism and Salt-Tolerance in Higher Plant Halophytes. *In* RL Jefferies, AJ Davy, eds, Ecological Processes in Coastal Environments. Blackwell Scientific Publications, Oxford, pp 211-227

Stewart GR, Lee JA (1974) The role of Proline Accumulation in Halophytes. Planta **120:** 279-289

Stewart GR, Lee JA, Orebamjo TO (1973) Nitrogen metabolism of halophytes. New Phytologist **72:** 539-546

Stewart GR, Rhodes D (1978) Nitrogen Metabolism of Halophytes. New Phytologist **80:** 307-316

Stirling CM, Davey PA, Williams TG, Long SP (1997) Acclimation of photosynthesis to elevated CO_2 and temperature in five British native species of contrasting functional type. Global Change Biology **3:** 237-246

Stitt M (1991) Rising CO_2 levels and their potential significance for carbon flow in photosynthetic cells. Plant, Cell and Environment **14:** 741-762

Stitt M (1999) Nitrate regulation of metabolism and growth. Current Opinion in Plant Biology **2:** 178-186

Stitt M, Krapp A (1999) The interaction between elevated carbon dioxide and nitrogen nutrition: the physiological and molecular background. Plant, Cell and Environment **22:** 583-621

Stitt M, Quick WP, Schurr U, Schulze ED, Rodermel SR, Bogorad L (1991) Decreased ribulose-1,5-bisphosphate carboxylase-oxygenase in transgenic tobacco transformed with 'antisense' *rbcS*. Planta **183:** 555-566

Storey R, Ahmed N, Wyn Jones RG (1977) Taxonomic and Ecological Aspects of the Distribution of Glycinebetaine and Related Compounds in Plants. Oecologia **27:** 319-332

Tognetti R, Minnocci A, Peñuelas J, Raschi A, Jones MB (2000 a) Comparative field water relations of three Mediterranean shrub species co-occurring at a natural CO_2 vent. Journal of Experimental Botany **51:** 1135-1146

Tognetti R, Raschi A, Jones MB (2000 b) Seasonal patterns of tissue water relations in three Mediterranean shrubs co-occurring at a natural CO_2 spring. Plant, Cell and Environment **23:** 1341-1351

Troll W, Lindsley J (1955) A photometric method for the determination of proline. Journal of Biological Chemistry **215:** 655-660

Tuba Z, Jones MB, Szente K, Nagy Z, Garvey L, Baxter R (1998) Some Ecophysiological and Production Responses of Grasslands to Long-Term Elevated CO_2 under Continental and Atlantic Climates. Annals of the New York Academy of Sciences **851:** 241-250

Tyree MT, Jarvis PG (1982) Water in tissues and cells. *In* OL Lange, PS Nobel, CB Osmond, H Ziegler, eds, Physiological Plant Ecology II. Springer-Verlag, Berlin, pp 35-78

Urban O (2003) Physiological impacts of elevated CO_2 concentration ranging from molecular to whole plant responses. Photosynthetica **41:** 9-20

Van Arendonk JJCM, Niemann GJ, Boon JJ, Lambers H (1997) Effects of nitrogen supply on the anatomy and chemical composition of leaves of four grass species belonging to the genus *Poa*, as determined by image-processing analysis and pyrolsis-mass spectrometry. Plant, Cell and Environment **20:** 881-897

Veteli TO, Kuokkanen K, Julkunen-Tiitto R, Roininen H, Tahvanainen J (2002) Effects of elevated CO_2 and temperature on plant growth and herbivore defensive chemistry. Global Change Biology **8:** 1240-1252

Walker DA (1993) Polarographic measurement of oxygen. *In* DO Hall, JMO Scurlock, HR Bolhar-Nordenkampf, RC Leegood, SP Long, eds, Photosynthesis and production in a changing environment. Chapman and Hall, London, pp 168-180

Wall GW (2001) Elevated atmospheric CO_2 alleviates drought stress in wheat. Agriculture, Ecosystems and Environment **87:** 261-271

Wall GW, Brooks TJ, Adam NR, Cousins AB, Kimball BA, Pinter Jr PJ, LaMorte RL, Triggs J, Ottman MJ, Leavitt SW, Matthias AD, Williams DG, Webber AN (2001) Elevated atmospheric CO_2 improved *Sorghum* plant water status by ameliorating the adverse effects of drought. New Phytologist **152:** 231-248

Walsh D (2000) Physiological studies of salt tolerance in *Plantago maritima* (L.). PhD. Trinity College, Dublin, Ireland

Wang Z, Pan Q, Quebedeaux B (1999) Carbon Partitioning into Sorbitol, Sucrose and Starch in Source and Sink Apple Leaves as Affected by Elevated CO_2. Environmental and Experimental Botany **41:** 39-46

Wang Z, Quebedeaux B, Stutte GW (1996) Partitioning of [^{14}C]glucose into Sorbitol and Other Carbohydrates in Apple under Water Stress. Australian Journal of Plant Physiology **23:** 245-251

Wang ZC, Stutte GW (1992) The role of carbohydrates in active osmotic adjustment in apple under water-stress. Journal of the American Society for Horticultural Science **117:** 816-823

Ward LK, Spalding DF (1993) Phytophagous British insects and mites and their food-plant families: total numbers and polyphagy. Biological Journal of the Linnean Society **49:** 257-276

Waterman PG, Mole S (1994) Analysis of Phenolic Plant Metabolites. Blackwell Scientific, Oxford

Weatherley PE (1982) Water uptake and flow in roots. *In* OL Lange, PS Nobel, CB Osmond, H Ziegler, eds, Physiological Plant Ecology II. Springer-Verlag, Berlin, pp 79-110

Weaver LM, Herrmann KM (1997) Dynamics of the shikimate pathway in plants. Trends in Plant Science **2:** 346-351

Whittaker JB (2001) Insects and plants in a changing atmosphere. Journal of Ecology **89:** 507-518

Wilson JB (1988) A review of evidence on the control of shoot:root ratio, in relation to models. Annals of Botany **61:** 433-449

Wolfe DW, Gifford RM, Hilbert D, Luo Y (1998) Integration of photosynthetic acclimation to elevated CO_2 at the whole plant level. Global Change Biology **4:** 879-893

Woodell SRJ, Dale A (1993) *Armeria maritima* (Mill.) Willd. (*Statice armeria* L.; *S. maritima* Mill.). Journal of Ecology **81:** 573-588

Wu H, Haig T, Pratley J, Lemerle D, An M (2000) Distribution and exudation of allelochemicals in wheat *Triticum aestivum*. Journal of Chemical Ecology **26:** 2141-2154

Wullschleger SD (1993) Biochemical limitations to carbon assimilation in C_3 plants - a retrospectiveanalysis of the A/C_i curves from 109 species. Journal of Experimental Botany **44:** 907-920

Wullschleger SD, Tschaplinski TJ, Norby RJ (2002) Plant water relations at elevated CO_2 - implications for water-limited environments. Plant, Cell and Environment **25:** 319-331

Wustman BA, Oksanen E, Karnosky DF, Noormets A, Isebrands JG, Pregitzer KS, Hendrey GR, Sober J, Podila GK (2001) Effects of elevated CO_2 and O_3 on aspen clones varying in O_3 sensitivity: can CO_2 ameliorate the harmful effects of O_3? Environmental Pollution **115:** 473-481

Wyn Jones RG (1980) An assessment of quaternary ammonium and related compounds as osmotic effectors in crop plants. *In* DW Rains, RC Valentine, A Hollaender, eds, Genetic engineering of osmoregulation. Impact on plant productivity for food, chemicals, and energy. Plenum Publishing Corporation, New York, pp 155-170

Wyn Jones RG, Gorham J (1983) Osmoregulation. *In* OL Lange, PS Nobel, CB Osmond, H Ziegler, eds, Physiological Plant Ecology III. Springer-Verlag, Berlin, pp 35-58

Yeo AR (1983) Salinity resistance: Physiologies and prices. Physiologia Plantarum **58:** 214-222

Yordanov I, Velikova V, Tsonev T (2000) Plant responses to drought, acclimation, and stress tolerance. Photosynthetica **38:** 171-186

Yoshiba Y, Kiyossue T, Nakashima K, YamaguchiShinozaki K, Shinozaki K (1997) Regulation of levels of proline as an osmolyte in plants under water stress. Plant and Cell Physiology **38:** 1095-1102