The effect of elevated CO_2 on *Phaseolus vulgaris* L. cv Contender

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IN MEMORY OF MY FATHER
LADUMA ANTHONY 1939-1968

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DEFINITION OF SYMBOLS

Term	**Unit**	**Definition**
A	$\mu mol\ m^{-2}\ s^{-1}$	Assimilation of CO_2 per unit leaf area
A_l	$\mu mol\ m^{-2}\ s^{-1}$	Assimilation in the presence of stomatal limitation
A_{max}	$\mu mol\ m^{-2}\ s^{-1}$	Maximum net assimilation
A_o	$\mu mol\ m^{-2}\ s^{-1}$	Assimilation in the absence of stomatal limitation
ACE	$\mu mol\ m^{-2}\ s^{-1}\ C_i^{-1}$	Apparent carboxylation efficiency, calculated from the initial slopes of A/C_i
APS	$\mu mol\ m^{-2}\ s^{-1}$	Apparent rate of photosynthesis
C_a	$\mu mol\ mol^{-1}$	CO_2 concentration in the ambient air
C_i	$\mu mol\ mol^{-1}$	CO_2 concentration in the leaf intercellular spaces
E	$mmol\ m^{-2}\ s^{-1}$	Rate of transpiration or evapotranspiration
g_b	$\mu mol\ m^{-2}\ s^{-1}$	Boundary layer conductance
g_c	$\mu mol\ m^{-2}\ s^{-1}$	Conductance to diffusion of CO_2
g_s	$mol\ m^{-2}\ s^{-1}$	Stomatal conductance
J_{max}	$\mu mol\ m^{-2}\ s^{-1}$	Light-saturated rate of electron transport
K_i	$\mu mol\ mol^{-1}$	Inhibitor constant at the site of carboxylation
$K_m\ (CO_2)$	$\mu mol\ mol^{-1}$	Michaelis constant of CO_2
$K_m\ (O_2)$	$mmol\ mol^{-1}$	Michaelis constant for O_2
PPFD	$\mu mol\ m^{-2}\ s^{-1}$	Photosynthetic photon flux density
Q	$\mu mol\ m^{-2}\ s^{-1}$	Photon flux of photons
QE	$\mu mol\ m^{-2}\ s^{-1}$	Quantum efficiency
R_d	$\mu mol\ m^{-2}\ s^{-1}$	Dark respiration rate
V_{max}	$\mu mol\ m^{-2}\ s^{-1}$	Maximum initial carboxylation velocity
$V_{max}\ (CO_2ase)$	$\mu mol\ m^{-2}\ s^{-1}$	Maximum RuBP saturated rate of carboxylation
$V_{max}\ (O_2ase)$	$\mu mol\ m^{-2}\ s^{-1}$	Maximum oxygenation rate
WUE	$mmol\ mol^{-1}$	Water use efficiency
Γ^*	$\mu mol\ m^{-2}\ s^{-1}$	CO_2 compensation point of photosynthesis in the absence of dark respiration
Γ	$\mu mol\ m^{-2}\ s^{-1}$	Light compensation point
Δ	‰	^{13}C Discrimination

DEFINITION OF ABBREVIATIONS

Abbreviation	Definition
ABA	Abscisic Acid
AGS	Amyloglucosidase
ATP	Adenosine triphosphate
BS	Bundle sheath cells
CF1	The coupling factor of the ATP synthase complex in thylakoid membranes
$[CO_2]$	Carbon dioxide concentration
DMSO	Dimethylsulphoxide
g	Gravity
GA	Gibberellic Acid
HI	Harvest index
HPLC	High performance liquid chromatography
IAA	Indole-3-acetic acid
NADPH	Nicotinamide adenine dinucleotide phosphate
NAR	Net assimilation rate
NSC	Non-structural carbohydrates
$[O_2]$	Oxygen concentration
PCA	Primary carbon assimilation cells
PCR	Photosynthetic carbon reduction cells
PCO	Photorespiratory carbon oxidation
PEP	Phosphoenolpyruvate
PEPCase	Phosphoenolpyruvate carboxylase
PGA	3-phosphoglyceric acid
P_i	Inorganic phosphate
RGR	Relative growth rate
RH	Relative humidity
RPP	Reductive pentose phosphate
Rubisco	Ribulose bisphosphate carboxylase oxygenase
RuBP	Ribulose-1,5 bisphosphate
SLA	Specific leaf area
TPH	Total plant height
WSD	Weighted stomatal density

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PREFACE

Presently, most botanical journals contain at least one article per issue on the question of elevated CO_2, indicating the importance placed by researchers on this topic world wide. Indeed, increasing CO_2 concentrations in the atmosphere has the potential to alter both the physiological functioning of plants and the structure of plant communities. Consequently, quantity and quality of food and fibre production may change leading to serious economic and ecological consequences. This then, may be the reason why there has been so much interest in the scientific community. Despite many investigations that have been carried out in the past to examine the implications of elevated atmospheric CO_2, there remain many gaps which need to be investigated.

Due to the wide range of topics covered and also to the complexity of the field of study, each chapter is presented in the format of a paper, with separate introduction, results, discussion and conclusion. The materials and methods used in this investigation for all chapters are presented in Chapter 2. The general introduction (Chapter 1) is limited to the general aspects of elevated CO_2 such as its origin, and how it enters the living system. Other general aspects are covered and the chapter is concluded by a section in which the aims and objectives of the thesis are outlined.

In this thesis I attempt to fill some of those gaps, and I believe the thesis extends our knowledge of the responses of plants to elevated CO_2. To achieve this goal the following key areas of plant performance were addressed and are presented in four research chapters, beginning with Chapter 3 which investigates effects of elevated CO_2 on the foliar concentration of a wide range of macro- and micronutrients. Part of this chapter was presented at the *X International Photosynthesis Congress, Montpellier, France, 1995*. This work has been published in *Physilogia Plantarum* journal (Mjwara *et al.* 1996 Photosynthesis, growth and nutrient changes in non-nodulated *Phaseolus vulgaris* grown under atmospheric and elevated carbon dioxide conditions *Physilogia Plantarum* 97:754-763). Chapter 4 examines changes of non-structural carbohydrates and nitrogenous compounds; Chapter 5 examines yield and productivity; and finally, Chapter 6 discusses water use efficiency and carbon isotope discrimination. Part of this Chapter 6 was also presented at the *2nd International Symposium on Irrigation of Horticultural Crops, Chania, Crete, Greece, 1996*. This work has since been accepted for publication (Mjwara and Botha 1996 Water use efficiency in *Phaseolus vulgaris* exposed to elevated CO_2 *Acta Horticulturae* (in press).

In Chapter 7, the general discussion and conclusion, I have attempted to integrate the results and to highlight and interpret significant findings. Where possible, cross-referencing between chapters has been used to allow easy transition by the reader and all references appear in the list of references at the end.

ABSTRACT

The response of *Phaseolus vulgaris* L. cv. Contender grown in controlled environmental conditions, at either ambient or elevated (360 and 700 μmol mol^{-1}, respectively) CO_2 concentrations ([CO_2]), was monitored from 10 days after germination (DAG) until the onset of senescence. Elevated CO_2 had a pronounced effect on total plant height (TPH), leaf area (LA), dry weight (DW) accumulation and specific leaf area (SLA). All of these were significantly increased by elevated [CO_2] with the exception of SLA, which was significantly reduced. Except for higher initial relative growth rates (RGR) in CO_2-enriched plants, RGR did not differ significantly between the two CO_2 treatments throughout the remainder of growth period. While growth parameters clearly differed between CO_2 treatments, the effects of CO_2 on many physiological processes including net assimilation rate (NAR), Rubisco activity, and some foliar nutrient concentrations were largely transient. For example, CO_2 enrichment significantly increased NAR, but from 20 DAG onward, NAR declined to levels measured on plants grown under ambient CO_2. Similarly, the decline in both foliar N concentration and Rubisco activity in CO_2-enriched plants after 20 DAG was significantly greater than the decline observed for ambient CO_2 plants.

Soluble leaf protein and total chlorophylls (*a*+*b*) were also significantly reduced in plants grown under elevated CO_2. Chlorophyll (*a*/*b*) ratios increased with time under elevated CO_2, indicating that the rate of decline of chlorophyll *b* was higher than that of chorophyll *a*. No significant changes in total carotenoid (*x*+*c*) levels were observed in either CO_2 treatment. Under enhanced CO_2, the foliar concentrations of K and Mn

were increased significantly, while P, Ca, Fe and Zn were reduced significantly. However, changes in Mg and Cu concentrations were not significant. High CO_2-grown plants also exhibited pronounced leaf discoloration or chlorosis, coupled with a significant reduction in leaf longevity.

The levels of non-structural carbohydrates (sucrose, glucose, fructose and starch) and nitrogenous compounds (nitrogen, total soluble proteins and free amino acids) were determined for leaves and developing seeds of *P. vulgaris*. Leaf tissue of elevated CO_2-grown plants accumulated significantly higher levels of both soluble sugars and starch. Leaf ultrastructure revealed considerable enlargement of starch grain sizes with surface areas more than five times larger compared to those of control plants. No apparent differences in structure and membrane integrity of chloroplasts in both CO_2 treatments were noted. Although ambient CO_2-grown plants had comparatively low levels of non-structural carbohydrates (NSC), they accumulated significantly higher levels of nitrogenous compounds. The levels of NSC were consistently higher in seeds of plants grown under elevated CO_2. In comparison to plants grown at elevated [CO_2], pods and seeds of ambient CO_2-grown plants had significantly larger pools of free amino compounds and N.

Stomatal conductance (g_s) declined significantly, as expected for plants grown under elevated CO_2. This was accompanied by a decline in transpiration rates (E). Reduced g_s and E led to high A/E ratio, which meant improved water use efficiency (WUE) values for CO_2-enriched bean plants. Leaf carbon isotope discrimination (Δ) against the heavier isotope of carbon (^{13}C), has been used to select for high WUE in C_3 plants. In plants grown at elevated CO_2 concentration, Δ was significantly reduced. Although Δ was negatively correlated with WUE in both CO_2 treatments, the correlation was steeper and highly negative for CO_2-enriched plants. These results indicate underlying differences in gas-exchange physiology, including stomatal responses between ambient and elevated CO_2-grown plants.

Photosynthetic acclimation was investigated using the response of assimilation to internal carbon dioxide concentration (A/C_i curves). At early stages of growth, the initial slope of the A/C_i response curve did not differ with CO_2 treatment. In contrast, CO_2-saturated photosynthetic rate (A_{max}) was significantly higher in plants grown under elevated versus ambient CO_2 at 15 DAG. However, at subsequent stages of growth both the initial slope and A_{max} declined in bean plants grown in elevated CO_2. Apparent carboxylation efficiency (*ACE*, estimated from the initial slope of A/C_i response) values followed a similar trend and were significantly reduced in CO_2-enriched plants. These results indicate that acclimation or negative adjustment of photosynthesis may have been caused by a combination of both stomatal and biochemical limitations.

Bean plants grown under conditions of elevated atmospheric CO_2 flowered 3 to 4 days earlier, and produced significantly more flowers and pods than plants grown at ambient conditions. Plants grown at elevated CO_2 aborted 22 and 20% more flowers and pods, respectively, than plants grown at ambient CO_2. Elevated CO_2 also significantly increased the number of tillers or lateral branches produced by plants, which contributed to a significant increase in pod number and seed yield in these plants. Although plants grown at elevated CO_2 produced on average 8 seeds per pod, while plants grown under ambient CO_2 conditions produced 5 seeds per pod, the greater number of seeds was offset by lower seed weights in plants grown under elevated CO_2. Thus, despite high seed yield in beans grown under elevated CO_2, the harvest index (HI) did not change significantly between CO_2 treatments.

CHAPTER 1: INTRODUCTION

1.1 The rising global atmospheric CO_2 concentration

The concentration of carbon dioxide in the atmosphere has increased by approximately 30% over the past 200 years since the industrial revolution (Bacastow *et al.*, 1985; Neftel *et al.*, 1985; Roeckner, 1992). The pre-industrial level of CO_2 concentration [CO_2] was about 280 μmol mol^{-1}. Current levels are rising at a rate of approximately 1.8 μmol CO_2 mol^{-1} yr^{-1} (Hendry, 1992). Terrestrial photosynthesis and oceanic dissolution take up a substantial amount of CO_2, but this is balanced by CO_2 evolution from respiration, decomposition, fires and oceanic release (Taylor and Lloyd, 1992; Bowes, 1993). While anthropogenic CO_2 sources such as burning of fossil fuels, cement manufacture and deforestation comprise only a small percentage of the planet's total carbon budget, these anthropogenic sources are the major components that are currently destabilising the global carbon balance (Bacastow *et al.*, 1985; Neftel *et al.*, 1985; Roeckner, 1992; Bowes, 1993).

Attempts by the Intergovernmental Panel on Climate Change (IPCC), a body set up in 1988 by the World Meteorological Organisation (WMO) and the United Nations Environment Programme (UNEP), to formulate policies directed at reducing CO_2 emissions have not yet produced the desired effect. This may be because too few countries have adopted these policies or lack the capacity to implement such policies, and thus little or no progress has resulted (Taylor and Lloyod, 1992; Roeckner, 1992;

Gail *et al.*, 1994). Consequently, if emissions of CO_2 are unabated, they will inevitably cause a doubling (with respect to pre-industrial levels) of CO_2 concentration by the turn of the next century (Neftel *et al.*, 1985; Taylor and Lloyod, 1992; Roeckner, 1992; Bowes, 1993). The rate of the estimated change in atmospheric [CO_2], is shown in Figure 1.1.

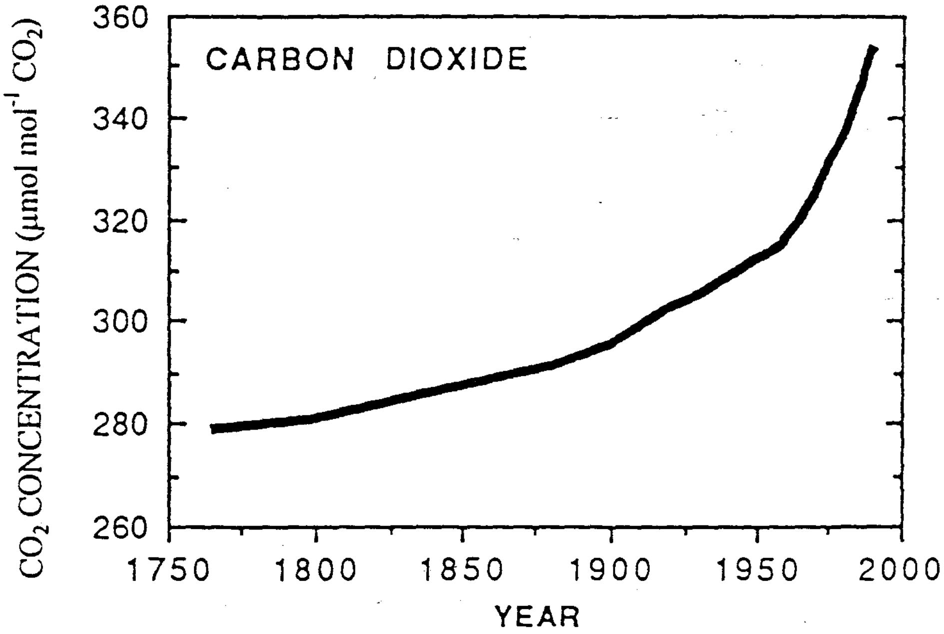

Fig. 1.1 Shows global rise of [CO_2] over the last 200 years (Roeckner, 1992).

1.2. Great global experiment

The greatest experiment in the world has begun, and through the rise of [CO_2], humans are changing the parameters that drive the planet's living system (Rogers *et al.*, 1994). As carbon dioxide is the first molecular link between the atmosphere and the biosphere, and is essential for photosynthesis, the entire food chain could be altered (Morell *et al.*, 1992; Rogers *et al.*, 1994). According to Woodrow (1994), the current rate of increase in atmospheric [CO_2] is probably occurring too fast for the process of plant species' evolution to keep pace with. Therefore plant scientists are faced with the challenge of predicting how plants and ecosystems will ecologically respond to a doubling of the current CO_2 levels over the next century.

1.3 Photosynthesis and rising CO_2

Carbon dioxide serves as substrate for photosynthesis and over half of the global flux of carbon passes through this process (Raven *et al.*, 1982; Galston, 1992). Consequently, whether plants can influence the rate of increase in atmospheric CO_2 by alterations in biotic storage capacity, or alternatively, how the rising CO_2 may impact on plants, crops and ecosystems are topics of major concern (Jarvis, 1989; Bazzaz, 1990; Dacey *et al.*, 1994). Most plants fix CO_2 through the C_3 pathway. The biochemistry of C_3 plants is physiologically limited by carbon at pre-industrial or even at current levels of [CO_2] (Zelitch, 1971; Wittwer, 1984; Bowes, 1991; Long, 1994).

The present $CO_2 : O_2$ ratio in the atmosphere encourages the competitive inhibition of ribulose-1,5-bisphosphate carboxylase oxygenase (Rubisco) enzyme by O_2 (Ogren, 1984; Chen and Spreitzer, 1992). Rubisco, being bi-functional, initiates both the photosynthetic carbon reduction (PCR) and photorespiratory carbon oxidation (PCO) cycles (Fig. 2). In addition, this enzyme has a low affinity for CO_2 and tends to favour oxygenation over carboxylation at low $CO_2 : O_2$ ratios (Andrews and Lorimer, 1987; Morell *et al.*, 1992; Long, 1994).

Badger (1992) also pointed out that the limitations imposed on photosynthetic efficiency by the interaction of CO_2 and O_2 with Rubisco are also manifested at limiting light intensities and high temperatures. At limiting light, the carboxylase and oxygenase reactions of Rubisco compete for the primary CO_2 acceptor, ribulose 1,5-bisphosphate, (RuBP) using ATP and NADPH generated by the light reactions of photosynthesis. Furthermore, the PCO cycle also consumes energy and evolves previously fixed CO_2 (Pearcy and Björkman, 1983). Therefore more quanta of light are required to fix CO_2 when conditions favour the RuBP oxygenase reaction and photorespiration. General circulation models (GCMs) also predict that the increase in atmospheric [CO_2], along with that of other radiatively active trace gases, will have significant effects on global climate (Raval and Ramanathan, 1989; Hogan *et al.*, 1991). As temperature increases, affinity of both CO_2 and O_2 decreases for carboxylase and oxygenase respectively (Badger, 1992). However there is a more rapid decrease in the carboxylase affinity, resulting in an increase in the ratio of oxygenation to carboxylation. In addition, this greater inhibition of carboxylation reaction may be due to the increased $CO_2 : O_2$ solubility ratio with increasing temperature (Edwards and Walker, 1983; Brooks and Farquhar, 1985), or a change in the kinetic constants for O_2 and CO_2 favouring reactions with O_2 (Edwards and Walker, 1983). The net result is an increase in photorespiration (Fig. 1.2).

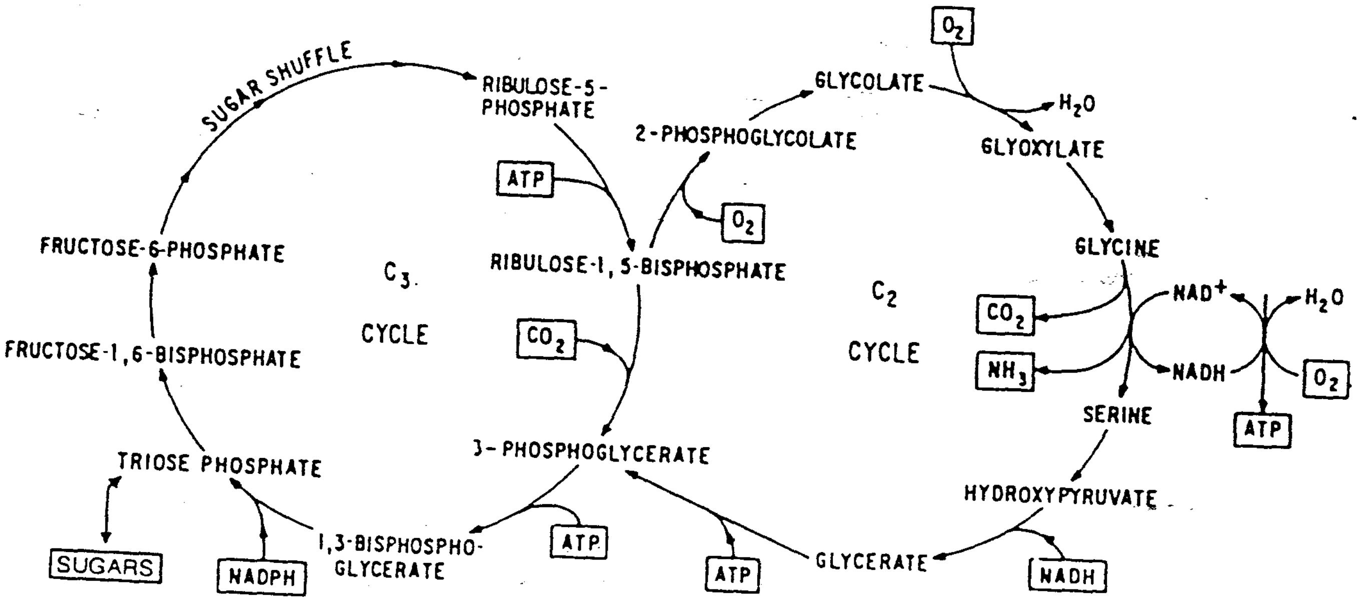

Fig. 1.2. Diagram showing the photosynthetic carbon reduction (PCR, sometimes referred as C_3 cycle) and photorespiratory carbon oxidation (PCO, sometimes referred as C_2 cycle) pathways. Rubisco catalyses both the carboxylation and oxygenation of RuBP, the initial reactions of the pathways (from Morell *et al.*, 1992).

Thus, at elevated temperatures, photosynthesis is limited by [CO_2] and inhibited by O_2. Consequently, the predicted increase in the concentration of CO_2 relative to O_2 will lead to reduction of oxygenation of Rubisco and thus increased rates of net photosynthesis. This response has been reported for a large range of crop and tree species. However, the increase in net assimilation rates (NAR) has been observed mostly in studies involving plants that have been exposed to short-term elevated [CO_2]. Critical reviews of this topic have recently been published (Bowes, 1993; Makino, 1994; Sage, 1994).

1.5 Potential benefits from improved photosynthesis at high [CO_2]

Some reports have postulated that with an increase in [CO_2], C_3 species will be able to compete successfully with the C_4 species (Badger, 1992; Israel and Nobel, 1994). In contrast to C_3 species, C_4 species utilise phosphoenolpyruvate carboxylase (PEPCase) as the first carboxylating enzyme. PEPcase is insensitive to O_2 concentrations [O_2], and little or no photorespiration is observed in C_4 plants. This response coupled with a suite of specialised anatomical features typical of C_4 plants helps to concentrate CO_2 at the site of carboxylation. This CO_2 concentrating mechanism effectively eliminates inhibitory effects associated with O_2-induced photorespiration (Hatch, 1987; Mjwara and Botha, 1993; Israel and Nobel, 1994). In addition, when other resources such as water, nutrients (particularly N and P), optimum light and temperature regimes are available (Larigauderie, 1988; Bazzaz and Fajer, 1992; Lavola and Julkunen-Tiitto, 1994), C_3 species may even become superior to C_4 plants (Badger, 1992; Rogers *et al.*, 1994 and references cited therein), as depicted in figure 1.3.

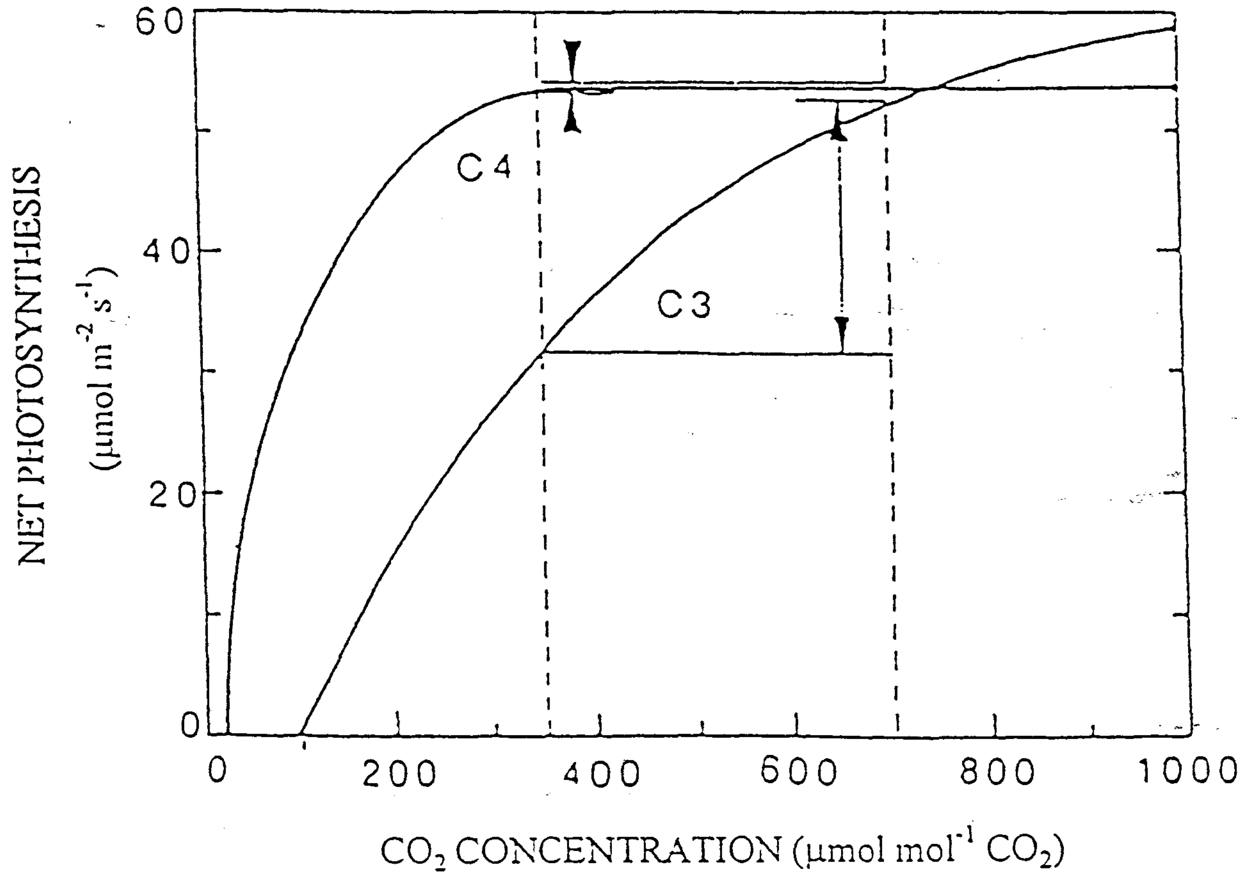

Fig. 1.3. Classical net photosynthesis curves for C_3 and C_4 species. Dashed vertical lines at 350 and 700 μmol mol^{-1} mark the current CO_2 level and the double concentration predicted to be reached sometime late in the next century. Arrows indicate incremental rise in net photosynthesis due to the CO_2 doubling (from Rogers *et al.*, 1994).

From the discussion above, it becomes apparent that in addition to increased photosynthetic rates, there are other potential benefits to increased [CO_2] such as; 1) an increased light efficiency as a result of the elimination of energy wastage associated with photorespiration; 2) greater nitrogen use efficiency, as a result of the reallocation of nitrogen from both Rubisco (Ziska and Teramura, 1992; Tissue *et al.*, 1993) and from components of the light harvesting protein complex (Badger, 1992; Lynch and González, 1992); 3) improved water-use efficiency as a result of the enhanced NAR at the same, or a decreased transpiration rate; and finally 4) broader niches for C_3 plants as they may spread from their traditional ecological niches (low light and temperature) to areas with higher temperatures and lower precipitation (Goldewijk *et al.*, 1994).

1.6 Too much of a good thing? Acclimation to elevated CO_2

While short-term exposure to elevated [CO_2] increases the rate of photosynthesis in terms of CO_2 assimilation per unit leaf area in C_3 plants (Figure 1.3), some studies show that this increase may be transitory and may not be maintained in the long term (Delucia *et al.*, 1985; Sage *et al.*, 1989; Van Oosten *et al.*, 1993; Xu *et al.*, 1994a). Numerous investigations have been undertaken recently in an attempt to clarify the causes of this acclimation phenomenon, often referred to as down-regulation. To date there is no consensus on the causes of down-regulation of net photosynthesis (Xu *et al.*, 1994a, and references cited therein). In fact, Bowes (1993) has deemed acclimation to elevated CO_2 more as a rule rather than the exception.

In addition to changes in Rubisco biochemistry and stomatal physiology (as the first parameters to come into direct contact with CO_2), differences in anatomy, morphology and phenology have also been observed (Bowes, 1993). These include reports of greater leaf area, increased leaf area index, leaf duration, leaf thickness, branching or tillering in stems, root length, total biomass and fruit size. In addition increased numbers of flowers, fruits and seeds, and alteration in the timing of developmental events have been reported (Calvert, 1972; Garbutt and Bazzaz, 1984; Enoch and Zieslin, 1988; Coleman *et al.*, 1993; Idso and Idso, 1994). Perhaps the most important point in this regard was raised by Bowes (1993), who stated that despite the decline in photosynthesis per unit leaf area, changes in other morphological features (listed above) may actually be improved under elevated CO_2.

In their review of the past 10 years of elevated CO_2 research, Idso and Idso (1994) argued that the "grand-average percentage plant growth enhancement" (i.e. percentage growth response of natural ecosystems to atmospheric CO_2 enrichment) suggests that wherever environmental conditions are less than ideal, the plant growth response to atmospheric CO_2 (expressed as a percentage) will probably be greater than it would have been under optimum growth conditions, and that this difference will continue to increase with the CO_2 content of the air. In contrast, other scientists have suggested that various resource limitations in combination with environmental stresses would reduce the growth stimulation provided by elevated CO_2 (Strain and Cure, 1985b; Lincoln *et al.*, 1986; Bazzaz and Fajer, 1992). It is apparent therefore that, we do not as yet fully understand the response of plants to elevated CO_2 particularly the long-term response.

1.7 Research objectives

The literature on elevated CO_2 suggests that species differ markedly in their response to elevated CO_2 (see reviews by Newton, 1991; Bowes, 1993; Rogers and Dahlman, 1993). Unfortunately, most of these studies have focused on the short-term effects of whole plant growth and enhanced photosynthetic rates (Newton, 1991; Lawlor and Mitchell, 1991). Longer-term studies which demonstrate acclimation or down-regulation also suggest large differences between species (Stitt, 1991; Baxter, 1994a).

An understanding of the mechanisms underlying the acclimation response to elevated [CO_2] is essential in order to predict accurately the impact of the future global rises of atmospheric CO_2. Consequently, the primary aim of this research was to investigate the cause(s) of acclimation, and at the same time, to incorporate other developmental aspects that could impact directly on plant productivity (e.g. yield quality and quantity). This information may also help us understand plants living in other ecosystems. Earlier researchers have reported reductions of nitrogen (Wong 1979; Wong and Osmond, 1991) and sometimes phosphorus content (Conroy *et al.*, 1992) in plants exposed to elevated CO_2. Very little information is available on a wide range of other macro- and micro-nutrients (Newton, 1991; Stulen and Den Hertog, 1993).

In an attempt to address the points raised above, I investigated a range of growth and physiological parameters of *P. vulgaris* exposed to a doubling of CO_2. The parameters I measured included: foliar nutrient concentrations, net assimilation rates, Rubisco activity, chlorophyll changes, leaf chlorosis, and several other growth attributes (Chapter 3). These parameters were examined in some detail. The use of this integrated approach should provide further insight into the dynamics of nutrient-plant relationships, and possibly shed some light on the functional relationships that may result due to CO_2-induced changes in these parameters induced by elevated CO_2. This data should enable us to make more reliable predictions regarding plant performance in CO_2-enriched environments, including productivity and yield quality.

It is expected that virtually all physiological processes in plants will be affected as carbohydrates levels change in response to CO_2 enrichment (Strain and Cure, 1985b; Farrar and Williams, 1991). Perhaps the most immediate process known is nutrient dilution, whereby non-structural carbohydrates (NSC) have been shown to increase in plants grown under elevated CO_2 (Kuehny *et al*, 1991). However, few studies have investigated the impacts of NSC on carbohydrate storage and subsequent translocation to seeds in plants grown under elevated CO_2. This and other aspects regarding changes in nitrogenous compounds are reported in Chapter 4.

While some results have been reported on changes in reproductive behaviour under elevated CO_2, there is still a dearth of information in this area (Newton, 1991;

Tremmel and Patterson, 1994; Rawson, 1995). Elevated [CO_2] almost always affects some aspects of plant development (Coleman *et al.*, 1993; Miglietta *et al.*, 1993b). Therefore factors affecting the timing of ontogenetic events such as tillering, flowering and seed set may also change (Tremmel and Patterson, 1994). Changes in reproductive patterns such as flower production and abortion, fruit and overall yield are presented in Chapter 5.

Most of the recent studies on elevated CO_2 have demonstrated that transpiration is reduced as a result of partial closure of stomata, which is clearly induced by elevated CO_2 (Bazzaz, 1990; Ceulemans and Mousseau, 1994; Goldewijk, 1994). This contributes to an increase in water use efficiency (WUE). Physiologically, increased WUE represents one of the most significant responses to elevated CO_2 identified thus far (Roger *et al.*, 1994). Some reports have indicated that elevated CO_2 increases plant size (due to an increase in leaf areas and sometimes number of branches, Coleman *et al.*, 1993; Miglietta *et al.*, 1993b), which leads to higher total losses of water through transpiration. This may counterbalance the reduction in water use per unit leaf area (Morison and Gifford, 1984; Jarvis, 1989; Wolfe and Erickson, 1993; Baker and Allen, 1994).

Still other reports have indicated that WUE is highest under elevated CO_2 where the leaf area index (LAI) is low. Tschaplinski *et al.* (1993) maintain that it is likely that the increased WUE of plants growing under elevated CO_2 will be able to compensate for water lost as a result of enhanced plant size. Samarakoon *et al.* (1995) demonstrated that a doubling of the atmospheric CO_2 level caused about a 30% reduction in transpiration, irrespective of soil water content and radiation level. Jarvis (1989) and Gorissen *et al.* (1995) pointed out that there is not enough information available to determine whether the changes in stomatal conductance in response to elevated CO_2 are merely a short-term transient response, or a long-term acclimation response. Thus the question of enhanced WUE under elevated CO_2 is still open to debate. This and other aspects of WUE including carbon isotope discrimination in relation to acclimation are addressed in Chapter 6.

It is a fact that all plant species and their communities will be affected by increasing levels of CO_2, yet it is impossible to study and determine these effects in all species. For this and other obvious reasons, Strain and Cure (1985a), in their executive summary (*Direct effects of increasing carbon dioxide on vegetation report by the United State Department of Energy , USDOE*), recommended several agriculturally important crop plants for immediate investigation. In order of decreasing priority, these were: Wheat, corn, white potato, soybean, rice, sweet potato, sorghum and cotton. In line with this, I chose to use the common bean *Phaseolus vulgaris* cv. Contender, an important source of protein in South Africa and other less developed countries.

CHAPTER 2: MATERIALS AND METHODS

2.1 Plant material, culture and growth conditions

2.1.1 Rationale of choosing Phaseolus vulgaris L. cv. Contender as an experimental plant

Phaseolus vulgaris L. cultivars have attracted the attention of researchers studying plant responses to environmental stresses, particularly atmospheric pollutants (Okamoto *et al.*, 1991). In addition to its economic importance resulting from its high protein content (Murray, 1984; Peña-Valdivia *et al.*, 1993), there are a number of features that favour the use of *P. vulgaris* particularly cultivar Contender as an experimental plant in this current research. Among those attributes are:

(i) *P. vulgaris* has an exceptionally short growth cycle and consequently reaches a marketable stage within 40-50 days under favourable conditions. This allows for multiple cultivations within a short space of time, as well as sufficient replication for both scientific verification and statistical purposes.

(ii) *P. vulgaris* has a small size (dwarf bean) and can be grown conveniently under confined conditions of controlled environment facilities such as the growth chambers used in this study.

(iii) The distinct separation of vegetative (roots, stem, leaves) and reproductive structures (flowers, pods and seeds) allows for unambiguous sampling of these organs for studies investigating assimilate partitioning among sources and sinks (Kostka-Rick and Manning, 1993).

These features make *Phaseolus vulgaris* L. cv. Contender a convenient experimental plant that is useful as a model species for studying the responses of crops to elevated concentrations of carbon dioxide.

2.1.2 Plant culture

Seeds of non-nodulating *Phaseolus vulgaris* L. cv. Contender were germinated in controlled environment cabinets either Conviron Ef-7H or Conviron S10H (Controlled Environments Ltd, Winnipeg, Canada), set at 360 or 700 μmol mol^{-1} atmospheric CO_2, respectively. Following germination, healthy seedlings of the same length and stages of development were transferred individually into 3 L plastic pots. This pot size has been shown to be adequate in preventing root restrictions (Arp, 1991). Pots were filled with a commercially obtained potting mixture with a pH of 6.2. Five g of slow-releasing fertilizer (NPK 2:3:2; Wonder Horticultural Products, Johannesburg, South Africa) was added to each pot prior to transplanting seedlings. Plants were watered as required, and every fifth day nutrients were added using a complete nutrient concentrate (Chemicult, Chemicult Products, Pty Ltd, Camps Bay, Cape Town, South Africa) containing the folowing: 6.5% N; 27% P; 13% K; 7.0% Ca; 2.2% Mg; 7.5% S; 0.15% Fe; 0.024% Mn; 0.024% B; 0.005% Zn; 0.002% Cu;

and 0.001% Mo. The concentrations of both macro- and micronutrients were essentially similar to those described by Yelle *et al.* (1987).

2.1.3 Growth conditions

Plants were grown continuously in the controlled environment chambers until the onset of senescence. The CO_2 concentration in each chamber was monitored and maintained at approximately 360 and 700 μmol mol^{-1} CO_2, respectively. Growth temperatures were set at 25/18°C day/night, and photoperiod was maintained at 16 h throughout the experiments. Light was provided by a combination of fluorescent tubes (F48T12.CW/VHO1500, Sylvania, USA) and frosted incandescent 60W bulbs (Phillips, Eindhoven, The Netherlands). The photosynthetically active radiation (PAR 400-700 nm) was approximately 500 μmol m^{-2} s^{-1} at the top of the plant canopy as measured with a Li-85A Quantum sensor (Li-Cor Inc., Nebraska, USA). To maintain constant vapour pressure deficits, relative humidity (RH) was set at 65/45% day/night respectively in both cabinets. The CO_2 concentrations in both cabinets were monitored using a LCA-2 portable infra red CO_2 gas analyser (Analytic Development Co. Ltd., Hoddesdon, Herts., U.K). There were slight insignificant fluctuations recorded in the ambient CO_2 chamber, mostly within 15 μmol mol^{-1} CO_2 limit above or below the average 360 μmol mol^{-1} level. In contrast, the enhanced CO_2 chamber maintained relatively constant levels of 700 μmol mol^{-1} CO_2 for the duration of the experiment. Plant growth conditions differed only with respect to CO_2 concentration.

2.2 Statistical analysis

Unless otherwise stated, all measurements were replicated three times. The following data were analyzed using a two-way analysis of variance to test for differences in the mean: growth attributes (LA, TPH, DW, RGR), foliar concentrations of macro- and micronutrients, gas exchange parameters (NAR, g_s, C_i, E), WUE, WSD, carbon isotope discrimination, reproductive parameters (flower numbers, pods, seed weight and numbers, harvest index), NSC, proteins, and amino acids. Treatments were considered statistically different at the 5% level of significance. In addition, I tested the significance between sampling days, as well as for the possible level of interaction between factors. Scheffe's multiple range test was used to establish where significant differences occurred. Differences in the gas exchange parameters were tested using the F-ratio statistic. Homogeneity of the variances using Levene's test was checked prior to ANOVA. Association among characters were examined by correlation and regression analysis.

2.3 Growth biomass and yield measurements

2.3.1 Growth measurements and observations

Various growth parameters such as leaf area (LA), dry weight (DW), total plant height (TPH) and relative growth rates (RGR) were measured 10 days post-germination, then following the emergence of the first trifoliolate leaf, and subsequently every fifth day until the plants reached senescence. Leaf areas of the same leaves used for gas exchange rates were measured using an AAC-400 area meter

(Hayashi Denkoh Co. Ltd, Tokyo, Japan). Leaf dry weight was measured using leaf material which had been oven-dried at 60°C for 72 hours, after which constant weight was attained.

In addition to visual examination of the leaf status such as chlorosis, leaf senescence and abscission, several newly formed trifoliolate leaflets were randomly tagged at different stages of growth and development in both CO_2 treatments, and monitored to determine leaf longevity (i.e. days till abscission). The number of tillers was also noted and recorded at similar time intervals. Relative growth rates were calculated as described by Hunt (1982) using the following equation (2.1):

$$\frac{\ln W_2 - \ln W_1}{t_2 - t_1} \qquad \text{Equation 2.1}$$

2.3.2 Reproductive parameters and yield measurements

Sampling and measurement of reproductive structures was performed as described by Rochat and Boutin (1989). Counting of days after anthesis (DAA) started when one fully opened flower appeared. Thereafter, monitoring and counting of all flower buds and opened flowers per plant was carried out every third day. Number of pods was also counted in the similar way. Pod length and the number of seeds per pod was recorded. Seeds could only be separated from their pods from 10 DAA, due to their small size before this date. Early immature seeds (soft and green) were collected from fully matured pods (which were marked by change in colour from green to pale yellow), while mature or late seeds were harvested from pods which were beginning to show brownish colour. The seeds at this late stage had lost all green colouring, resembling ordinary mature seeds. Seed dry weights were measured from ten seeds from each CO_2 treatment after drying at 70°C until constant weights were achieved. Harvest index (seed biomass/total plant biomass, HI) was determined according to Donald and Hamblin (1976).

2.4 Leaf gas exchange measurements

2.4.1 Experimental setup and calibration

A portable ADC LCA-2 infra red CO_2 gas analyser (IRGA) coupled to an ADC DL-2 datalogger (Analytic Development Co. Ltd., Hoddesdon, Herts., U.K) was used to measure net assimilation rates. Calibration and operation has been described elsewhere (Mjwara and Botha 1993). In order to reduce interplant variability, all gas exchange measurements were performed on recently-expanded leaves of the same age and position on plants from each CO_2 treatment. All measurements were made between 1100 and 1200 h (i.e. 4-5 hrs into the light period). ADC GD-600 gas diluters (Analytic Development Co. Ltd. Hoddesdon, Herts., U.K.) were used to reduce the concentration of the CO_2 to the same level as that of the CO_2 chamber, and to ensure that similar conditions in the leaf chamber were maintained during photosynthetic measurements. The flow rate of the gas stream was kept constant at 350 ml per minute using an ADC ASUM mass flow meter (Analytic Development Co. Ltd. Hoddesdon, Herts., U.K.).

After steady conditions were achieved, gas exchange measurements were taken at 15 min intervals using an LCA-2 portable infra-red CO_2 gas analyser (IRGA) coupled to an ADC DL-2 datalogger (Analytic Development Co. Ltd. Hoddesdon, Herts., U.K.). The IRGA was set up in differential mode in open circuit. The IRGA was calibrated weekly using bottled gas (Fedgas Alrode, Port Elizabeth, S.A) with a known CO_2 concentration (400 µmol mol^{-1} CO_2).

2.4.2 Light supply and temperature control.

Actinic light was provided by a Phillips (SON-T) 400 Watt high pressure sodium lamp with maximum light intensity in excess of photosynthetic photon flux density (PPFD) of 2000 µmol $m^{-2}s^{-1}$. To achieve the required light intensity, fine shade cloth frames were mounted under the light source, above the leaf chamber. The temperature of the leaf chamber was closely monitored and regulated using a Lauda RM-3 (Optolabor, Johannesburg, South Africa) Multitemp water circulator. Preliminary light response measurements indicated that the optimum light intensity point for the common bean was at ≈800 µmol $m^{-2}s^{-1}$ PPFD at a leaf temperature of 25°C. Therefore all photosynthetic measurements were made at 800 µmol m^{-2} s^{-1} PPFD at a leaf temperature of 25°C.

2.4.3 Humidity Control

The ADC LCA-2 requires that all gas exchange measurements are taken on a dry-air bypass setting. A pre-humidifier with wet-and-dry bypass was incorporated into the gas flow system, between the pump and the ADC LCA-2 datalogger. In order to prevent stomatal closure in response to the dry airstream, the leaf was allowed to acclimatise between readings for 15 min on a humid air bypass. Relative humidity of the humid air bypass setting was maintained at approximately 68-70%.

2.4.4 Assimilation (A) versus internal CO_2 (A/C_i) experiments

The A/C_i curves were constructed using bottled CO_2 gas with less than 1% O_2 (Fedgas Alrode, Port Elizabeth, South Africa) balanced against nitrogen. Response of A to changes in C_i was measured as described by Sage *et al.* (1988). Leaves were first exposed to their respective growth CO_2 concentrations (either 360 or 700 μmol mol^{-1} CO_2) until steady state conditions were achieved (usually between 10-15 min). Two in-line ADC GD-600 gas diluters were used to reduce the concentration of the CO_2 in the leaf cuvette to approximately 0 μmol mol^{-1} CO_2, before the first measurements were taken. Subsequent readings were taken as the CO_2 concentration in the leaf cuvette was increased in predetermined steps to a maximum of 1000 μmol mol^{-1} CO_2. Apparent carboxylation efficiency (*ACE*) was calculated from the initial slope of the curve relating A to C_i according to von Caemmerer & Farquhar (1981; also see parameter calculations in Appendix B).

2.4.5 Principle of the photosynthesis measurements and related calculations

The concept that photosynthesis is measured with a system, rather than a single instrument, is an important one. The system concept emphasises the fact that there is no discrete photosynthetic sensor. Photosynthesis is always a calculated parameter, determined from measurements of internal CO_2 concentrations, gas flow, leaf area, temperature, humidity, stomatal conductance and transpiration (Field *et al.* 1989). It was therefore essential to use calculations incorporating these parameters, similar to those described by von Caemmerer and Farquhar, (1981). However, the calculations (Appendix B) in this case were adapted by Botha and Brown (1991) in their software package, IRCAL (Version 2.0), (Copyright by C.E.J. Botha and B.J.L. Brown, Rhodes University, Grahamstown, 1991).

2.5 Carbon isotopic discrimination measurements

Carbon isotope composition was determined from leaves of a similar morphological age as those used for gas exchange measurements. Leaves were dried at 70°C for 72 hrs, and then finely ground and passed through #30 mesh screen. This process ensures homogeneity and improves combustion. Carbon isotope composition was measured at the Ematek Laboratory, C.S.I.R, Pretoria, SA. Sub-samples of oven-dried leaves (5 mg) were combusted in special sealed quartz vessels in a Carlo Erba CHN elemental analyser under pure atmospheric O_2 at 860°C. To determine the $\delta^{13}C$ content from the CO_2 resulting from combustion, the delta E isotope ratioing mass spectrophotometer (Finnigan MAT) was used. Leaf carbon isotope discrimination was then calculated using equation 2.2 (Farquhar and Richards 1984) assuming carbon isotope composition in ambient air was -8‰ with respect to sample and Pee Dee Belemnite (PDB) standard, respectively:

$$\Delta = \frac{\delta_a - \delta_p}{1 + \delta_p} \qquad \text{Equation 2.2}$$

where δ_p and δ_a refer to the carbon isotope ratios of leaf material and source air, respectively. While the PDB standard was applicable for plants grown under ambient atmospheric air, a value of -22‰ was used for CO_2-enriched plants, obtained from analysis of source air (as was suggested by G.D. Farquhar, *pers. comm.*, 1994). The determination of leaf Δ was repeated at least two times. Other related parameters and their equations are described below. Farquhar *et al* (1982) and later (1989) defined Δ in C_3 plants as being directly related to the ratio of intercellular to ambient CO_2 concentration (Ci/Ca):

$$\Delta = a + (b - a)\frac{c_i}{c_a} \qquad \text{Equation 2.3}$$

where a is the fractionation occurring as a result of CO_2 diffusion in air (4.4‰), b is the net fractionation caused by Rubisco during carboxylation (27‰), and C_i/C_a the ratio of intercellular to ambient CO_2. Farquhar *et al.* (1989) argue that WUE is also closely correlated with C_i/C_a in the relationship:

$$W = \frac{A}{E} = \frac{C_a\left(1 - \frac{C_i}{C_a}\right)}{1.6v / P} \qquad \text{Equation 2.4}$$

where v is the water vapour pressure difference, *1.6* is the factor which arises because the binary diffusivity of water vapour and air is 1.6 times than that of CO_2 and air.

2.6 Determination of tissue elements

2.6.1 Tissue preparation and extraction for macro- and micronutrients

After determining dry weights, leaf material was finely ground using a Casella Grain Mill (to pass through a #30 mesh sieve/screen). Sub-samples of dry leaf material were then ashed at 490°C in a Muffle Furnace (Gallenkamp, Laboratory Equipment, U.K). After cooling, the ash samples were collected and treated with 1N HNO_3, reheated to 90°C to ensure complete oxidation and to dehydrate silica in accordance with the methods of Hasses (1971) and Allen *et al.* (1974). After heating, 1 ml of concentrated HCl was added to the residue which was then dissolved in 10 ml distilled H_2O, and further warmed to facilitate complete dissolution. The solution was filtered through a No. 44 filter paper into 50 ml volumetric flask and further diluted to volume. Hereafter, this solution is referred to as original solution (OS).

2.6.2 Phophorus determination

Three dilutions were prepared from the OS. The first dilutions were performed using automatic diluters. In triplicate, 3 ml aliquots of OS were mixed with 27 ml of vanadomolybdophosphoric yellow reagent (V.M.N.) and transferred into Kimble vials for P determination.

2.6.3 Preparation of Vanadomolybdophosphoric yellow reagent (V.M.N.) and P determination

The V.M.N. was prepared using method described by Hasses (1971) and Allen *et al.* (1974). The reagent is comprised of two solutions in a ratio of 1:1 (v/v). Solution A (50 g of AR ammonium molybdate was dissolved in 800 ml of deionised H_2O) and poured into a 2 litre volumetric flask containing solution B (2.5 g of AR ammonium meta-vanadate dissolved in 600 ml of boiling deionised H_2O). After cooling, 500 ml of conc. AR nitric acid was added, and then cooled again prior to addition of deionised H_2O to bring the mixture to volume. All chemicals were obtained from Merck chemical Co., Darmstadt, Germany.

Phosphorus was measured using a Shimadzu UV-160A UV visible recording spectrophotometer (Shimadzu Corporation, Analytical Instruments Division, Kyoto, JAPAN) set at 446nm. Absolute amounts of P concentrations were determined from a standard curve prepared from dilutions of stock solution (0.1295 g dissolved in 400 ml deionised H_2O stored in polythene bottle) of dipotassium hydrogen phosphate anhydrous (K_2HPO_4) with V.M.N. stock to give various concentrations from 0 to 25 ppm.

2.6.4 Dilutions from OS for remaining macronutrients

For determination of the remaining macronutrients, 1ml OS was mixed with 1 ml of 20 ml of 5% lanthanum chloride and made to volume in 50 ml volumetric flask with deionised H_2O.

2.6.5 Preparation of 5% Lanthanum stock solution

The reagent was prepared by dissolving 58.65 g AR La_2O_3 (Merck) in 250 ml of concentrated HCl and deionised H_2O made to volume in a 500 ml volumetric flask. This stock solution was then used to prepare the diluted lanthanum chloride (316 ml stock/1 litre deionised H_2O, v/v) for dilution with OS.

2.6.6 Preparation of OS for micronutrients

The balance of the remaining OS was transferred into Kimble vials for determination of the trace elements Fe, Cu, Mn and Zn without further dilutions.

2.6.7 Determination of macro- and micronutrients

Concentrations of macronutrients (Ca, Mg, and K) and micronutrients (Cu, Mn, Fe and Zn) were determined using a Varian AA 1275 atomic absorption spectrophotometer (Varian Techtron, Pty Ltd, Australia). Concentrations were determined from the absorbance readings after careful calibration of the AA instrument with appropriate AAS standards for all the elements investigated (AAS standards were commercially obtained from Clinical Sciences Diagnostics, Division of C.S.M.L (PTY) LTD. Booysens, S.A).

2.6.8 Tissue preparation and extraction for Nitrogen

Sub-samples (0.250 g) of dry leaf material were digested using a KB 12S Micro-Kjeldatherm, Kjeldahl digestion system (G. Gehardt & Co., KG Fabrik Fu Larburgerate, Federal Republic of Germany) using the protocols of Hasses (1971) and Allen *et al.* (1974). Two grams of AR potassium sulphate was added to each flask, prior to the addition of 2.5 ml concentrated H_2SO_4 (A.R, SG of 1.84 Kg, 98.08 g/mol) and 0.5 ml of mercury catalyst (prepared from 10 g of red mercury (II) oxide (HgO) dissolved in 100 ml of deionised H_2O containing 11 ml of concentrated H_2SO_4 stored in a polythene bottle). The samples were allowed to boil for 35 min to digest. After clearing, the temperature was reduced. The mixture was allowed to simmer for a further 20 min, but the total digestion did not last more than 1 h to prevent possible loss of N due to the decomposition of the ammonium sulphate formed in the reaction (Allen *et al.*, 1974). After completion of digestion, the mixture was quantitatively transferred to a steam-distillation apparatus after addition of 10 ml deionised H_2O and 10 ml of caustic/hypo solution (prepared from 25 g sodium thiosulphate solution dissolved in 200 ml deionised H_2O and 400 g NaOH, made to volume in 1 litre volumetric flask).

2.6.9 Nitrogen determination

The titration of the distillate was carried out with standard 0.015 *M* HCl solution (12.5 ml conc. HCl to 10 litres of deionised H_2O, standardised with 10 ml aliquots of 0.01 *M* NaOH and two drops of screened indicator) to the grey end-point or the first tinge of pink in 250 Erlenmeyer flasks containing 1% boric acid indicator (0.2% methyl red solution and 0.2% methylene blue solution in 95% ethanol which was mixed in 2:1 ratio stored in an amber glass container). Blank determinations were performed on collected distillates in which the above procedure was followed without any leaf material. Volumes of standard HCl solution were recorded, and after subtraction of mean blank readings, foliar N content was calculated.

2.7 Carbohydrate analysis

2.7.1 Leaf sample preparation and extraction of non-structural carbohydrates

Sampling was performed 10 DAG and every fifth day thereafter until senescence. The extraction and analytic procedures of Bucker and Ballach (1992) were used in this investigation with slight modifications to suit our experimental conditions. Mature leaves were harvested (between 13 and 15h00) and quickly frozen in liquid nitrogen. Pulverized, lyophilized (for 36 hr) samples (250 mg) were extracted 3 times each for 20 min (in shaking water bath set at 90°C) with 5 ml of hot 80% (v/v) ethanol/H_2O solution. The homogenates were combined and centrifuged at 1 500 *g* using the RC-Sorvall Superspeed Refrigerated Centrifuge (Sorvall Du Pont Instruments, USA) at room temperature for 15 min. In order to remove the contaminants, the supernatants were transferred into 500 ml separating funnels and separated into hydrophobic (mostly oils and chlorophylls) and hydrophillic (largely water soluble carbohydrates) fractions by adding diethylether (60 ml) and distilled H_2O (3 X 100 ml, Austenfeld, 1986).

The hydrophobic fraction was discarded and the hydrophillic fraction was evaporated to dryness *in vacuo* using a Buchi Rotavapor-RE (Savant Instruments, Inc., Farmingdale, NY, USA) set at 40°C, and equipped with a refrigerated condensation trap (Lauda RM-3) operating at -23°C. Samples were resuspended in 5 ml xylitol (1 mg ml^{-1} (w/v) solution Merck). This solution was used as the internal standard. To ensure ultra-purity, the samples were first passed through Sep-pak C_{18} cartridges (Waters Chromatography Division, Millipore Corporation, Milford, MA, USA) before finally vortexed using Beckman TJ-6 Tabletop centrifuge (Beckman Instruments, Inc., Irvine, CA, USA) through a 0.45 mm membrane filter (Acro LC 13, Gelman Sciences) at 10 000 rpm for 10 min. The samples were then stored in small sterile vials at -20°C until they could be quantified using high performance liquid chromatography (HPLC). The pellet was also evaporated to dryness and then stored in a glass desiccator over an anhydrous desiccant until further analysis for starch.

2.7.2 Pods and seeds sample preparation and extraction of non-structural carbohydrates

Extraction procedure of oven-dried samples for soluble and insoluble carbohydrates fractions were exactly the same as that of leaf samples.

2.7.3 Analysis and quantification of NSC

Changes in the pool sizes of the primary non-structural carbohydrates (starch, sucrose, glucose and fructose) usually present in large amounts in bean plants (Buysse and Merckx, 1993) were investigated. Two methods of analysis of NSC were employed, one for 80% ethanol-soluble fraction (section 2.7.3.(ii)) and one for non-soluble fraction (section 2.7.3.(ii)).

2.7.3. (i) Soluble carbohydrates assay

Soluble carbohydrates analysis and quantification was performed according to the method described by Bucker and Ballach (1992). Sucrose, glucose and fructose were separated and quantified using a Spectra-Physics high performance liquid chromatography (HPLC) system that was equipped with an Isochrom LC pump, a Supelcogel^{TM} C-611 (weak ion exchange gel), 30 cm X 7.8 mm ID column, and an Erma ERC-7515A differential refractometer index detector which was connected to an SP-4290 integrator. Prior to injection of approximately 200 μl of the sample, the column was eluted with ultra pure H_2O, followed by 10^{-4}M NaOH which was the running eluent throughout the analysis. The flow rate was 0.5 ml min^{-1}, and column temperature was maintained at 60°C. Sugars were identified and quantified on the basis of retention time and peak areas of authentic sugar standards (i.e. sucrose, glucose and fructose, see appendix A Figs. 8.1 & 8.2 for examples of HPLC traces).

2.7.3 (ii) *Starch assay*

To determine starch concentration, the starch UV-method for biochemical and food analysis, Cat. No. 207748 (Boehringer Manheim, GMBH Biochemica) was followed. Starch content was first hydrolysed to D-glucose at pH 4.6 in the presence of the enzyme amyloglucosidase (AGS, EC 3.2.1.3) as shown in equation 2.5.

$$Starch + (n-1)H_2O \xrightarrow{AGS} nD - glu\cos e \qquad \text{Equation 2.5}$$

The principle of the glucose assay is that in the presence of hexokinase (EC 2.7.1.1), glucose is converted to glucose 6-phosphate (Equation 2.6), which reduces NADP$^+$ to

$$D - glu\cos e + ATP \xrightarrow{HK} G - 6 - P + ADP \qquad \text{Equation 2.6}$$

NADPH in the presence of glucose 6-phosphate dehydrogenase (EC 1.1.1.49), as shown in equation 2.7.

$$G-6-P+NADP^{+}\xrightarrow{G6P-DH}gluconate-6-phosphate+NADPH+H^{+}$$

Equation 2.7

The amount of NADPH formed is stoichiometric with the amount of glucose and is determined spectrophotometrically at 340 nm.

The particulate that remained after extraction with alcohol was solubilized by adding to it 5 ml of 8 *M* HCl and 20 ml DMSO. It was then incubated for 1 h in a shaking water bath set at 60°C. To prevent clotting, the mixture was vigorously mixed with a glass rod at intervals. The solutions were then cooled to room temperature and the pH set to 4-5 with a saturated solution of NaOH. The solution was transferred to 100 ml volumetric flask and adjusted to volume with distilled H_2O. At this stage the extract was filtered and 0.2 ml was used for the starch assay as follows (Boeringer Mannheim, 1984):

Step 1.

Analyses were conducted in 10 mm pathway Pecsa quartz cuvettes (Pecsa Analytical Co., Johannesburg, SA) (Table. 2.1).

Table 2.1 Protocol followed in the preparation for starch assays

Pipette into cuvettes	reagent blank (A_1)	sample (A_2)	sample blank
solution 1	200 µl	200 µl	-
sample solution	-	100 µl	100 µl
Distilled H_2O	100 µl	-	-

Solution 1 contained 100 mg lyophilisate, consisting of citric acid buffer, pH 4.6; AGS, 84 U (Unit definition: One unit will liberate 1.0 mg of glucose from starch in 3 min at pH 4.6 at 55°C) and stabilizers. at this stage At this stage the cuvettes were closed, mixed and incubated for 15 min at 55°C (water bath). Thereafter the following was added as indicated in table 2.2:

Table 2.2 Post incubation protocol for individual cuvettes for starch assay.

Pipette into cuvettes	reagent blank	sample	sample blank
solution 2	1 ml	1 ml	1 ml
Distilled H_2O	1 ml	1 ml	1.2 ml

Solution 2 contained 5 g powder mixture consisting of triethanolamine buffer, pH 7.6; NADP, 75 mg; ATP, 190 mg; $MgSO_4$; and stabilizers. The contents were then mixed by gently swirling. After 3 min the absorbance of the solution (A_1) reagent blank was read at 340 nm. The reaction was then started by adding the following as indicated in table.2.3.

Table 2.3 Final steps in the protocol followed in the preparation for starch assays

Pipette into cuvettes	reagent blank	sample	sample blank
suspension 3	200 μl	200 μl	200 μl

After 15 min the absorbancies were read and final absorbance was determined by the difference (A_2-A_1) for both reagent blank and sample.

Concentrations were calculated as follows:

$$c = \frac{V \times MW}{\varepsilon \times d \times v \times 1000} \times \Delta A[g/l] \times F \qquad \text{Equation 2.8}$$

where, V final volume (ml); v volume of sample, MW molecular weight of the substance assayed (g mol^{-1}) for starch: $MW_{glucose}$-MW_{H2O}, d light path (cm), ε absorption coefficient of NADPH at 340 nm = 6.3 [1 x mmol^{-1} x cm^{-1}] and F dilution factor.

2.8 Microscopy and morphometric measurements of starch grains in leaves

2.8.1 Electron microscopy

Fully expanded mature leaves of 25 day old plants from both treatments were harvested for transmission electron microscope (TEM), using the procedure described by Evert *et al.* (1996). Leaf tissue was fixed and trimmed to approximately 1 mm x 2 mm pieces under cold 3% glutaraldehyde in 0.05 *M* Sodium-cacodylate buffer, and transferred to vials containing the above fixative-buffer. Vials were placed in a refrigerator set at 4°C for 6 h, and the solution replaced with fresh fixative buffer at 2 h intervals, and then post-fixed in 2% osmium tetroxide (Merck) overnight in a refrigerator. Post-fixed tissue segments were rinsed in buffer and dehydrated in cold, graded ethanol series, followed by two changes of propylene oxide (1 h total). The tissue was then infiltrated and embedded in Spurr's low viscosity epoxy resin (Spurr, 1969). Ultrathin sections approximately 50-60 nm (silver-god) were cut from several blocks (5-10 from each of the leaf samples from both CO_2 treatment) using glass knives mounted on an LKB UM-3 ultramicrotome (Bromma, Sweden). Sections were collected on 200-mesh copper grids and stained in uranyl acetate and followed by lead citrate, then examined and photographed with a Jeol 100-CX-2 (Tokyo, Japan) transmission electron microscope at an accelerated voltage of 80 and 100 kV.

2.8.2 Morphometric measurements

To compare the starch grains quantitatively, simple morphometric measurements were performed as follows. TEM photomicrographs were scanned into the computer using Sigma Scan Image Program (Jandel Scientific, San Rafael, CA, USA) and recalled as digitized TIF computer images. When recalled to the image analyser for measurements, a suitable calibration was set which took into consideration the photomicrograph magnification, and light areas showing starch grains on the image were identified and coded by pseudo-colours (see Fig. 4.2). Areas and other measurements such as numbers, widths and lengths of starch grains were then calculated from the images..

2.8.3 Stomatal measurements

Fully expanded mature leaves of similar morphological age as those used for gas-exchange measurements were used to estimate stomatal density in plants exposed to both CO_2 treatments. Leaf epidermal impressions of adaxial and abaxial surfaces were obtained using nail polish (Guehl *et al.*, 1994), and were examined with a light microscope at X400 magnification. All impressions were taken midway between the midrib and leaf margin and halfway between the apex and base of the leaf to avoid variability in sampling due to uneven distribution of stomata in the leaf of *P. vulgaris* (O'Leary and Knecht, 1981). Total numbers of stomata were counted within a 0.5 X 0.5 mm area for both adaxial and abaxial surfaces. Since both sides of the leaf were involved in gas exchange measurements, a weighted stomatal density (WSD, stomata mm^{-2}) value was calculated: (WSD = $ADSD^2 + ABSD^2$)/(ADSD + ABSD) as outlined by El-Sharkway *et al* (1985), where *ADSD* and *ABSD* are adaxial and abaxial stomatal density, respectively. Measurements were carried out using five different leaves per sampling from plants grown under ambient and elevated [CO_2].

2.9 Total amino acid analysis

2.9.1 Sample preparation and extraction

Dried leaf or seed sample material (500 mg) was homogenized and fractionated into soluble (amino acid containing fraction) and insoluble fractions in 25 ml of 80% (v/v) ethanol at 0°C as described by Lewis and Chadwick (1983). Extraction was performed for 24 hrs in a cold room at 0°C using a shaking water bath. The extract was filtered and reduced under an air stream to a final volume of 10 ml. The extract was then filtered again and the precipitate added to the original residue (bound N fraction). Portions of the samples were then stored in small sterile vials at -20°C until quantification using ninhydrin for total amino acids and high performance liquid chromatography (HPLC) for individual separation of amino compounds.

2.9.2 Determination of total amino acids: ninhydrin method

The procedure followed was originally described by Yemm and Cocking (1955), as adapted by Herridge (1984). To prepare the ninhydrin reagent, 5 g of 2,2-dihydroxyinden-1,3 dioxin (ninhydrin, Merck) were dissolved in 10 ml of 0.01 *M* potassium cyanide (65 mg in 100 ml distilled H_2O, stable for 3 months at 20°C) and 590 ml of methoxy-ethanol (Merck). This was prepared 24 hrs before use to ensure its stability, and was kept in a refrigerator at 4°C in a brown-glass reagent bottle.

The stock solution containing the amino acid standards was prepared each day using a 2.5 μmol ml^{-1}, 50:50 L-asparagine and L-glutamine (Merck, 16.5 mg and 18.2 mg, respectively) dissolved in 100 ml distilled H_2O. L-asparagine and L-glutamine are the most common amino compounds in plant material (Lewis, 1986). Sequential dilutions (in 10 ml distilled H_2O) were made from this stock solution for the preparation of a standard curve (0.1, 0.2, 0.4, 1.0 and 2.0 ml to produce 12.5, 25, 50, 125 and 250 nmole concentrations, respectively). For analysis, a sample of 1:9 dilution was mixed with 1.2 ml ninhydrin reagent and further diluted with 1 ml distilled H_2O. In addition, with the preparation of the amino acid standard curve, 0.5 ml of each standard and a duplicate 0.5 ml H_2O blank was used. The contents were thoroughly mixed and placed in a boiling water bath for 10 min. After boiling, the samples were cooled and absorbance measured at 570 nm using a UV visible recording spectrophotometer. Total amino acid content of samples was determined from the standard curve.

Sampling consisted of three sample replicates, and at least two analyses were run from each sample.

2.9.3 Analysis and quantification of amino acids: HPLC

The individual amino compounds in 50-200 µl of sample solution were separated and quantified using an HPLC at University of Cape Town, Medical School, South Africa. The amino acids separated were: phosphoserine, aspartic acid, glutamic acid, hydroxyproline, serine, asparagine, glycine, glutamine, histidine, threonine, arginine, alanine, proline, tyrosine, valine, methionine, cystine, isoleucine, leucine, phenylalanine, tryptophan, lysine and ammonia.

2.10 . Spectrophotometric determination of chlorophylls and carotenoids

2.10.1 Sample preparation, extraction and determination

Fresh leaf material (500 mg) was quickly frozen in liquid nitrogen. Following the method of Lichtenthaler (1987), frozen samples were homogenized in 10 ml ice cold 100% acetone and acid washed sand using a cold mortar and pestle (BDH chemicals Ltd. Pole, UK. The homogenates were then filtered through Whatman No. 1 filter paper in a Buchner funnel under vacuum, and the residue was washed with an additional 5 ml acetone. Since carotenoids and chlorophylls are extremely light sensitive and are easily photobleached (Lichtenthaler, 1987), the extraction procedures were performed in dim light. The concentrations of chlorophylls (*a* and *b*) and carotenoids were determined spectrophotometrically using a UV visible recording spectrophotometer.

Quantitative determination of chlorophylls and carotenoids was carried out immediately after preparation of the leaf extract. Concentrations of chlorophyll *a* (C_a), chlorophyll (C_b), total chlorophylls (C_{a+b}), and total carotenoids (C_{x+c}) were calculated as outlined by Lichtenthaler (1987). Chlorophyll concentrations were expressed on a leaf weight basis (mg g^{-1} fresh weight). The calculations are shown in Table 2.4.

Table.2.4. Equations for the determinations of concentrations of chlorophyll *a* (C_a), chlorophyll *b* (C_b), total chlorophylls (C_{a+b}), and total carotenoids C_{x+c}) in leaf pigment extract using acetone, 100% (pure solvent). Absorbance was read using a UV visible recording spectrophotometer. The pigment concentrations were obtained by inserting the measured absorbance values in the equations indicated by subscripts and then multiplied by the dilution factor.

	Solvent:-100% Acetone
C_a	$11.24A_{661.6} - 2.04A_{644.8}$
C_b	$20.13A_{644.8} - 4.19A_{661.6}$
C_{b+c}	$7.05A_{661.6} + 18.09A_{644.8}$
C_{x+c}	$\dfrac{1000A_{470} - 190C_a - 63.14C_b}{214}$

2.11 Total rubisco activity and total protein determination

Total rubisco activity was determined as described by Friedrich and Huffaker (1980). Rubisco activity was determined as the ribulose 1,5-bisphosphate (RuBP)-dependent incorporation of [^{14}C] bicarbonate into acid-stable products.

2.11.1 Sample preparation and extraction

Leaflets were removed, the midrib vein excised, and 250 mg of leaf material was obtained for the extraction of rubisco and total protein determination. Leaf samples were frozen in liquid N_2 and homogenized using a cold mortar and pestle with acid washed sand in 1.5 ml cold buffer containing 0.04% v/v β-mercaptoethanol in 0.2 *M* Tris-(hydroxymethyl)-aminomethane, and pH was corrected using concentrated H_2SO_4 (thus referred to as Tris-SO_4 pH 8.0, Merck). Insoluble polyvinylpyrrolidone (PVP, 100 mg, Merck) was added to each leaf sample prior to homogenization. After homogenization, the slurry was passed through three layers of cheesecloth and the filtrate centrifuged at 27 000 *g* for 10 min at 4°C using the RC-Sorvall Superspeed Refrigerated Centrifuge. Duplicate 0.1 ml aliquots of the supernatant were mixed with 5 ml of 5 % trichloroacetic acid (TCA, Merck) and stored at 4°C until assayed for soluble proteins (Bradford 1976). Duplicate aliquots were diluted (1:5) with Tris-SO_4 buffer for determination of rubisco activity.

2.11.2 Rubisco activity assay

Duplicate aliquots were incubated at 30°C for 10 min with 0.1 ml of 44 mM $MgCl_2$, 22 mM $NaH^{14}CO_3$ (0.5 µCi/µmol, Amersham International, Amersham, UK), and 55 mM Tris-SO_4 buffer (final pH 8.0). The reaction was initiated by the addition of 0.02 ml of freshly prepared 10 mM ribulose1,5-bisphosphate (Merck). The reaction was terminated after 2 min by addition of 0.05 ml of 2 N HCl. Duplicate 0.05 ml aliquots from each assay mixture were mixed with 10 ml Pico-Fluor™ 40, a universal liquid scintillation cocktail (Packard Instruments, B.V. Chemical Operations, The Netherlands) in scintillation vials. The acid stable ^{14}C-radioactivity was determined with a Beckman LS 5801 scintillation spectrophotometer/counter (Beckman Instruments, Fullerton, CA, USA) programmed for automatic quench correction.

2.2.3 Total soluble protein assay

Soluble proteins from leaf extract were determined colorimetrically following the dye-binding procedure described by (Bradford, 1976). To 0.1 ml of protein solution or any dilution thereof, 5.0 ml of Bradford reagent (100 mg Coomasie Brilliant Blue G-250, Sigma Chemical Co., St Louis, MO, USA) dissolved in 50 ml 95% ethanol was added to 100 ml (w/v) phosphoric acid and the solution diluted to a volume of 1 L with distilled H_2O. The sample was thoroughly mixed and absorbance was read at 595 nm against a reagent blank using a UV visible recording spectrophotometer. Protein concentrations were determined from a standard curve prepared from bovine serum albumin (BSA, Sigma).

CHAPTER 3: PHOTOSYNTHESIS, GROWTH AND FOLIAR NUTRIENT CHANGES

3.1 Introduction

Atmospheric CO_2 has increased dramatically since the industrial revolution, and its present concentration is predicted to double by the middle of the next century (Neftel *et al.* 1985, Bazzaz 1990). This has inspired a large number of researchers world-wide to examine the responses of plants to CO_2 enrichment (see Bowes 1993 and references cited). There has been steady growth in research focused on the effect of CO_2 enrichment on selected nutrients, particularly nitrogen (Curtis *et al.* 1989; Hocking and Meyer, 1991) and phosphorus (Conroy *et al.*, 1992; Morin *et al.*, 1992). Few papers deal with the wide spectrum of both macro- and micronutrients (see Overdieck, 1993 and literature cited). However, as was pointed out by Newton (1991) and Stulen and Den Hertog (1993), little information is available to assess the effect of CO_2 enrichment on leaf nutrient concentrations. This deficiency is surprising, given the extensive number of studies examining the overall interaction of enhanced CO_2 on plants grown at different levels of nutrient concentrations (Norby *et al.*, 1986; Woodin *et al.*, 1992).

Researchers have noted that CO_2 enrichment leads *inter alia* to accelerated photosynthetic rates, which in turn translates into increased overall plant growth (Radoglou *et al.*, 1992). Enhanced plant growth rate often leads to low nutrient status (Chapin, 1980), due to the "dilution" of minerals and nitrogen-based compounds by higher concentrations of carbon-based compounds or increased C/N ratios (Akey and Kimball, 1989). Some authors have argued that high C/N ratios (particularly high starch/N ratios) observed in long-term CO_2 studies are a major cause of the observed reduction in NAR or what is termed "acclimatization" (Ehret and Jolliffe 1985b). Other researchers have suggested that factors such as reduction in Rubisco content, its activity and/or its regeneration capacity are responsible for acclimation (Rowland-Bamford *et al.*, 1991; Bowes, 1993; Sage, 1994).

Despite adequate demonstration in CO_2 studies of the close interdependence of plant nutrient status (mainly N and P) and physiological processes (such as photosynthesis and Rubisco physiology), there is a relative paucity of data on how CO_2 influences the concentration of most other nutrients, which may impact on a wide spectrum of other biochemical and morphological processes. Physiological, growth, and ecological aspects that are likely to be affected by CO_2-induced changes in tissue nutrient concentration include leaf discoloration, herbivory, host-pathogen relationships and nutrient cycling (Bazzaz, 1990; Johnson and Lincoln, 1991; Nederhoff and Buitelaar, 1992). The CO_2-induced changes in these processes may alter the natural capacity of plants to deal with physical or biotic stresses (Waterman and Mole, 1989; Rogers *et al.*, 1994). The net result may be acclimatization to elevated CO_2 that is accompanied by reduction in yields, thus rendering CO_2 fertilization less beneficial in both glasshouse and field crops (Tissue *et al.*, 1993).

In order to predict the potential impacts of elevated CO_2 on vital physiological and ecological parameters, there is a need for further qualitative and quantitative independent assessment of plant tissue, particularly concentrations of both macro- and micronutrients, under elevated CO_2 conditions. The present study was undertaken to contribute in this regard by quantifying the effect of elevated CO_2 on foliar concentrations of macro- and micronutrients in *Phaseolus vulgaris* L., and characterising how nutrient concentrations influence net assimilation rate, leaf chlorosis and other growth attributes.

3.2 Results

No transformation of the data was necessary since the variances of all the growth attributes, NAR, Rubisco activity, chlorophylls, carotenoids, macro- and micronutrients were found to be homogeneous at the 5% level of significance ($P>0.05$).

3.2.1 Growth parameters

Bean plants grown under elevated CO_2 exhibited enhanced overall above-ground growth. Total plant height (TPH) under both CO_2 treatments increased exponentially over the growth period, but the mean TPH for bean plants grown at 700 μmol mol^{-1} was significantly greater than plants grown under 360 μmol mol^{-1} CO_2 ($P<0.0001$; Fig. 3.1). During most of the growth period, height differences were, on average, 8 cm. However, from 30 DAG, TPH in the elevated CO_2 treatment was 12 cm greater than in ambient CO_2.

As observed with *P. vulgaris* by Porter and Grodzinski (1984), leaf area was significantly greater in plants grown under elevated CO_2 than those grown at ambient CO_2 ($P<0.0001$; Fig. 3.2). Between 35 and 40 DAG, the leaf area of CO_2-enriched plants was almost twice that of ambient CO_2-grown plants. This pattern of CO_2-induced difference in leaf area increasing with time was also observed by Cure *et al.* (1989) in their study of soybean leaf development under elevated CO_2.

CO_2-enriched plants had significantly greater leaf dry weights throughout the growth period compared to ambient CO_2-grown plants ($P<0.01$; Fig. 3.3). Although LDW values were approximately 50% of those observed by Porter and Grodzinski (1984) for *P. vulgaris*, the proportions were very similar. LDW initially increased in both treatments, with CO_2-enriched plants reaching a peak at 20 DAG while the LDW continued to rise through to 30 DAG in ambient CO_2-grown plants. In both instances this peak was followed by a decline in LDW.

Mean specific leaf area (SLA; leaf area per unit leaf weight) increased with time in both treatments, but values observed in CO_2-enriched plants were significantly lower than ambient CO_2-grown plants ($P<0.0001$; Fig. 3.4). Treatment differences continued up to 35 DAG, where a slight insignificant increase was observed (Fig. 3.4). Similar patterns for SLA have been reported for White clover (Ryle *et al.*, 1992) as well as many other species (see Nederhoff *et al.*, 1992 and Baxter *et al.*, 1994).

Relative growth rates did not differ significantly for most of the growth period in both CO_2 treatments except at early stages of growth (10-15 DAG). This was despite a large, consistent, significant difference between CO_2 treatments in total plant biomass (Table 3.1). In a study involving 156 species, Poorter (1993) found that CO_2-induced differences in RGR are variable and largely species specific.

Table 3.1. Mean relative growth rates between stages indicated and total plant biomass (at 15, 25 and 35 DAG) of bean plants. Data are means of three replicates. (n=12; $^{*}P<0.05$; $^{**}P<0.01$; ^{ns} not significant $P>0.05$ as tested by Scheffe's multiple test)

DAG	Treatment µmol mol^{-1} CO_2	RGR g g^{-1} d^{-1}	Total dry weight g
10-15	360	0.15	6.8
	700	0.18*	13.7**
20-25	360	0.12	25.2
	700	0.11^{ns}	36.8**
30-35	360	0.03	37.9
	700	0.02^{ns}	52.0**

There were more patches of leaf discoloration or chlorosis in leaves grown under elevated CO_2 than those at ambient CO_2. Although the incidence of leaf discoloration was high in plants grown under elevated CO_2, not all leaves including the ones that were tagged exhibited these signs. Similar CO_2-induced patterns of chlorosis were reported by Delucia *et al.* (1985) studying soybean, and by Nederhoff and Buitelaar (1992) studying eggplant leaves. For *P. vulgaris* in the present study, this phenomenon seemed to be more pronounced in the leaves at later stages of plant growth (i.e. 25-40 DAG). As a result, leaf longevity was significantly reduced in CO_2-enriched bean plants ($P<0.0001$; Fig. 3.5).

3.2.2 Net assimilation rate

Net assimilation rate was significantly higher in bean plants grown at 700 μmol mol^{-1} ($P<0.0001$; Fig. 3.6) than plants grown under 360 μmol mol^{-1} CO_2. As has been observed in a number of studies involving the same and other species (see Peet, 1984; Delucia *et al.*, 1985; Baker and Allen, 1993 and refences cited), NAR increased during the initial growth stages (10-20 DAG, with the average of 22 μmol m^{-2} s^{-1}), followed by a noticeable decline to levels almost similar to those observed in plants grown under ambient CO_2. The NAR of plants grown at elevated CO_2 from 20-40 DAG was on average 17.3 μmol m^{-2} s^{-1} while that of ambient grown plants was 15.3 μmol m^{-2} s^{-1}, indicating a much smaller average difference (2 μmol m^{-2} s^{-1}) between the two CO_2 treatments at this stage.

3.2.3 Rubisco activity and chlorophylls and carotenoids

Rubisco activity, measured as % incoporation of labelled ^{14}C into acid-stable products, did not differ significantly for most of the growth period in either CO_2 treatment. However, after 25 DAG, a sharp decline in Rubisco activity was observed in plants grown under elevated CO_2 (Fig. 3.7, $P<0.01$). Many other studies of elevated CO_2 have reported this pattern of Rubisco response, as well as its correlation to a reduction in NAR (Peet *et al.*, 1986; Porter and Grodzinski, 1984; Yelle *et al.*, 1989b; Besford, 1993).

Chlorophyll *a* content was always higher in both treatments compared to Chlorophyll *b* (Figs. 3.8**A** and **B**). However, the total Chlorophyll ($a+b$) declined significantly in plants grown under elevated CO_2 (Fig. 3.9, $P<0.01$). No consistent response of changes in chlorophyll concentration has been reported in C_3 species grown under elevated CO_2. Norby *et al.* (1991) reported a significant reduction in total chlorophyll concentration which was in agreement with the results obtained in this study in bean plants exposed to elevated CO_2. In contrast to these findings, a significant increase in chlorophyll concentration was observed for bean, tomato and wheat, respectively (Kavari-Nejad, 1986; *Sandhu et al.*, 1992; Greiner de Mothes and Knoppik, 1994). Chlorophyll a/b ratios did not differ significantly for most of the growth period. However, there were significant increases in chlorophyll a/b ratios during 20-25 DAG and at 40 DAG in high CO_2-grown plants (Fig. 3.10, $P<0.10$ and $P<0.05$, respectively). This period corresponded to the decline in NAR. Total carotenoid ($x+c$) levels did not differ significantly between CO_2 treatments throughout the growth period (Fig. 3.11, $P>0.05$). In an attempt to determine a relationship between changes in chlorophyll and carotenoids, the chlorophyll ($a+b$): carotenoid ($x+c$) ratio was established for both CO_2 treatments. The chlorophyll:carotenoid ratio was significantly reduced in plants grown under elevated CO_2 (Fig. 3.12; $P<0.01$).

3.2.4 Element concentrations in the leaf

The foliar concentrations of the minerals measured from plants grown under both CO_2 treatments were generally within the range reported for beans and other herbaceous plants (Geraldson *et al.*, 1973). Nutrient concentration data is presented in Figs. 3.13-15.

3.2.4(i) Nitrogen and other macronutrients

Leaf nitrogen concentration in CO_2-enriched plants were initially greater than in ambient CO_2 plants, but from 20 DAG, N levels in CO_2-enriched plants declined to levels below those measured in ambient CO_2 plants ($P<0.01$; Fig. 3.13). Similar CO_2-induced effects on foliar N concentrations have been reported in other studies (Hocking and Meyer, 1991; Conroy *et al.*, 1992).

Foliar concentrations of macronutrients are presented in Fig. 3.14 8**A-D**. Ca and P were significantly reduced, while K was significantly increased in CO_2-enriched plants. Although there was a noticeable decline in Mg concentrations in plants grown under elevated CO_2, the overall changes were not significant. The macronutrient concentrations followed the order Ca>K>P>Mg.

3.2.4(ii) Micronutrients

The foliar concentrations of the micronutrients examined are presented in Fig. 3.15 **A-D**. Fe and Zn were significantly reduced, while Mn was significantly increased in CO_2-enriched plants. Cu concentrations showed transient changes. During the first 10-15 DAG, the concentration increased significantly in elevated CO_2 plants. However, from 20 DAG until maturity, Cu concentrations declined steadily in these plants. Despite these transient changes, analysis of variance showed no significant difference in Cu uptake between CO_2 treatments. The micronutrient concentrations followed the order Fe>Mn>Zn>Cu.

Foliar concentration of both micro- and macronutrients were generally reduced in plants grown under elevated CO_2. However, when the data was corrected for leaf starch, reductions in N, P, and Fe were not significant. (Appendix A, Table 8.1). Kuehny *et al.* (1991) also observed a similar effect when their data was expressed on a starch-free dry weight basis.

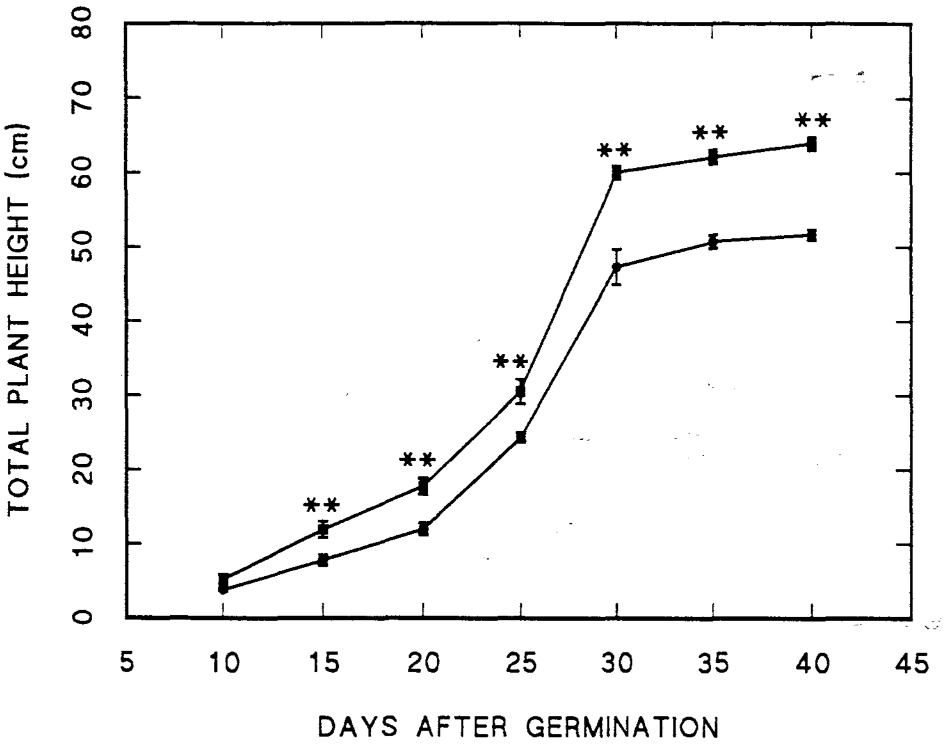

Fig. 3.1. Effect of [CO_2] on total plant height (TPH) of bean plants grown at 360 (●) and 700 (■) µmol mol^{-1} CO_2. (n=9 per each data point; Error bars denote ± SE; ** indicates significant differences at $P<0.01$ as tested by Scheffe's multiple range test).

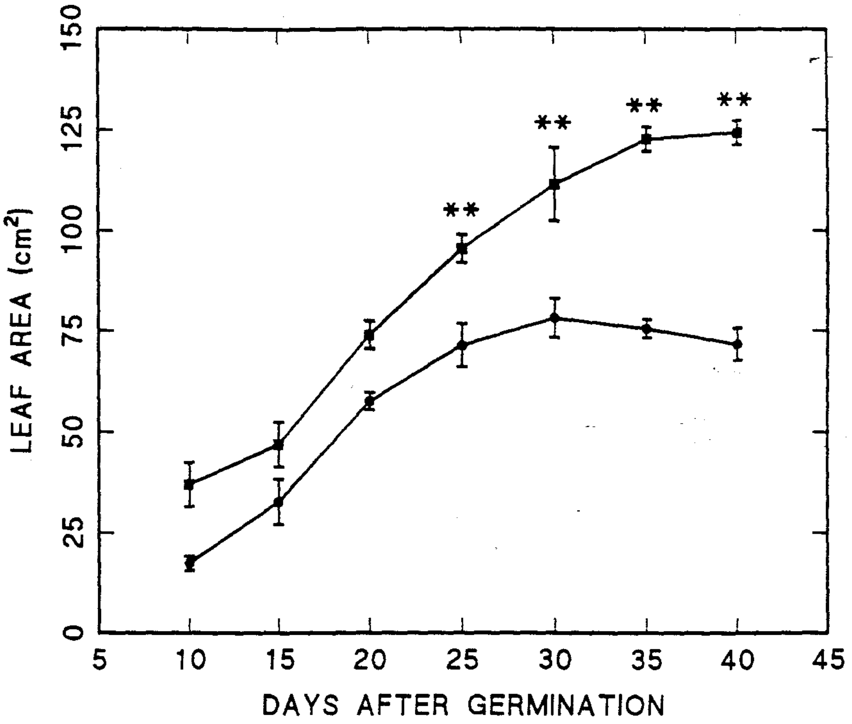

Fig. 3.2. Changes in leaf areas (LA) in bean plants grown at 360 (●) and 700 (■) μmol mol^{-1} CO_2. (n=9 per each data point; Error bars denote ± SE; ** indicates significant differences at $P<0.01$ as tested by Scheffe's multiple range test).

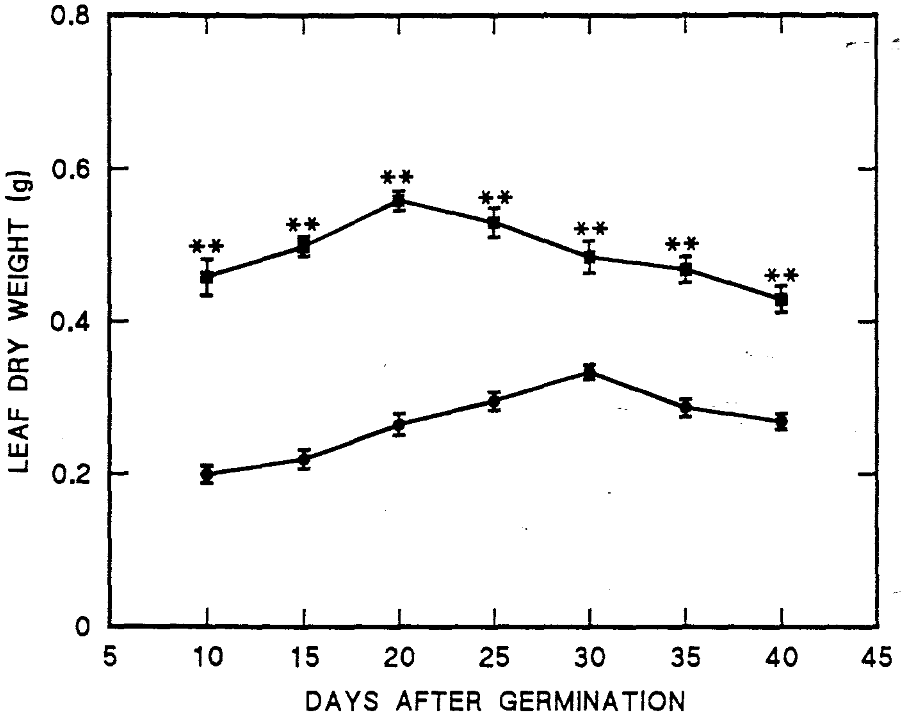

Fig. 3.3. Leaf dry weight (LDW) accumulation in bean plants grown at 360 (●) and 700 (■) μmol mol^{-1} CO_2. (n=9 per each data point; Error bars denote ± SE; ** indicates significant differences at $P<0.01$ as tested by Scheffe's multiple range test).

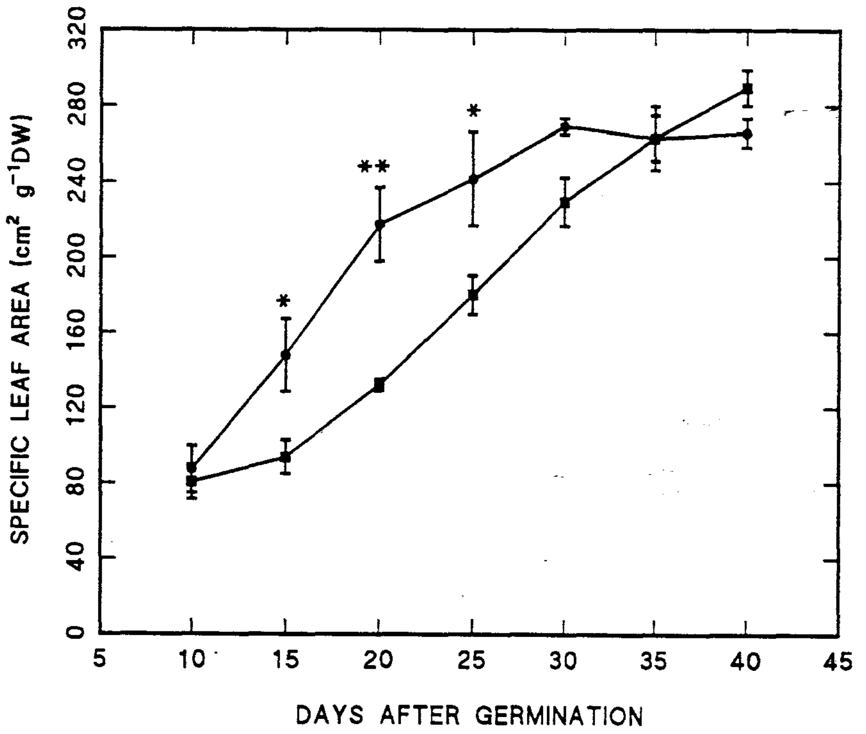

Fig. 3.4. Calculated specific leaf areas (SLA) from LDW and LA data of bean plants grown at 360 (●) and 700 (■) μmol mol^{-1} CO$_2$. (n=9 per each data point; Error bars denote ± SE; * and ** indicate significant differences at $P<0.05$ and $P<0.01$ respectively as tested by Scheffe's multiple range test).

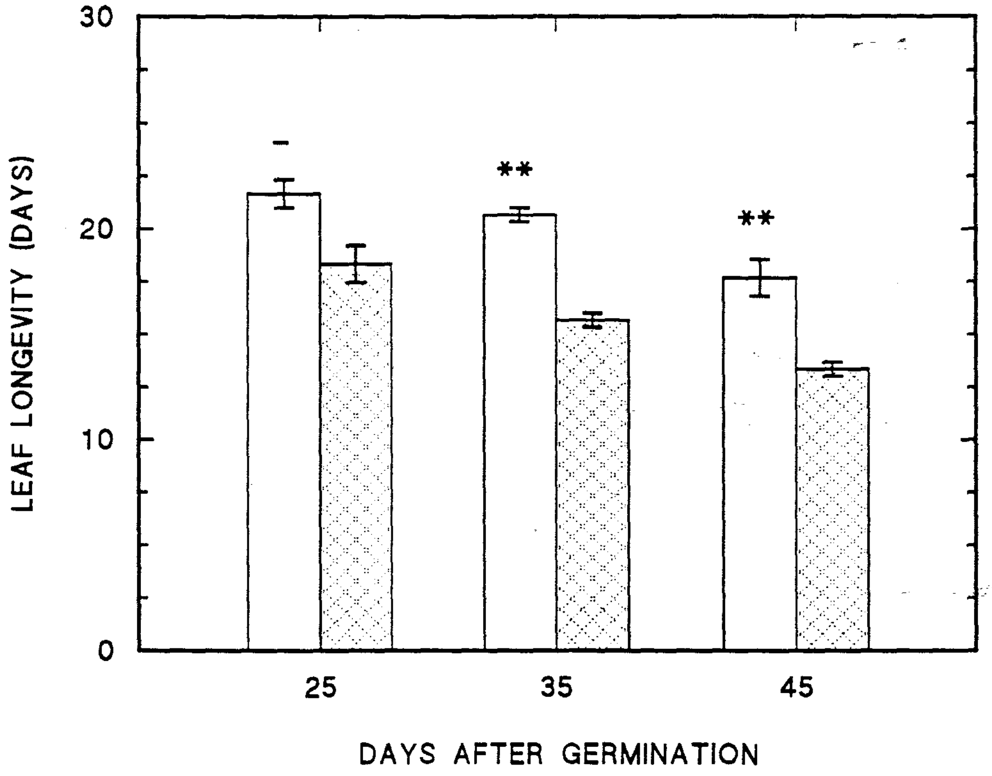

Fig. 3.5. Effect of [CO_2] on leaf longevity expressed as time in days prior to leaf senescence of bean plants grown at 360 (open bars) and 700 (hatched bars) µmol mol^{-1} CO_2. (n=9 per each data point; Error bars denote ± SE; $^{-}$ $P<0.10$, ** $P<0.01$ indicates significant differences as tested by Scheffe's multiple range test).

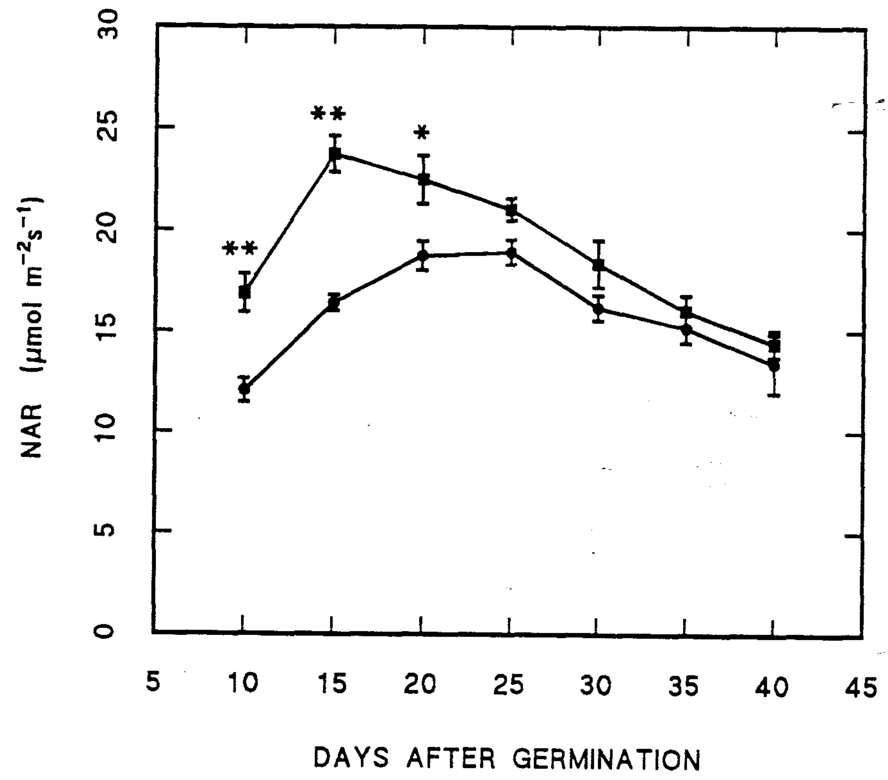

Fig. 3.6. Time course of net assimilation rate (NAR) response measured at incident light of 550 μmol m^{-2} s^{-1} in bean plants grown at 360 (●) and 700 (■) μmol mol^{-1} CO$_2$. (n=9 per each data point; Error bars denote ± SE; * and ** indicate significant differences at $P<0.05$ and $P<0.01$ respectively as tested by Scheffe's multiple range test).

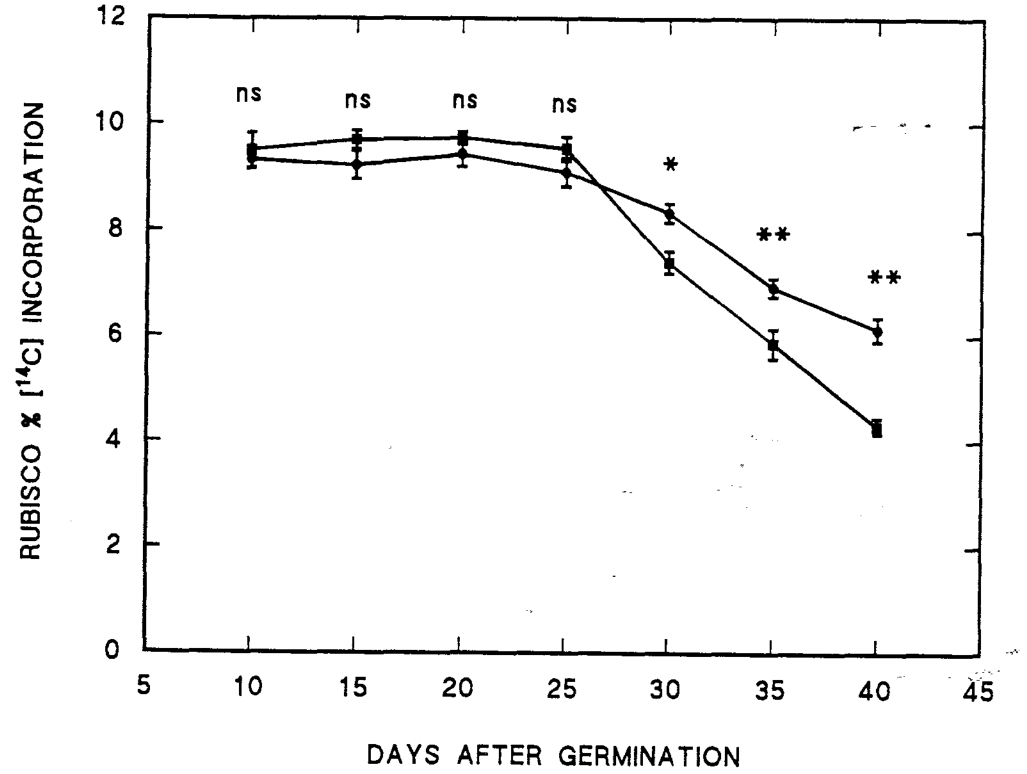

Fig. 3.7. Changes in Rubisco activity in bean plants grown at grown at 360 (●) and 700 (■) µmol mol^{-1} CO$_2$. Rubisco activity was determined by measuring the rate of incorporation of [^{14}C] into acid-stable products. (n=3 per each data point; Error bars denote ± SE; * and ** indicate significant difference at $P<0.05$ and $P<0.01$ respectively, as tested by Scheffe's multiple range test following a one-way ANOVA since interaction was found significant).

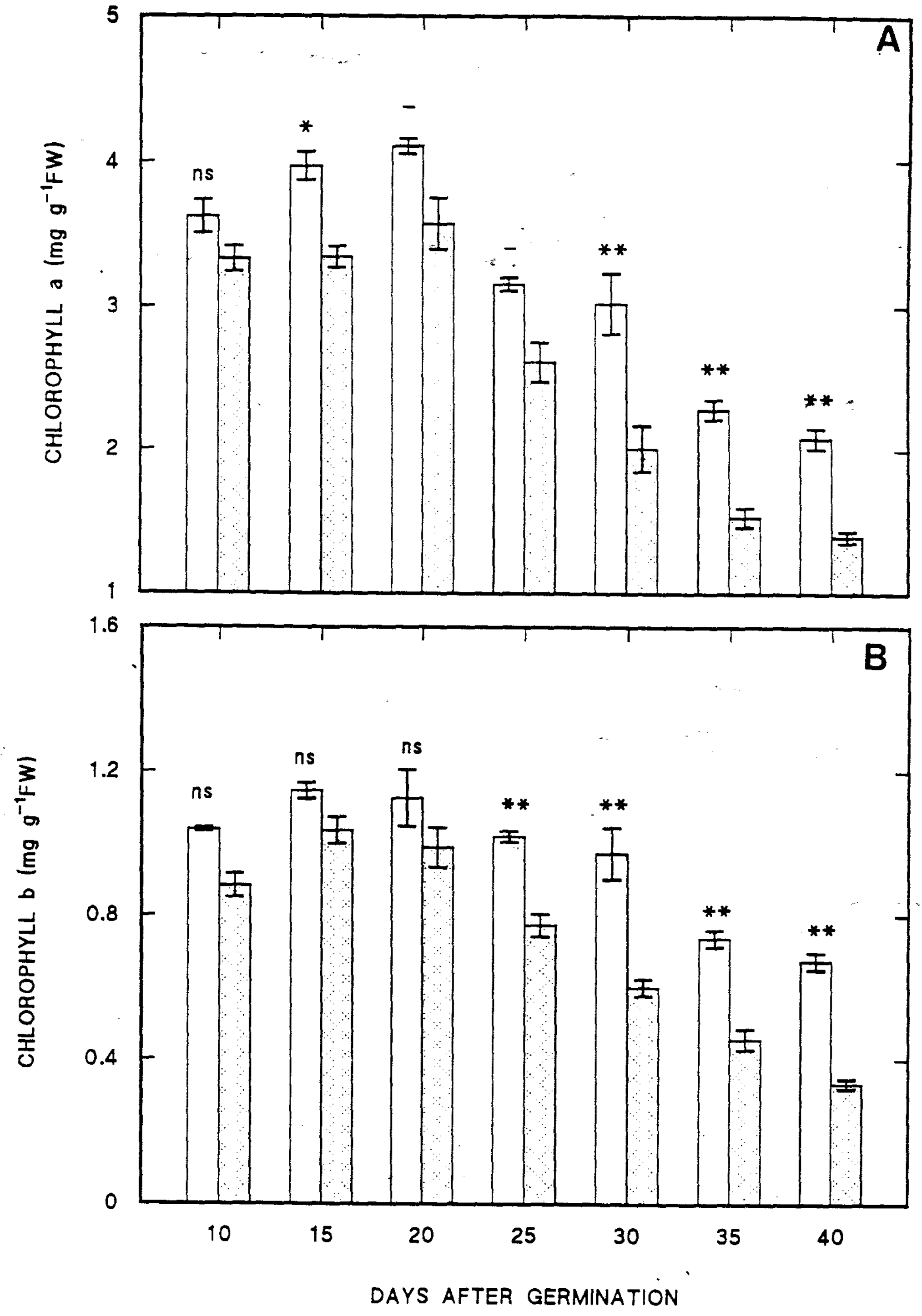

Fig. 3.8. Changes in chlorophyll *a* (**A**) and *b* (**B**) content at various stages of development in bean plants grown at 360 (open bars) and 700 (hatched bars) µmol mol^{-1} CO$_2$. (n=9 per each data point; Error bars denote ± SE; $^{-}$P<0.10; *P<0.05; and **P<0.01 indicate significant difference as tested by Scheffe's multiple range test).

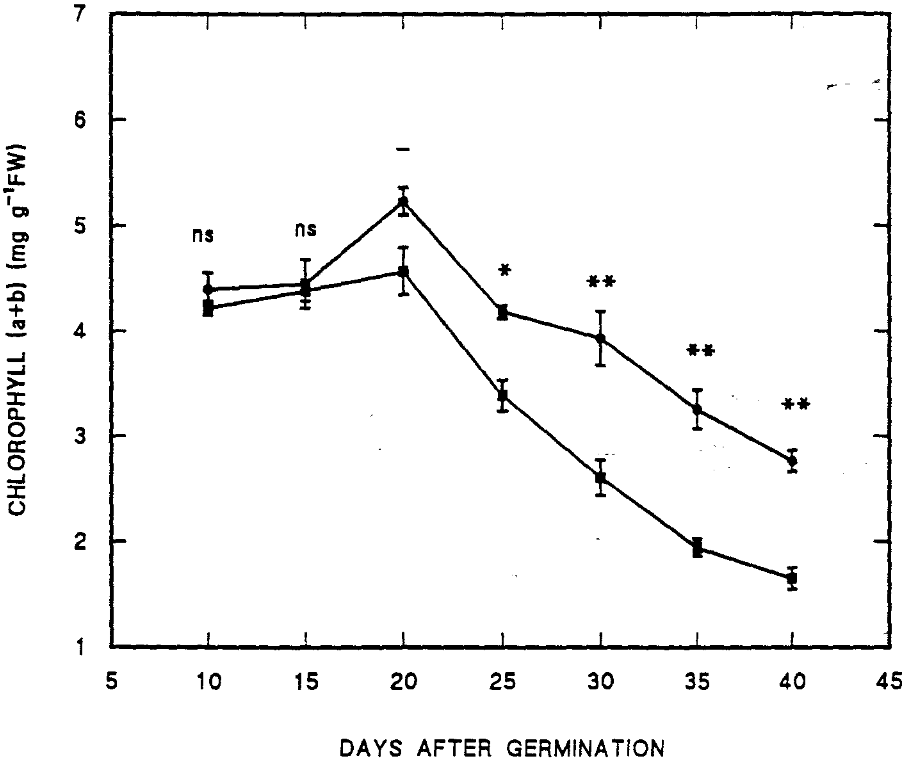

Fig. 3.9. Changes in total chlorophyll (*a*+*b*) content in bean plants grown at 360 (●) and 700 (■) μmol mol^{-1} CO_2. (n=3 per each data point; Error bars denote ± SE; ⁻P<0.10; *P<0.05; and **P<0.01 indicate significant difference as tested by Scheffe's multiple range test).

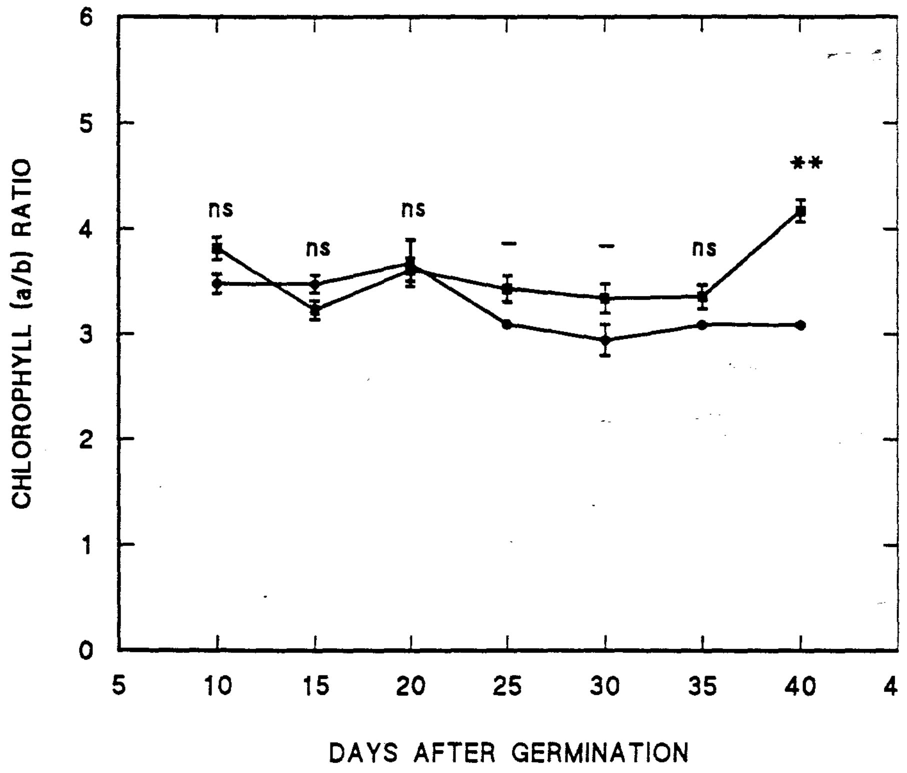

Fig. 3.10. Changes in chlorophyll (*a*/*b*) ratio in bean plants grown at 360 (●) and 700 (■) μmol mol^{-1} CO$_2$. (n=3 per each data point; Error bars denote ± SE; $^{-}$P<0.10; *P<0.05; and **P<0.01 indicate significant difference as tested by Scheffe's multiple range test).

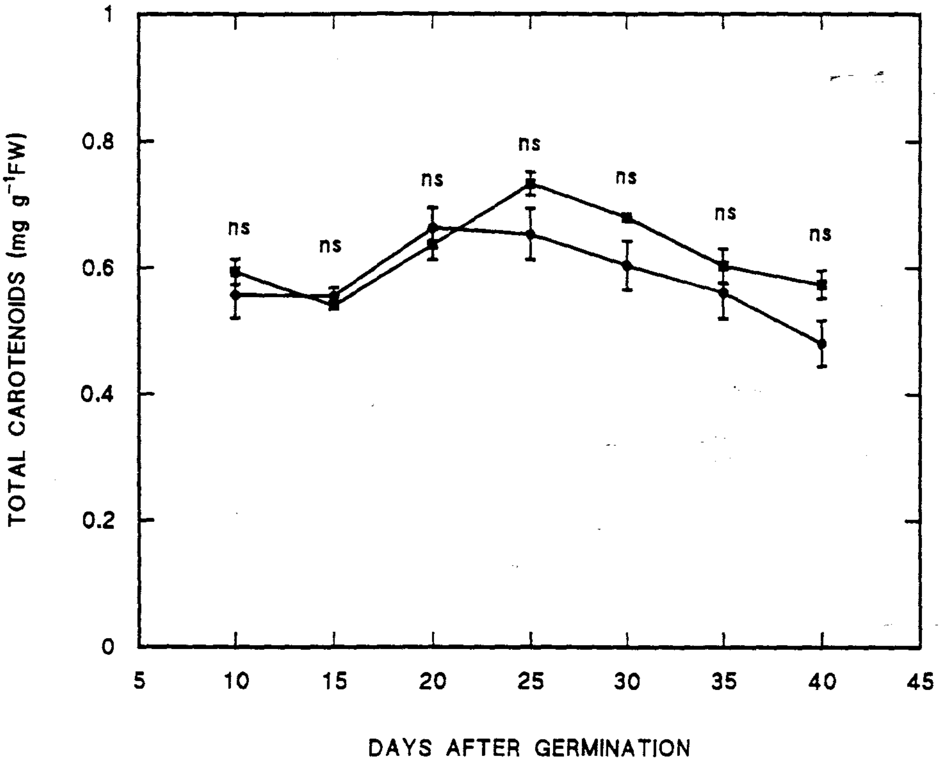

Fig. 3.11. Changes in total carotenoid content (*x*+*c*) in bean plants grown at 360 (●) and 700 (■) μmol mol^{-1} CO$_2$. (n=3 per each data point; Error bars denote ± SE; ^{ns} indicates non-significant differences as tested by Scheffe's multiple range test).

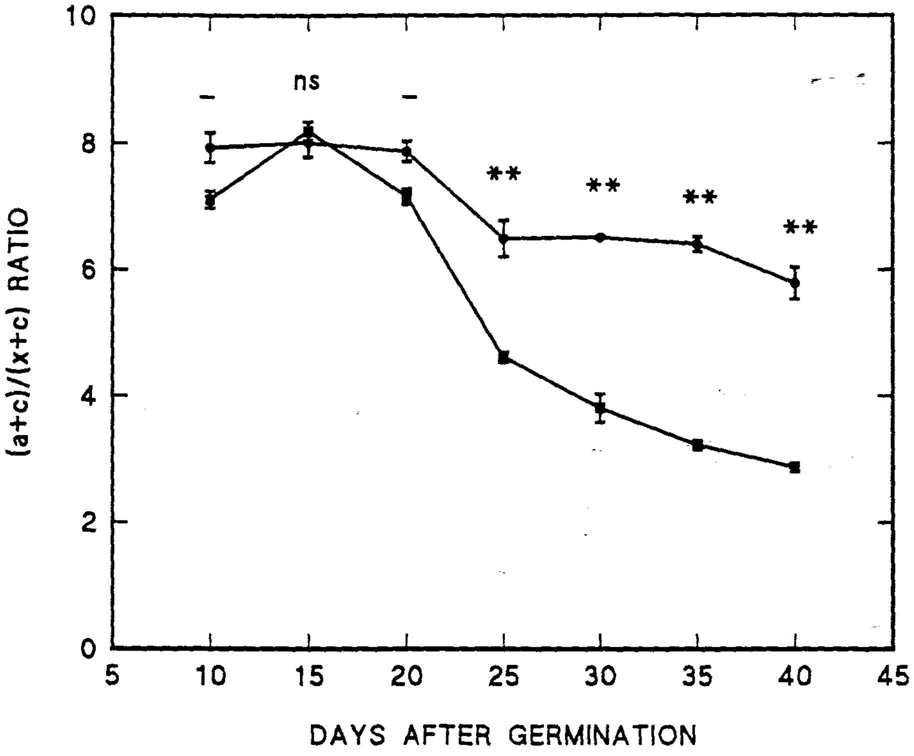

Fig. 3.12. Changes in total chlorophyll (a+b)/total carotenoid (x+c) ratio in bean plants grown at 360 (●) and 700 (■) µmol mol^{-1} CO$_2$. (n=3 per each data point; Error bars denote ± SE; $^{-}$P<0.10; *P<0.05; and **P<0.01 indicate significant difference as tested by Scheffe's multiple range test).

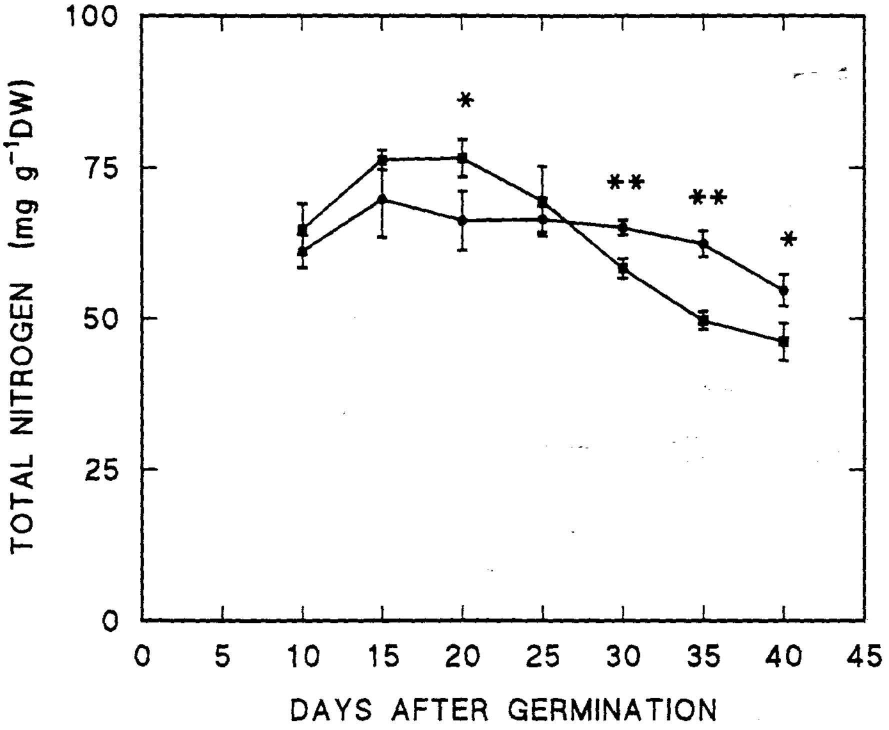

Fig. 3.13. Changes in leaf nitrogen content in bean plants grown at 360 (●) and 700 (■) μmol mol^{-1} CO_2. (n=3 per each data point; Error bars denote ± SE; (n=9 per each data point; Error bars denote ± SE; * and ** indicate significant differences at $P<0.05$ and $P<0.01$ respectively as tested by Scheffe's multiple range test

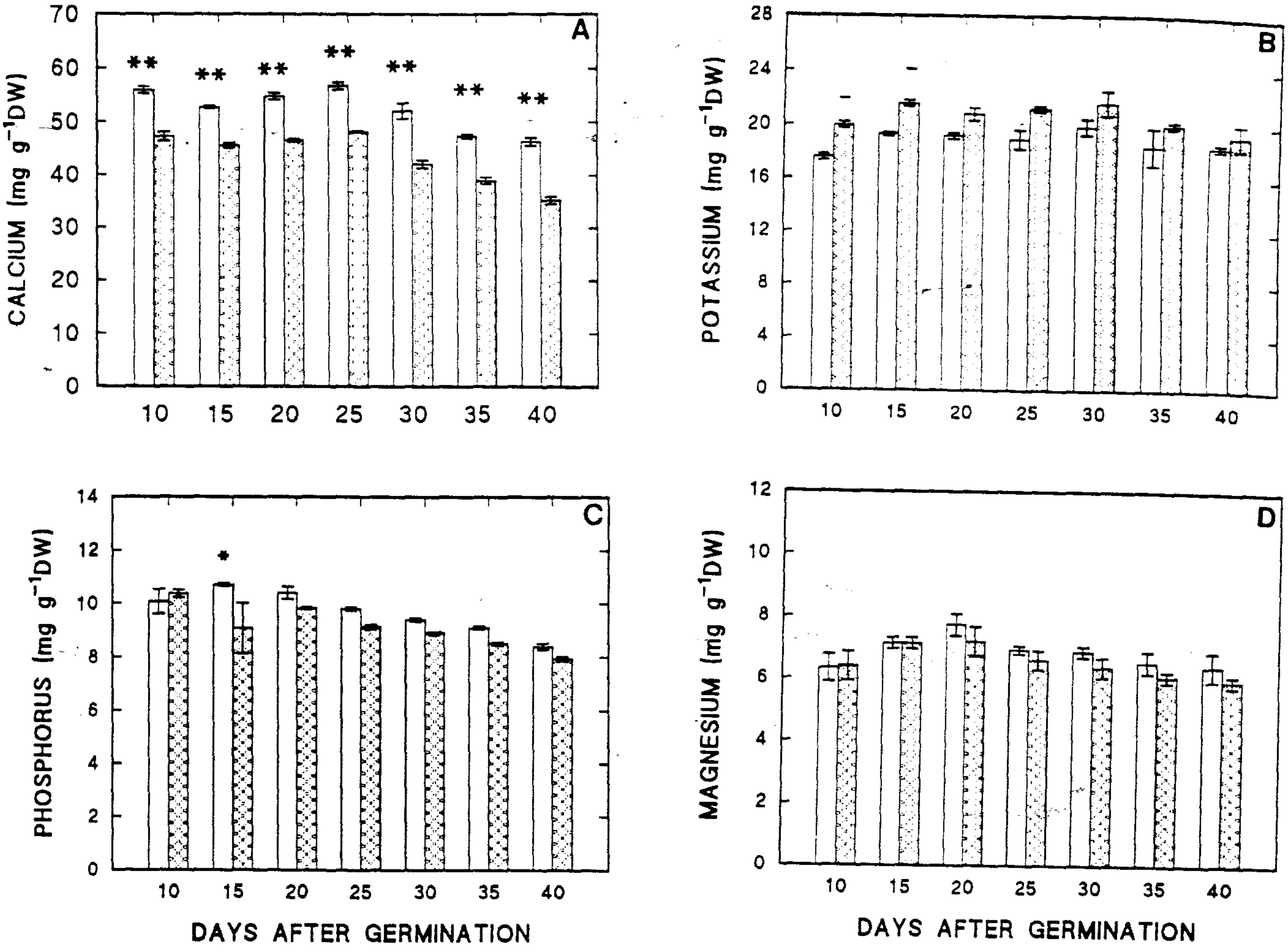

Fig. 3.14. Influence of two levels of CO_2 concentrations on leaf macronutrient status: **A** - Calcium; **B** - Potassium; **C** - Phosphorus & **D** - Magnesium in bean plants grown at 360 (open bars) and 700 (hatched bars) μmol mol^{-1} CO_2. Expressed as mg g^{-1} dry weight. (n=3; Error bars denote ± SE; $^{-}$P<0.10, *P<0.05, **P<0.01 indicate significant differences as tested by Scheffe's multiple range test).

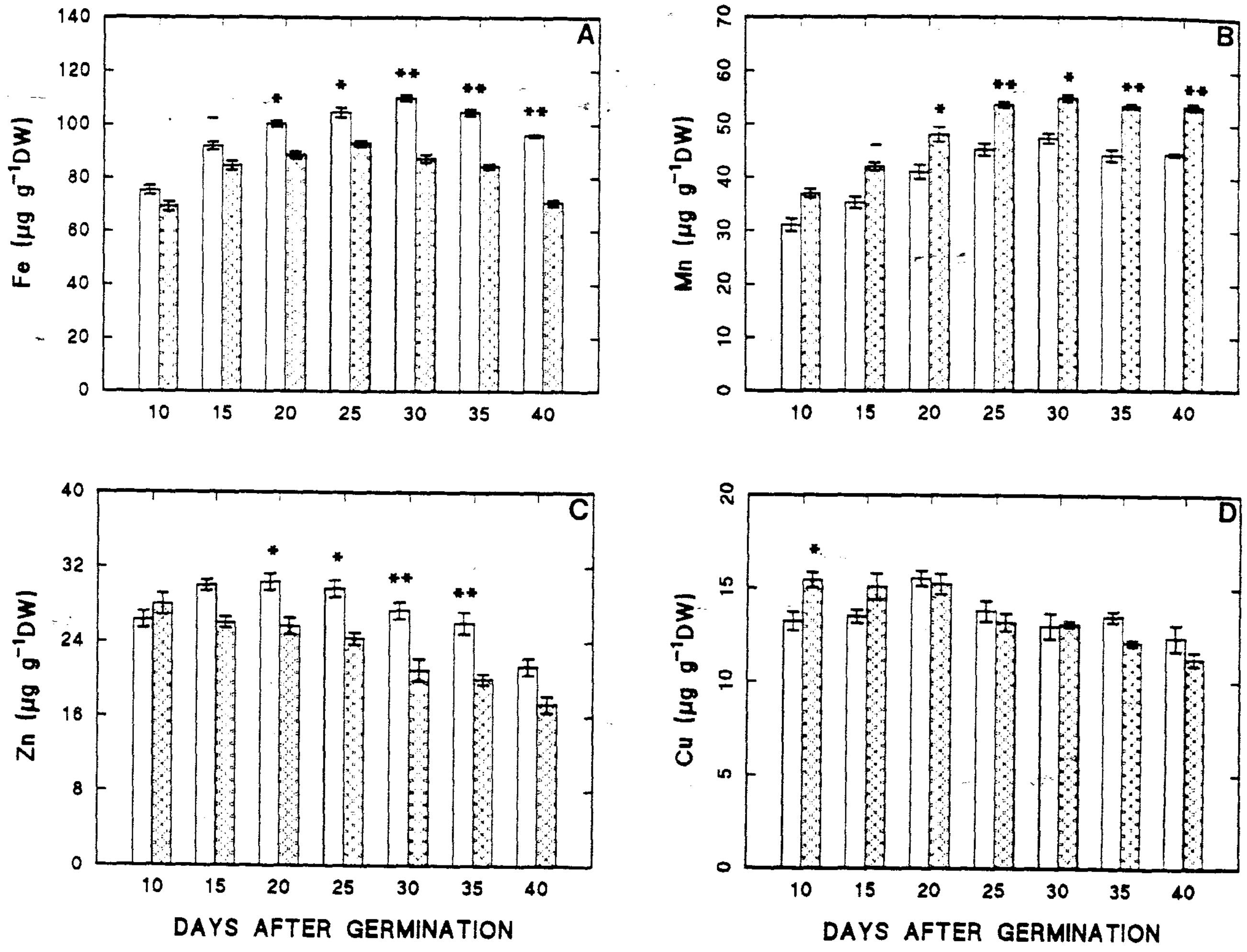

Fig. 3.15. Influence of two CO_2 concentrations on leaf micronutrient status: **A** - Iron; **B** - Mn; **C** - Zinc & **D** - Copper in bean plants grown at either 360 (open bars) or 700 (hatched bars) μmol mol^{-1} CO_2. (n=3; Error bars denote ± SE; $^-$ P<0.10, * P<0.05, ** P<0.01 indicate significant differences as tested by Scheffe's multiple range test. For Cu * indicates significant difference at P<0.05 following a one-way ANOVA since interaction was found significant).

Table 3.2. Summary of the F statistics obtained from the two-way analysis of varience tests ($^{***}P \le 0.001$; $^{**}P \le 0.01$; $^{*}P \le 0.05$; $^{n.s}$ not significant $P > 0.05$).

	Source of variation		
Variables	Treatment	DAG	Interaction
TPH	520.21^{***}	2773.92^{***}	2773.92^{***}
LA	415.90^{***}	232.39^{***}	15.81^{***}
DW	2272.71^{***}	30.27^{***}	22.23^{***}
SLA	58.79^{***}	180.04^{***}	12.35^{***}
LD	76.05^{***}	29.85^{***}	$1.05^{n.s}$
NAR	142.70^{***}	66.06^{***}	11.30^{***}
N	4.07^{**}	31.82^{***}	8.37^{***}
Ca	579.18^{***}	82.41^{***}	$1.69^{n.s}$
P	13.52^{***}	12.52^{***}	$1.85^{n.s}$
K	31.67^{***}	3.45^{*}	$0.43^{n.s}$
Mg	$3.45^{n.s}$	4.32^{**}	$0.30^{n.s}$
Fe	406.66^{***}	95.18^{***}	15.32^{***}
Zn	75.47^{***}	27.04^{***}	4.68^{**}
Mn	240.39^{***}	95.36^{***}	$0.86^{n.s}$
Cu	$0.06^{n.s}$	11.91^{***}	3.93^{**}

3.3 Discussion

Plants exposed to elevated CO_2 showed a significant increase in TPH, LA, and LDW. NAR was initially stimulated by CO_2 enrichment, but from 20 DAG, a decline in NAR stimulation was observed. Such responses to high CO_2 are well documented for a wide range of plant species (Peet, 1984; Delucia *et al.*, 1985; Baker and Allen, 1993). Arp (1991) attributed the decline in NAR observed in plant species exposed to long term elevated $[CO_2]$, in part, to source-sink imbalances resulting in end-product feedback inhibition. However, in this study, I noted a consistent expansion in LA, an enhanced LDW, total plant biomass and TPH. These enhanced growth parameters suggest that there is a change in sink pattern or sizes which are manifested in expansion of LA and possibly enhanced TPH. Such morphological changes are presumed to be due to the formation or stimulation of additional active sinks (Paul and Stitt, 1993). These sinks might be expected to utilize and store the increases and accumulation of photosynthates induced by elevated $[CO_2]$. However, despite these morphological changes, NAR continued to decline to levels similar to those observed in ambient-CO_2 grown plants.

Specific leaf areas were consistently lower, while NAR were higher in CO_2-enriched plants during most of the early stages of growth. At later stages of growth, the reverse effects of CO_2 occurred with respect to SLA and NAR in these plants. A negative correlation between NAR and SLA has been reported previously (Lambers *et al.*, 1989). In their study of the effects of elevated CO_2 and temperature on white clover, Ryle *et al.* (1992) reported the same response of SLA, however no clear explanation was cited. Other researchers ascribed reductions in SLA to an increase in the starch content of leaves

(Hirose, 1987; Ehret and Jolliffe, 1985b; Poorter, 1993). Enhanced starch content in leaves has been a topic of debate, and some contention, especially in studies of the long-term effects of elevated CO_2. It has been postulated that acclimatization or down-regulation of photosynthetic capacity may be due to starch accumulation in the leaves (Potter and Breen, 1980; Ehret and Jolliffe, 1985a). Arp (1991) attributed the decline in NAR to the inability of plants to create new additional active sinks to cope with the increases of photosynthates and accumulation of carbohydrates generally observed in CO_2-enriched plants. Still other researchers point to other factors which negatively affect carboxylation, such as decreases in the quantity and or activity of Rubisco and ribulose 1,5-bisphosphate (RuBP) regeneration capacity (Sage *et al.*, 1989; Rowland-Bamford *et al.*, 1991; Sage, 1994). This could have been the case in this study, as Rubisco activity was reduced significantly at later stages of growth in plants exposed to elevated [CO_2] (Fig. 3.7).

Rubisco constitutes more than 60% of the total protein in a mature leaves (Besford *et al.*, 1990; Wojcieska, 1994). Therefore the bulk of total leaf N is invested in Rubisco, since N is a major constituent of all proteins, including enzymes. The analysis of foliar N concentrations showed an increase during the first 20 DAG in the bean plants exposed to elevated CO_2, but from this point onward, a significant decline of foliar N was observed (Fig. 3.13). Similar results have been reported in crop plants grown under elevated CO_2 (Hocking and Meyer, 1991; Conroy *et al.*, 1992). At a similar growth stage, NAR and Rubisco activity declined in CO_2-grown plants. This suggests a very delicately balanced system, in which a slight decline in N is immediately manifested in a sharp drop in NAR. Such a close relationship between foliar N concentration and Rubisco activity was recently demonstrated by Wojcieska (1994). In addition, it has been shown that the effects of limited N supply on NAR are amplified by limitations of other nutrients, in particular phosphorus (Conroy, 1992; Thomas *et al.*, 1994).

Leaf phosphorus content exhibited a significant reduction in bean plants when grown in a high CO_2 environment, which corroborates results reported by Morin *et al.*, (1992) in a similar study using clover plants. Deficiency of orthophosphate (Pi) reduces NAR (Fredeen *et al.*, 1989) by limiting the regeneration rate and/or capacity of RuBP (Sage *et al.*, 1989; Guidi *et al.*, 1994). The inhibition of regeneration of Pi induced by elevated CO_2 during sucrose and starch synthesis limits the production of ATP during photosynthesis, leading to "feedback" inhibition of photosynthesis (Thomas *et al.*, 1994). One may conclude that down-regulation of NAR observed in CO_2-enriched plants may result, in part, from the adverse effects of low P content on the capacity to regenerate RuBP and Pi, both vital for CO_2 fixation.

Although a number of elements such as Fe, Mn, Zn and Ca play a crucial role as functional or structural regulatory cofactors including enzyme activation and osmoregulation in photosynthetic process (Hipkins, 1983; Davis, 1994), Mg has been singled out as one of the most important elements intimately associated with the CO_2 fixing enzyme, Rubisco (Marschner, 1986; Dreyer *et al.*, 1994). Apart from its function as a central atom in the chlorophyll molecule, and as a pH regulator within the cell, Mg plays a vital role in modulation of Rubisco. Binding of Mg^{2+} to this enzyme increases its affinity K_m for the substrate CO_2 and the turnover rate V_{max} (Hewitt, 1983; Marschner, 1986). Bean plants grown under elevated CO_2 were unable to sustain the required increases in Mg uptake necessary to maintain critical concentrations of this element for Rubisco activation. Consequently, bean exposed to elevated CO_2 showed reduction in NAR. Because of its similarity to Mg, Mn (both divalent cations, Mg^{2+} and Mn^{2+} respectively) can compete and successfully replace this element for the same site in a number of cofactors and enzymes (Hewitt, 1983) including Rubisco (Raven, 1990). Although Mn uptake was significantly increased in CO_2-enriched bean plants, its positive role (possible Rubisco activator) may have been subdued by the general reduction of other elements such as N, P, Mg, Ca, Zn, and Fe.

Zn, Fe, and Mn are associated with electron transport and photophosphorylation at PSII (Raven, 1990; Geider and La Roche, 1994), while Cu is mainly bound with plastocynins linked to PSI (Marschner, 1986). These components are essential in CO_2 fixation. The general reduction of these elements (with the exception of Mn which was significantly increased) in high CO_2-grown plants coupled with the significant reduction in total chlorophylls, prompts the speculation that the light harvesting systems may have been adversely affected (Fig. 3.9). In fact, Siefermann-Harms (1985) and Juhler *et al.* (1993) pointed out that the photosynthetic pigments are not uniformly distributed among the components of the photosynthetic apparatus. Chlorophyll *b* and carotenoids, for example, are enriched in the light harvesting complex of PSII, whereas chlorophyll *a* and β-carotene are typical of pigments of the reaction centre complex PSI. Therefore, the decline of these pigments in plants grown under elevated CO_2 could have adversely affected the light harvesting systems.

Apart from a rapid decline in chlorophyll *b* compared to chlorophyll *a* in CO_2-enriched plants, the increase in chlorophyll *a*/*b* ratio at later stages of growth (Fig. 3.10) suggests a decline in the proportion of PSII : PSI, PSII being localized mainly in photosynthetic membranes forming grana (Darmency *et al.*, 1992). These factors may have, in part, contributed in the decline in NAR observed at later stages of growth in high CO_2-grown plants. Despite the decline in total chlorophyll, total carotenoids did not differ significantly in between the treatments (Fig. 3.11). Carotenoids have been implicated in the photoprotective de-excitation of chlorophyll in the light-harvesting pigment bed when light is excessive (Demmig-Adams and Adams, 1992), and may also assist in light harvesting under certain conditions (Sieferman-Harms, 1985).

In addition, down-regulation of NAR may have been exacerbated by the limited supply of foliar Fe to the chlorophyll synthesis, since Fe is an essential element in coproporphyrinogen oxidase, an enzyme involved in catalyzing chlorophyll synthesis (Geider and La Roche, 1994; Fodor *et al.*, 1995). This, coupled with the general decline in chlorophylls, may have led to leaf discoloration or chlorosis (Misra and Bansal, 1992) which was observed in the CO_2-enriched bean plants. This phenomenon may, in addition, have been compounded by Ca shortages which have been shown to cause disintegration of cell walls and loss of tissue structure (Maschner, 1986), resulting in injury with similar pigments in plants grown under elevated CO_2 could have adversely affected the light harvesting systems.

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Furthermore, elevated CO_2 usually results in larger plants compared to plants grown in ambient CO_2, as was observed in this study and other studies (Coleman *et al.*, 1993). The present study is based on a comparison of leaf nutrient concentrations in plants of similar ages. As a result, the foliar nutrient levels of ambient and elevated CO_2 treated plants could differ because of potential ontogenetic changes as was demonstrated for nitrogen by Coleman *et al.* (1993). It is also important to point out that this study was undertaken using non-nodulating bean plants, and nodulation has been suggested to affect nutrient concentrations (Binkley and Richter, 1987; Waterman and Mole, 1986). Clearly, debate and research on the effects of elevated CO_2 remains a topic of great interest, and further detailed studies involving the analysis of other plant parts, such stems and roots, will provide a complete picture of the response of plants to future elevated CO_2.

CHAPTER 4: CHANGES IN NON-STRUCTURAL CARBOHYDRATES AND NITROGENOUS COMPOUNDS IN LEAVES AND SEEDS

4.1 Introduction

Although it has been shown that partitioning of compounds in the plant may be largely controlled by innate genetic programs (Bewley and Black, 1994), some modifications may result from agronomic practices or changes in environmental conditions (Müntz 1982; Peña-valdivia *et al.*, 1994; Bewley and Black, 1994). The current increase in atmospheric CO_2, an environmental parameter, is fast altering various physiological, morphological, and other important aspects of plant performance (see review by Bazzaz, 1990). Amongst other things, non-structural carbohydrates (NSC) are most substantially increased in the leaves of plants exposed to elevated CO_2 (Peet *et al.*, 1986; Wong, 1990, Farrar and Williams, 1991). The drastic accumulation of NSC will not only affect bulk transport to various sinks in the plant but may also interfere with the way in which the assimilates are distributed (Schulze *et al.*, 1994). Changes in allocation patterns under such conditions have been reported (Krapp *et al.*, 1993; Schulze *et al.*, 1994) and it is highly possible that substantial shifts of these compounds may be channeled towards the fruits during the period of fruit/seed development.

Several studies have investigated changes in carbohydrates pools under elevated CO_2 (Vu *et al.*, 1989; Yelle *et al.*, 1989a; Rowland-Bamford *et al.*, 1990). However, few studies have looked at changes in NSC throughout the entire course of the growth period, so as to assess their potential effects on reproductive structures such as flowers, pods and seed filling (Chu *et al.*, 1992; Cipollini *et al.*, 1993). Unfortunately, most published studies on the effect of $[CO_2]$ on fruit yield and productivity, have mainly reported the quantitative aspects such as changes in numbers, sizes and weights (Sionit *et al.*, 1980; Cure, 1986; Baker *et al.*, 1992). This is despite the fact that several authors have reported nutrient dilution effects resulting from unfavorable shifts in carbon/nutrient ratios in plants grown under elevated CO_2 concentrations (Akey and Kimball, 1989; Kuehny *et al.*, 1991; Hocking and Meyer, 1991) which are bound to affect yield including fruit quality (Barnett and Pearce, 1983; Schulze *et al.*, 1994; Conroy *et al.*, 1994).

It has been established that before flowering and fruit set, most of the metabolites from source leaves are invested in dry matter, new leaf formation and roots, but after flowering the partitioning and distribution pattern is diverted to fruits, sometimes at the expense of other vegetative plant organs (Pate, 1984). Consequently, fruits are the main active sinks at later stages of development (Pate, 1984). Remobilization of reserve nutrients including carbohydrates has been shown to contribute significantly to yield in plants (Gifford and Evans 1981; Hewitt and Marrush, 1986; Bar-Zur and Schaffer, 1993).

While leaves are regarded as the primary source of food material, other organs may also contribute essential products to the developing seeds. For example, in bean and pea plants, the quantitative studies of carbon and nitrogenous compounds transfer from the leaves and photosynthetic pericarps (pod walls) have shown that pods are also committed to export to fruits, thus becoming a significant part in provision of food reserves to the developing seeds (Flinn and Pate, 1970; Macnicol, 1983; Lewis, 1986; Lewis *et al.*, 1971; Rochat and Boutin, 1989).

Legume seeds are an important source of dietary protein, particularly in South Africa and the rest of the Third world countries (Murray, 1984; Peña-Valdivia *et al.*, 1993). It is difficult to speculate that the associated high nutritive value will still be maintained under the impact of elevated [CO_2]. To date very little published data appear on the quality aspect, *inter alia*, relative measure of nitrogenous compounds and NSC (Newton, 1991; Conroy *et al.*, 1994). I believe that a better understanding of these changes and/or remobilization to seeds are of primary importance, as they may have a direct impact on agricultural industry as yield quantity and quality may be affected by these processes. However, the most important question, is whether the mature seeds produced by plants grown in elevated [CO_2] are able to retain sufficient high quality food reserves to sustain subsequent embryo development. Undoubtedly, the implications for the survival of future progeny, which is largely dependent on viability of previously produced seeds, becomes even more important (Murray, 1984; Lewis, 1986; Bewley and Black, 1994). Therefore, the seed occupies a critical largely be determined by the physiological and biochemical features of the seeds. Of key importance to this success are the seed's responses to the environment and the food reserves it contains (Lewis, 1986; Bewley and Black, 1994).

Consequently, the results presented in this chapter attempt to establish to what extent the mobilization of NSC from leaves affects the final fruit yield and quality in *P. vulgaris*. Therefore, changes in pool sizes of nitrogenous compounds (such as total nitrogen, amino compounds and total soluble proteins) and NSC (e.g. sucrose, glucose, fructose and starch) were investigated.

Several researchers have reported damaged chloroplasts due to enlarged starch grain sizes (Cave *et al.*, 1981, Gucci *et al.*, 1991). This and other potential adverse effects on the delicate photosynthetic machinery as a result of enlarged starch grains, were also investigated. Furthermore, as pods may also be an important source for additional assimilates for the developing seeds, particularly at early stages of development, and may exhibit some similarities to the leaves in terms of carbon fixation including synthesis and reallocation of NSC, it was essential to sample them too. This was performed early after anthesis before the seeds could be separated from the pods due to their small size before this stage.

4.2 Results

4.2.1 Starch and soluble sugars

Plants exposed to elevated CO_2 exhibited a significant increase in total NSC in both leaves and pods. The leaf starch content (major component of NSC) showed an average increase of more than 40 mg per g leaf dry weight compared to plants grown in ambient CO_2. (Fig. 4.1, $P<0.0001$). This observation was in agreement with results reported in similar studies (Wong, 1990; Kuehny *et al.*, 1991). The pools of soluble sugars, especially sucrose, were significantly higher in leaves exposed to elevated CO_2. The average difference being 15 mg per g leaf dry weight higher than the controls across all growth stages (Table 4.1).

Table 4.1 Changes in soluble carbohydrates in leaves of plants exposed to either 360 or 700 µmol mol^{-1} CO_2. Values presented are the means calculated from three replicates. (n=3; $^{**}P<0.01$; $^{*}P<0.05$; ^{ns} not significant $P>0.05$).

DAG	Treatment µmol mol^{-1} CO_2	Soluble carbohydrates mg g^{-1} DW			
		Sucrose	Fructose	Glucose	**Total**
10	360	6.57	1.37	0.11	8.11
	700	18.97^{**}	3.31^{*}	1.01^{**}	23.29^{**}
20	360	14.07	2.13	0.91	17.11
	700	25.46^{*}	4.23^{*}	1.98^{**}	31.67^{**}
30	360	18.07	2.89	1.03	21.99
	700	33.01^{*}	3.88^{*}	1.91^{*}	38.8^{**}
40	360	17.15	1.56	0.68	19.39
	700	28.76^{*}	2.27^{*}	1.15^{**}	32.18^{**}

Three stages of pod development were identified, which will be referred to hereafter as:

I. Stage marked by pod and embryo formation or *sensu stricto* embryogenesis,

II. pod growth and filling, and

III. seed development and maturation (as described by Rochat and Boutin, 1989).

Soluble NSC and starch were determined from early stages of pod development up to the seed stage (Figs. 4.2 and 4.4, respectively) under normal and elevated CO_2 treatments. Starch and soluble sugars fraction were greatly increased during the initial stages of pod development (I and II). Stage III was typified by a significant decline in both starch and soluble sugars. However, the rate of decline was more pronounced for soluble sugars than for starch. Several authors have reported this type of response (Slack and Browse, 1984; Rochat and Boutin, 1989; Bewley and Black, 1994). Despite the similar response observed in *P. vulgaris* under both CO_2 treatments, the levels of starch and soluble sugars remained consistently higher in bean plants exposed to elevated CO_2.

4.2.2 Chloroplast ultrastructure and morphometry

Electron micrographs revealed considerable enlargement in starch grain sizes in bean plants exposed to elevated CO_2 (Fig. 4.3). Calculations using digitized TIF images indicated an average of 5-fold increase in starch grain surface area in bean leaves exposed to enhanced CO_2 (Table 4.2). Several authors have reported similar observations for bean and other plant species (Cave *et al.*, 1981; Vu *et al.*, 1989). There was no significant difference ($P>0.05$) in the numbers of starch grains per chloroplast, which remained within the range of 4-6 on average in both CO_2 treatments. Despite considerable enlargement in grain sizes, there was no visible damage of chloroplasts or apparent malformation of thylakoid membranes. In their ultrastructural analysis of chloroplasts of soybean leaves exposed to elevated CO_2, Vu *et al.* (1989) reported a similar apparent general stability of membranes while Cave *et al.* (1981) reported contrary results. Although not employed in this study, chlorophyll fluorescence which is a measure of the intrinsic process of leaf photosynthesis, is now regarded as a most reliable, sensitive and useful non-destructive indicator of thylakoid stability (Seeton and Walker, 1990; Ceulemans *et al.*, 1995).

Table 4.2 Morphometric measurements of starch grains (numbers and relative sizes such as long axis or length, short axis or width and total surface area) in leaves of plants exposed to either 360 or 700 μmol mol^{-1} CO_2. Measurements were determined in plants of 25 DAG. Values presented are the means calculated from 6 TIF images of leaf micrographs for each [CO_2] treatment; $^{***}P<0.001$; $^{**}P<0.01$; $^{*}P<0.05$; ^{ns} not significant $P>0.05$.

Treatment μmol mol^{-1} CO_2	Starch grain number	Grain length (μm)	Grain width (μm)	Grain surface area (μm)
360	4-5	1.17	0.42	0.50
700	4-6^{ns}	2.12*	1.16**	2.61***

4.2.3 Nitrogenous compounds

The quantity of N in both leaves (Chapter 3; Fig. 3.13) and pods (Fig. 4.6) was significantly reduced in plants exposed to elevated CO_2. The leaf total soluble protein content initially increased and then declined to levels significantly lower when compared to bean plants grown under ambient CO_2 (Fig. 4.5; $P<0.001$). Total soluble protein content in the developing pods (stage I) initially increased steadily, followed by a steep rise in stage II. However, the rate of increase fell in stage III, and a slight decline was observed in both CO_2 treatments (Fig. 4.7). The levels of protein was significantly higher ($P<0.0001$) in bean plants grown under ambient CO_2 when compared to plants grown in elevated [CO_2].

Table 4.3 summarizes the composition of the free amino compounds in the seeds in both CO_2 treatments. During early seed development, the amino-acid pool is dominated by glutamate, alanine, arginine and to a certain extent asparagine. Not only did the concentration of these amino acids decline with maturity, but the general amino acid pool size declined significantly from approximately 5000 to 2000 μmol per g seed dry weight on average in both CO_2 treatments. Several authors have observed similar trends and reported reduced levels of amino compounds in mature seeds (Müntz, 1982; Macnicol, 1983; Rochat and Boutin; 1989).

According to Rochat and Boutin (1989) free amino acids occurring in the seed during the filling stages (I and II) appear to play various roles according to their nature and seed development. The initial levels of amino acids, represent a temporary reserve materials in the embryo sac fluid that are subsequently translocated to the apoplast of the seedcoat and the cotyledon, where they are used for synthesis of proteins. Subsequently, as seed development nears completion, free amino acids such as asparagine and arginine represent nitrogen reserve materials which will be probably used at the early stages of seed germination. Indeed, bean plants investigated showed arginine to be the one of the dominant amino acid in the mature seed. While free amino acids declined in both treatments, the levels were consistently and significantly higher in plants grown under ambient CO_2 (Table 4.3).

Table 4.3. Free amino acids (µmol g^{-1} DW) in seeds of plants exposed to either 360 or 700 µmol mol^{-1} CO_2. Values presented are the means calculated from three replicates for each [CO_2] treatment; harvested at three stages of development ($^{***}P<0.001$; $^{**}P<0.01$; $^{*}P<0.05$; ^{ns} not significant $P>0.05$).

	Days after anthesis (DAA)					
	24	24	30	30	36	36
Compound	CO_2 treatment (µmol mol^{-1})					
µmol g^{-1} DW	360	700	360	700	360	700
Phosphoserine	54	-	96	116	81	58
Aspartic	45	35	120	98	78	48
Glutamic	1045	905	319	245	177	117
Hydroxyproline	58	55	-	-	-	-
Serine	138	121	183	124	130	113
Asparagine	590	516	95	88	265	143
Glycine	69	50	67	55	34	-
Glutamine	69	29	20	-	35	-
Histidine	98	77	45	32	35	-
Threonine	-	-	-	-	-	-
Arginine	656	542	910	721	603	498
Alanine	845	727	430	305	371	347
Proline	55	50	33	16	20	24
Tryosine	89	75	29	26	29	21
Valine	317	288	87	65	74	63
Methionine	76	59	66	32	42	46
Cystine	-	-	15	-	-	-
Isoleucine	219	197	84	45	48	-
Leucine	699	643	334	219	279	247
Phenylalanine	89	122	53	44	54	44
Tryptophan	-	29	-	-	14	7
Lysine	36	20	42	29	38	26
Ammonia	156	138	166	152	129	119
TOTALS	5403	4678^{***}	3194	2415^{**}	2501	1921^{**}

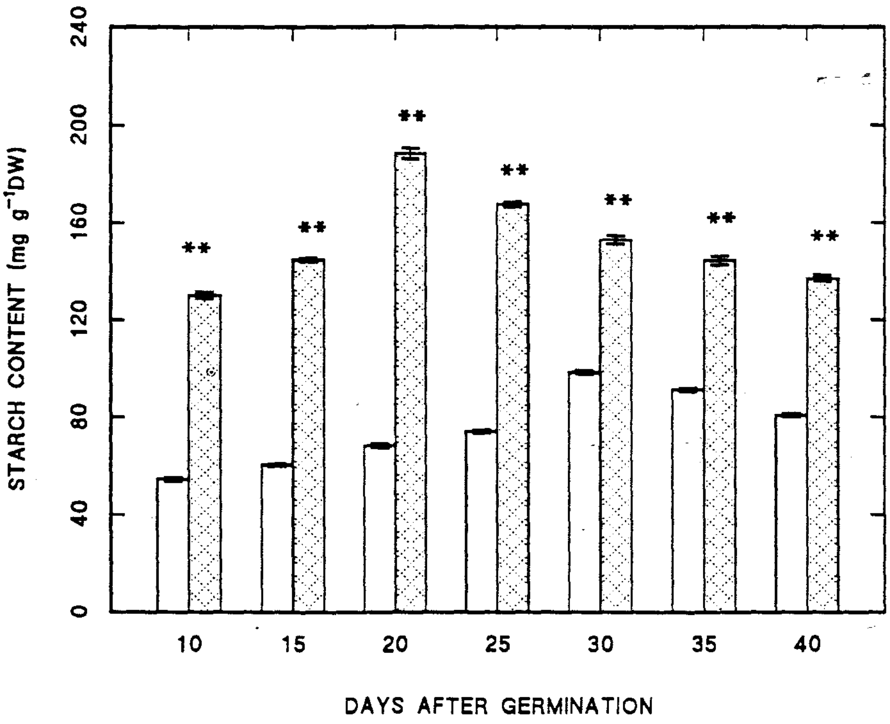

Fig. 4.1 Analysis of starch content of the leaf tissue of bean plants grown at 360 (open bars) and 700 (hatched bars) μmol mol^{-1} CO_2. The concentrations expressed as mg starch per g dry weight of leaf sample. (n=3 per each data point; Error bars denote ±SE; ***P<0.001; **P<0.01; *P<0.05; ^{ns} not significant P>0.05).

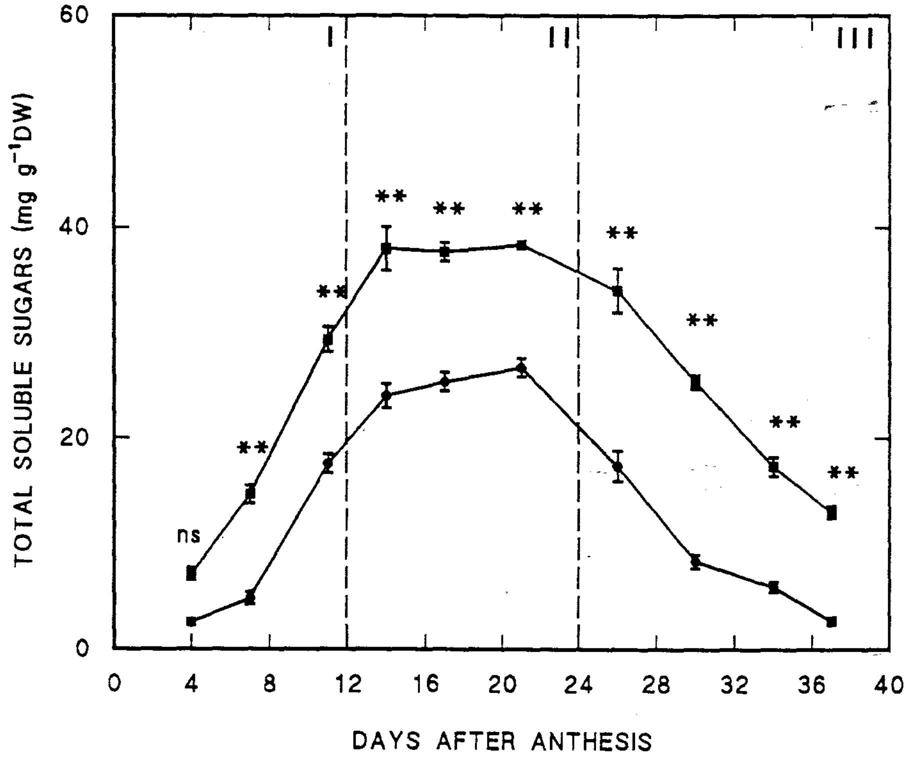

Fig. 4.2 Changes in the total soluble sugars during the pod and seed development stages. Three marked stages are: I, stage of pod and embryo formation or *sensu stricto* embryogenesis II, pod growth and filling and III seed development and maturation (Rochat and Boutin, 1989). The plants were grown at 360 (●) and 700 (■) μmol mol^{-1} CO_2. The concentrations are expressed as mg per g dry weight of leaf sample. (n=3 per each data point; Error bars denote ±SE; $^{***}P<0.001$; $^{**}P<0.01$; $^{*}P<0.05$; ^{ns} not significant $P>0.05$).

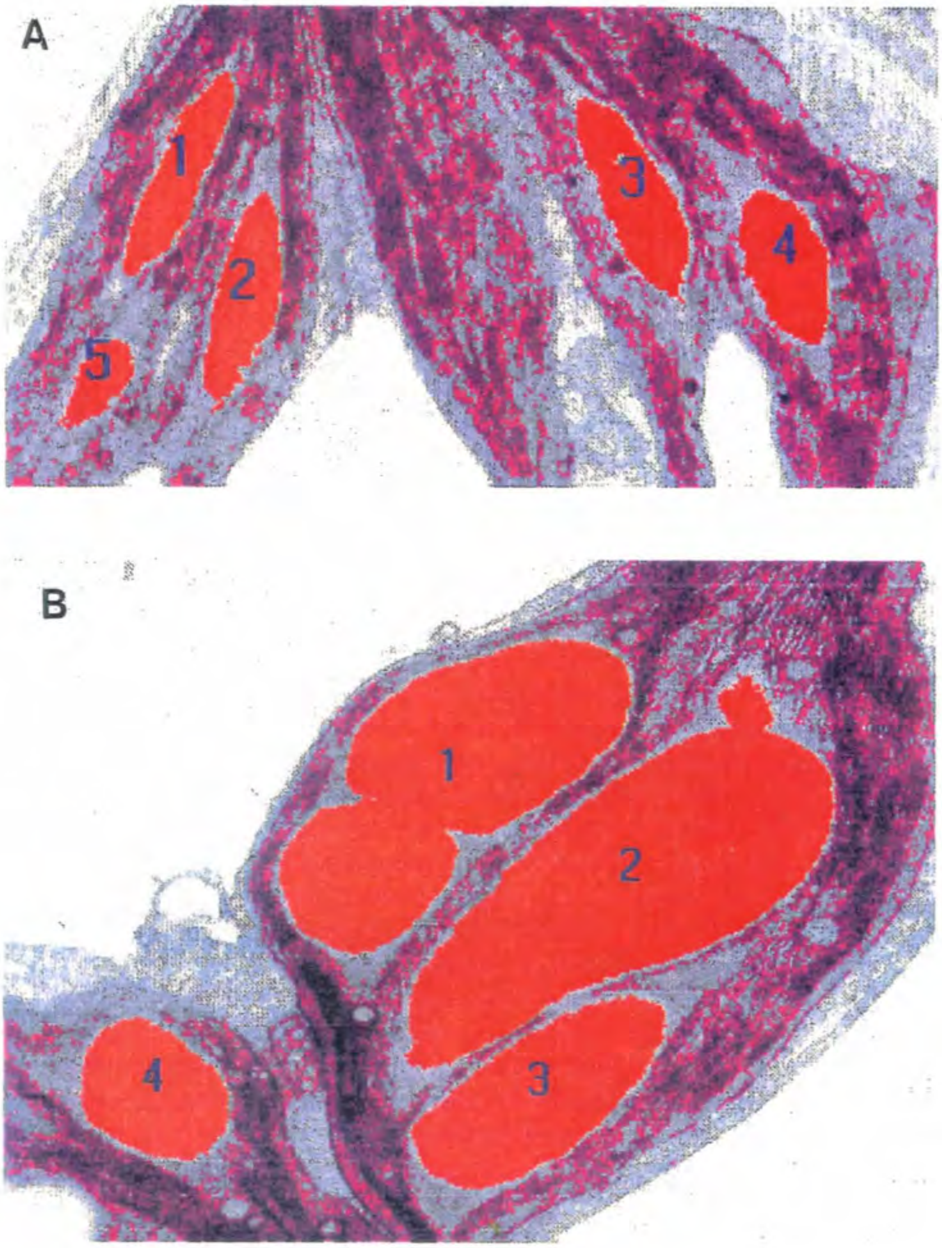

Fig. 4.3 TIF images of digitized electron micrographs of chloroplasts showing different anatomical structures observed in bean plants of 25 DAG grown at 360 (**A**) and 700 (**B**) μmol mol^{-1} CO_2. Note, enlarged starch grains (**B**) induced by elevated CO_2 indicated by numbers printed on the individual starch grain in both images. Pseudo coloring was used for morphometric measurements (see Chapter 2).

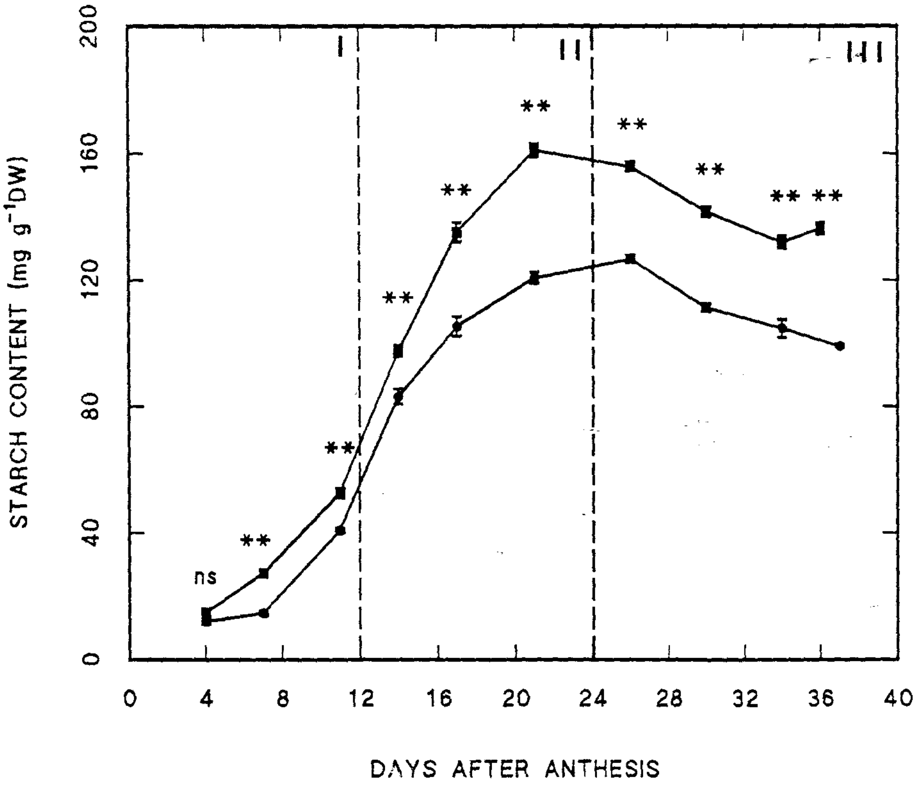

Fig. 4.4 Changes in the starch content during the pod and seed development of bean plants grown at 360 (●) and 700 (■) μmol mol^{-1} CO_2. See legend of Fig.4.2 for other details.

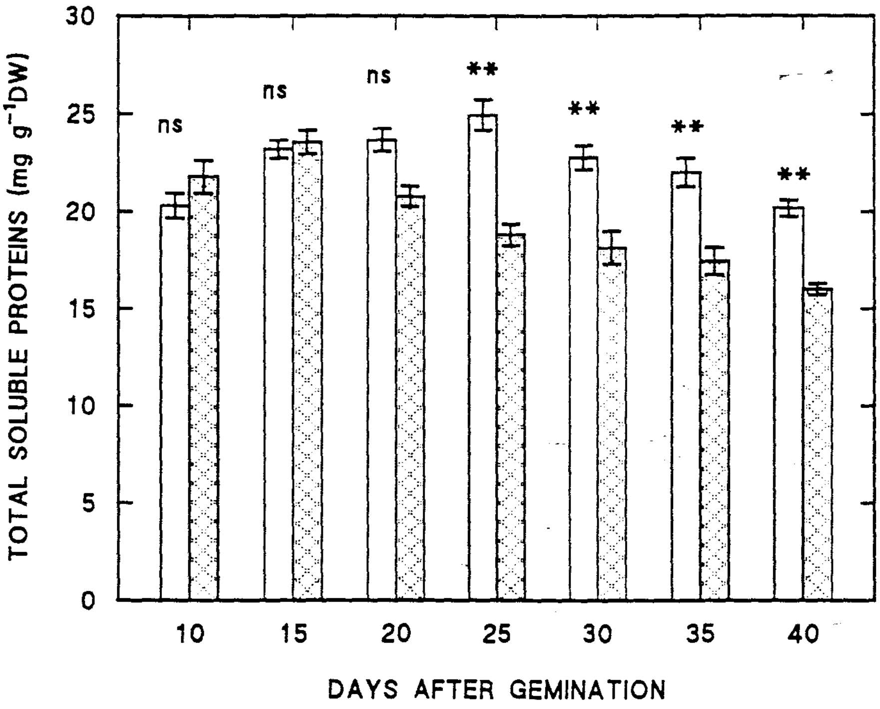

Fig. 4.5 Analysis of total soluble protein content of the leaf tissue of bean plants grown at 360 (open bars) and 700 (hatched bars) μmol mol^{-1} CO_2. The concentrations expressed as mg total soluble protein per g dry weight of leaf sample. (n=3 per data point; Error bars denote ±SE; $^{***}P<0.001$; $^{**}P<0.01$; $^{*}P<0.05$; ^{ns} not significant $P>0.05$).

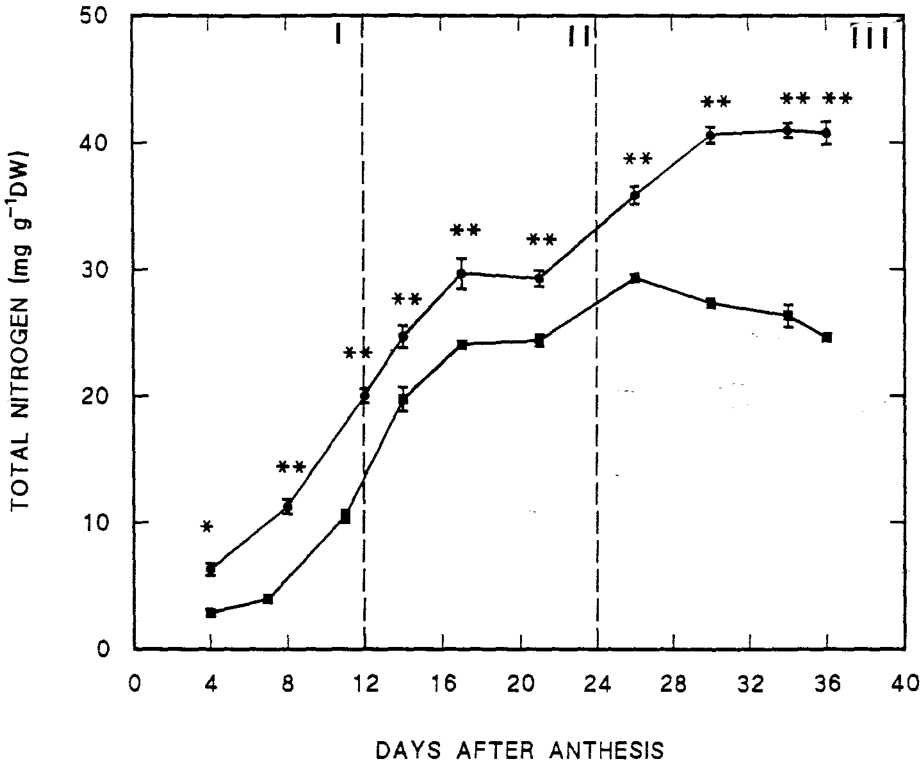

Fig. 4.6 Changes in the total nitrogen content during the pod and seed development of bean plants grown at 360 (●) and 700 (■) μmol mol^{-1} CO_2. See legend of Fig. 4.2 for other details.

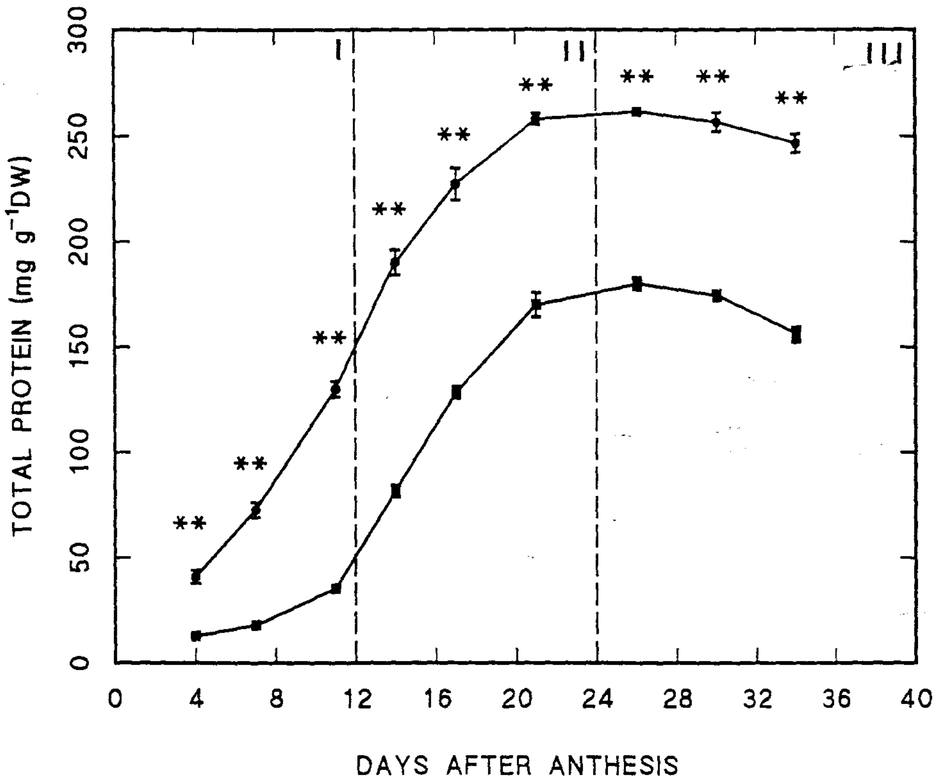

Fig. 4.7 Changes in the total soluble protein content during the pod and seed development of bean plants grown at 360 (●) and 700 (■) μmol mol^{-1} CO_2. See legend of Fig. 4.2 for other details.

4.3 Discussion

The starch content increased significantly in leaves of bean plants grown in elevated CO_2 (Fig. 4.1). Several authors have reported similar increases in starch content (Peet *et al.*, 1986; Wong, 1990; Farrar and Williams, 1991). In this study, changes in concentrations of soluble carbohydrates, differ from findings of Finn and Brun (1982) and Ingvardsen and Veierskov (994) who reported insignificant changes in soluble carbohydrates in soybean and barley exposed to elevated CO_2, respectively. However, Huber *et al.* (1984), Vu *et al.* (1989) and Yelle *et al.* (1989a) showed that leaves of soybean and tomato plants, respectively, accumulated large amounts of soluble carbohydrate (sucrose, fructose and glucose) and starch compared to plants grown at ambient CO_2. These observations are consistent with the results obtained in this study for *P. vulgaris* where both starch and other soluble carbohydrates contents increased under elevated CO_2 (Table 4.1).

Abnormally-enlarged starch grains in plants grown under enhanced [CO_2] have been singled out as the main cause of chloroplast disruption including the deformation of the membranous systems of the thylakoids (Cave *et al.*, 1981; Gucci *et al.*, 1991). This invariably leads to adverse effects on the photosynthetic machinery which in turn results in reduced NAR (Milford and Pearman, 1975; Ehret and Jolliffe, 1985b; Cure *et al.*, 1991). Although bean leaves investigated under elevated CO_2 exhibited large starch grains (see TIF images, Fig. 4.3), almost five times larger than those observed under low CO_2 concentrations (Table 4.2), no physical damage to the chloroplasts integrity or deformation of thylakoid membrane was evident. This is despite the fact that NAR was reduced with time in these plants (Chapter 3). Several workers have found no precise correlation between starch grain sizes or content accumulation and NAR, thus casting doubt on the regulation of NAR by leaf starch levels (Yelle *et al.*,1989a; Vu *et al.*, 1989; Gucci *et al.*, 1991). This suggests the involvement of other factors in down-regulation of NAR (see review by Sage, 1994)

In contrast to the leaves, NSC in the developing pods exhibited a transient response irrespective of CO_2 treatment. The sharp decline of the NSC observed in the seed stage has been reported elsewhere (Matheson, 1984; Slack and Browse, 1984; Bewley and Black, 1994). These authors suggested that sucrose, particularly in legume seeds, increases during early stages of pod filling but declines significantly during the seed stage or seed maturation stage because sucrose (major component of the soluble fraction) is in particular thought to be converted into acetyl-CoA and subsequently to high energy compounds and fatty acids. This is consistent with the present study where elevated CO_2 led to an increase in total soluble NSC content in pods (stage I and II) and a sharp decline during seed development (stage III) in both [CO_2] treatments (Fig. 4.2). As mentioned, total starch concentration in pods and seeds followed the same trends (Fig. 4.4), the rate of decline was not as steep as that observed for total soluble sugars.

According to Matheson (1984) and Slack and Browse (1984) the storage of fatty acids as seed reserve rather than carbohydrates may be an attempt to maximize the quantity of stored energy in a small volume of tissue. Furthermore, the carbon in fatty acids is more highly reduced than in carbohydrate complexes and consequently, the amounts of energy released during oxidation of the two compounds is about 38 and 17 kJ g^{-1}, respectively (Slack and Browse, 1984). One other factor which may also explain the sharp drop in NSC is that sucrose (being the major component of mobile fraction) is a major amino acid precursor, supplying the basic carbon skeletons in the synthesis of amino compounds (Lewis and Pate, 1973; Macnicol, 1983; Huber *et al.*, 1992). Furthermore, the steep decline in NSC coincides with the stage of rapid pod filling and development, which prompts the speculation that at this stage vast amounts of assimilates are utilized. This is consistent with previous results (Flinn and Pate 1970; Peña-Valdivia and Ortega-Delgado, 1991) which demonstrated that young developing pods are major sinks before they are fully mature and capable of photosynthesis . Recently, Schulze *et al.* (1994) also showed that large sinks are created during flower induction and even more so at subsequent growth stages of fruit development which were marked by massive utilization of freely available soluble sugars.

A substantial fraction of soluble NSC could have been used in respiration and lost as CO_2 by both flowers and young pods (Matheson, 1984). Perhaps the most important point in this regard is that the largest percentage of NSC particularly in plants grown under elevated CO_2 was locked up as immobile storage starch (Fig. 4.1) and was not available for transport (Matheson, 1984; Poorter, 1993). In plants grown under elevated CO_2, this could be exacerbated by the general reduction in leaf longevity. At this stage most leaves in these plants were already showing chlorotic symptoms and others had senesced (Chapter 3, Fig. 3.5), therefore the rate of mobilization of soluble NSC from leaves could have been reduced by this phenomenon (Table 4.1), thus placing more pressure on the pools available in the pods. Under these conditions no substantial reserves in pods could have been maintained.

All these reasons may have contributed to the drastic decline in NSC observed in both CO_2 treatments. Starch pools also declined but not as steeply as was observed for the soluble fraction. Nevertheless the overall rate of decline in NSC was more pronounced in plants grown under ambient CO_2 and the net results were significant low levels of NSC including starch in these plants.

Although exposure of plants to higher CO_2 concentrations usually results in an initial stimulation of photosynthesis, rates may decline in some species as plants acclimate (Sage *et al.*, 1989; Van Oosten *et al.*, 1993). The reasons for this decline are not fully understood, but sink capacity for storage and/or utilization may strongly influence its extent amongst other things (Sharkey, 1985; Bazzaz, 1990). Studies involving manipulation of sink strength under elevated CO_2 atmosphere support this hypothesis (Thomas and Strain, 1991). Nevertheless, there have been numerous reports which conclusively demonstrate that an increase in carbohydrate content leads to nutrient dilution, particularly in leaves of plants exposed to elevated CO_2 (Lincoln *et al.*, 1986; Akey and Kimball, 1989; Kuehny *et al.*, 1991). Consistent with these results, this study showed that, leaf, pod and seed N declined in bean plants grown in elevated CO_2 (Chapter 3, Fig. 3.13; and Fig. 4.6, respectively).

Nitrogen is a main factor that is limiting photosynthetic capacity and seed yields in plants. Final seed yields often depend on the proteolysis of stored foliar N and its translocation to the seed (Dalling *et al.*, 1975; Huffaker, 1982). Furthermore, N is essential for the synthesis of proteins and amino compounds (Lewis, 1986; Bewley and Black, 1994). For instance, an arginine (one major amino acid component of legume seed) molecule contains 4 times as much nitrogen as the other amino acid molecules e.g. glutamate, glycine, alanine or aspartate (O.A.M. Lewis, *pers. comm.*). The decline in the content of total soluble proteins both in the leaves (Fig. 4.5) and pods (Fig. 4.7) of high CO_2-grown plants may have been caused by reduced N concentrations in these organs (Chapter 3, Fig. 3.13 and Fig. 4.6, respectively). The reduction of N and total soluble proteins in the leaves of these plants occurred during stage I and II, and that may have interfered with the storage protein synthesis and amino acids mobilization to seeds in stage III. This is manifested by a drastic decline in storage amino compound pools in the seeds (Table 4.3) of bean plants exposed to elevated CO_2.

As explained in the introduction, there is little published data with which the effects of elevated CO_2 on changes in free amino acids levels observed here can be compared. In their study of the changes in nitrogenous compounds in the developing pod and seed filling, Macnicol (1983) and Rochat and Boutin (1989) observed similar fluctuations and reported reduced levels of amino compounds in later stages of seed development (Table 4.3). These fluctuations were attributed to various processes, *inter alia*, resorption of the liquid endosperm, variable rate of translocation of sucrose and asparagine (major amino acid precursors) into the seed, changes in the capacity of the cells for amino acid biosynthesis and end-product regulation of amino acid synthesis.

Most of these processes are not yet fully understood (Lewis, 1986). It is more than likely that one or more of these processes may have been affected by elevated CO_2, this in addition to reductions in levels of N in these plants, resulting in a complex interaction. Thus, I conclude that reduced content and complete lack of some amino compounds - a feature more prevalent in elevated CO_2 grown plants - may have been as a result of enhanced [CO_2]. Such inferences may only be proven once additional data on the changes in amino compounds and seed protein induced by elevated CO_2 concentrations are available.

4.4 Conclusions

Partitioning and allocation of assimilates play an essential role in plant development and crop yield (Gifford and Evans 1981; Hewitt and Marrush, 1986; Schulze *et al.*, 1994). While this study did not employ any specific techniques such as radiolabelling to trace the patterns of mobilization of both NSC and nitrogenous compounds to reproductive structures and seeds, sufficient research and data exists in this regard to base our arguments on (Lewis and Pate, 1973; Macnicol, 1983; Ingvardsen and Veierskov, 1994; Vivin *et al*, 1995). Thus, observed changes in allocation of assimilates in various plant organs could be explained in terms of these reported patterns.

During pod and seed filling, N and C are essential in ensuring proper development of the seed. As mentioned in the foregoing discussion, both N and sucrose are essential precursors for synthesis of protein and other amino compounds. Enhanced soluble NSC with the concomitant decline in N content in both the leaf and reproductive structures of plants grown under elevated CO_2, is not an ideal situation, and presumably this inhibited amino acid synthesis. This was manifested in the significant decline in total soluble proteins and amino compound pools observed in plants grown under elevated CO_2.

These observations highlight the subtle but vital co-ordinated mechanisms underlying the acquisition and regulation of nitrogen and carbohydrates by plants. It is highly likely that there were disruptions induced by increased $[CO_2]$ which may well have interfered with these mechanisms. The reduction in nitrogen and nitrogenous compounds observed in *P. vulgaris* grown under elevated CO_2 must have been (primarily) due to those changes, which had deleterious effects on seed quality reducing the quantity of amino compounds and total proteins. However, whether these changes were as a direct or indirect result of dilution of nitrogenous compounds, due to enhanced accumulation of NSC, or as a result of alteration of the actual biochemical mechanisms responsible for protein synthesis induced by elevated CO_2 remain speculative at this stage.

CHAPTER 5: EFFECT OF ELEVATED CO_2 ON FLOWER, FRUIT ABSCISSION, AND SEED YIELD

5.1 Introduction

As a result of photosynthetic stimulation, elevated [CO_2] can accelerate plant growth and could potentially increase agricultural productivity. Since flowers, fruit, and seeds are of great economic importance and because these invariably determine yields, a number of researchers have reported on the response of these reproductive structures to increasing atmospheric CO_2 (Hesketh and Hellmers, 1973; Sionit *et al.*, 1981; Garbutt and Bazzaz, 1984; Acock and Allen, 1985; Mortensen and Ulsaker, 1985; Spencer and Bowes, 1986; Newton, 1991; Tremmel and Patterson, 1994; Rawson, 1995). Yield in crops is the result of an integration of many processes that occur during growth and development. These processes are expressed at the morphological, phenological, physiological, and biochemical levels of organization and to a large extent are controlled by growth regulators and genes (Begonia and Aldrich, 1990; Frederick and Hesketh, 1994).

Most of the factors controlling yield may be influenced by external environmental parameters. Phenology for instance, is primarily determined by temperature, light intensity and photoperiod (Lawn and Byth, 1973; Hadley *et al.*, 1984). Floral initiation is primarily a photoperiodic response but may be regulated by sugar concentrations (Aloni *et al.*, 1991), while floral growth is regulated mainly by temperature. In addition, both temperature and photoperiod influence post-flowering development (Hodges and French, 1985; George *et al.*, 1990; Ephrath and Hesketh, 1991). Other variables such as changes in endogenous growth regulators, for example IAA, ABA, and kinetins, (Subhadrabandhu *et al.*, 1978; Guinn and Brummett, 1988) or mineral nutrition, water, and [CO_2] (Acock and Allen, 1985) can also influence phenology.

Enhanced [CO_2] has been reported to delay flowering in some species (Hesketh and Hellmers, 1973; Marc and Gifford, 1984). Other authors report earlier anthesis and accelerated seed filling in plants grown under elevated CO_2 (Mortensen and Ulsaker, 1985; Spencer and Bowes, 1986). Timing of flowering may be of particular importance to insect-pollinated species which could find themselves out of synchrony with their pollinators (Garbutt and Bazzaz, 1984; Newton, 1991). Apart from changes in flower number (as direct result of elevated [CO_2]), patterns of flower sexes may change, as was observed for cucumber, which predominantly produced female flowers (Enoch *et al.*, 1976). Similarly the rate of flower abortion or abscission before or after fertilization has been noted to be affected by elevated [CO_2]. Hansen and Shibles (1978) reported high production of flowers in soybean grown in elevated CO_2 but that most later either abscised or aborted before developing into fruits.

Seed size and number has been reported to increase in some species growing under elevated CO_2 (Sionit *et al.*, 1981; Miglieta *et al.*, 1993), but decreasing seed size has been noted (Wulf and Miller-Alexander, 1985). Whilst Garbutt and Bazzaz (1984) reported no significant changes in seed number in *Abutilon theophrasti* and a slight decrease in *Datura stramonium*, they pointed out that the decline was offset by the increase in individual seed weight in this species. Seed yield has been observed to increase as a result of increased tillering which is normally common in plants grown under elevated CO_2 and not as a result of increase in fruit/seed weight (Havelka *et al.*, 1984; Oechel and Strain, 1985; Chaudhuri *et al.*, 1990; Nederhoff, 1994).

Despite these controversies, an increasing number of authors have demonstrated a general increase of between 33-40% of marketable or harvestable yields, particularly in C_3 species (Fig. 5.1) exposed to elevated CO_2 concentrations (Kimball, 1983; Allen *et al.*, 1987; Wheeler *et al.*, 1994). In addition, studies demonstrate that there is a wide range in the specific nature of responses, as a large variability exists among species, with respect to their response to elevated CO_2. Based on these conflicting results, the importance of elevated CO_2 on reproductive patterns remains uncertain. In addition, studies were preliminary (see review Newton, 1991), and report largely on the comparative changes, few have examined the underlying implications as a result of these responses. Clearly, the basic question whether the global rise in [CO_2] will contribute to yield (Manderscheid and Weigel, 1995) still has to be answered. With the above considerations in mind, I decided to undertake a detailed analysis of the changes and patterns of yield components of *P. vulgaris* exposed to elevated CO_2.

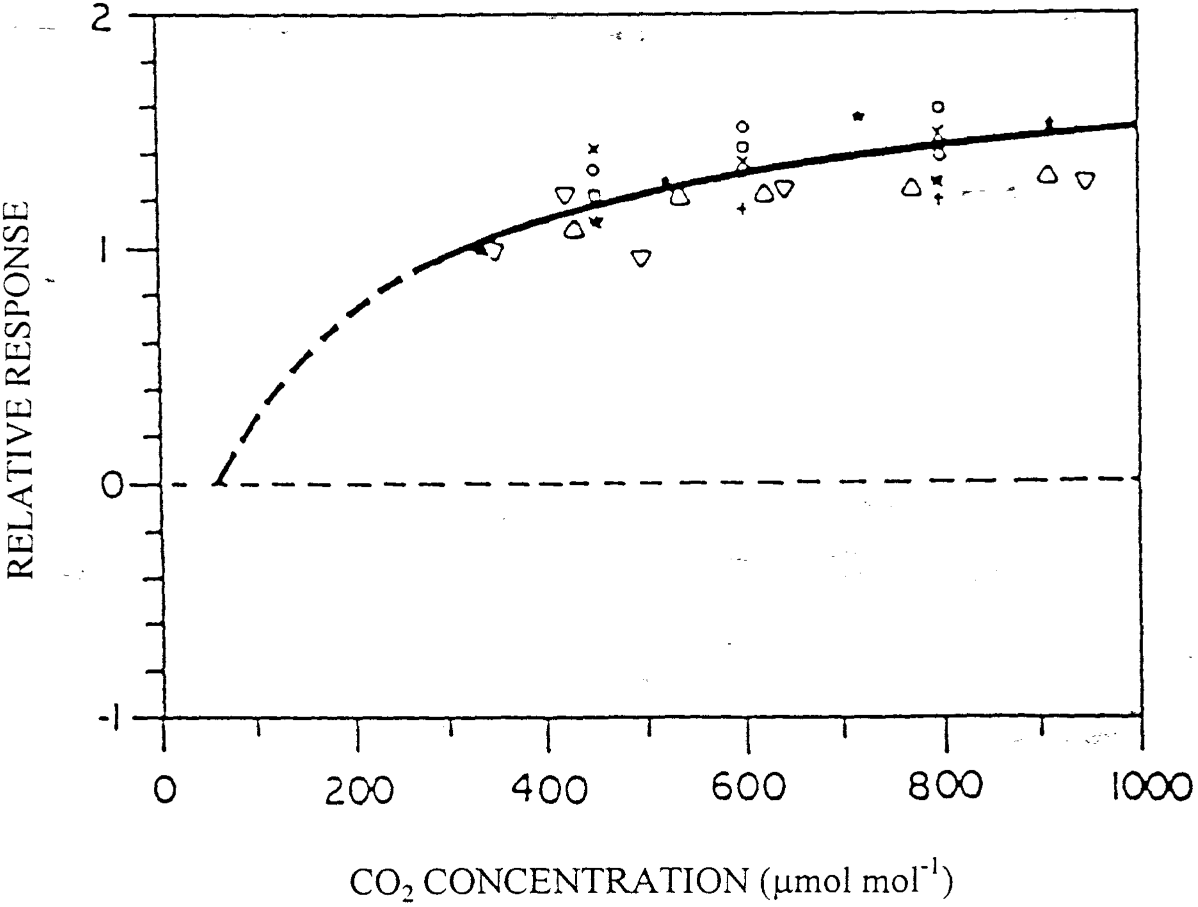

Fig. 5.1 Shows a rectangular hyperbola response of normalized soybean seed yield data plotted against carbon dioxide concentration for Ransom, Bragg, and Forrest cultivars. Data obtained from outdoor controlled environment chamber experiments at three locations: Gainsville Florida, 1981 [squares]; 1982 [crosses], Mississippi State, Mississippi, 1981 [diamond]; 1982 [pluses]; Clemson, South Carolina, 1982[stars]; and from open top chamber experiments at Raleigh, North Carolina, 1980 [stars]; 1981 [up open triangles]; 1982 [down open triangles]. All data were normalized to 340ppm =1.0 Source: Allen *at al.*(1987).

5.2 Results

5.2.1 Flower and pod initiation and abortion

Elevated [CO_2] induced early flowering in bean plants by 4 days (Fig. 5.2). A number of studies on the effects of elevated [CO_2] have reported early floral induction (Mortensen and Ulsaker, 1985). Early flowering was accompanied by massive and significant production of flowers and pods in bean plants grown under elevated CO_2. However, abscission of both flowers and small pods also set in quickly in high CO_2-grown plants where percentages of 75 and 55%, respectively were observed, while abscission claimed 55 and 35% of flowers and pods in plants grown under ambient CO_2, respectively (Table 5.1). Several authors have reported similar findings with respect to fruit abortion in soybean (Hansen and Shibles, 1978; Seddigh and Jolliff, 1984).

Table 5.1 Shows cumulative total numbers of flowers and pods initiated, aborted/abscised in bean plants grown at 360 or 700 µmol mol^{-1} CO_2. (n=12; **P<0.01; *P<.0.05; ^{ns} not significant P>0.05)

Variable	CO_2 Treatment	Initiated	Aborted	% Difference
Number of flowers	360	92	50.1	53
	700	155^{**}	112.5^{**}	75
Number of pods	360	41	15	35
	700	78**	44^{***}	55

5.2.2 Yield and yield components

Tillers or lateral branches increased significantly in bean plants grown under elevated CO_2 (Fig. 5.3; $P< 0.001$). A similar increase in the number of tillers induced by CO_2 enrichment has been reported elsewhere in bean and other crop plants (Acock and Allen, 1985; Conroy *et al.*, 1994; Manderscheid and Weigel, 1995). As a result of the high number of tillers, enriched CO_2-grown plants produced a significantly higher number of pods than ambient CO_2-grown plants (Table 5.2).

Table 5.2 Seed yield, components of yield such as total number of pods, seed yield (total number of seeds) per plant, dry weights per 10 seeds (g), total dry weight of seed biomass (g) and harvest index (defined as the ratio of seed yield to total above-ground biomass, HI) in bean plants grown at 360 or 700 μmol mol^{-1}.CO_2 (${}^{-}P<0.10$, ${}^{*}P<0.05$, ${}^{**}P<0.01$ indicate significant differences).

CO_2 Treatment	Total pods	Seed yield per plant	Mass per 10 seeds (g)	Mass total seeds (g)	HI
360	21	120	5.6	63.6	1.67
700	33**	252***	3.8**	95.7**	1.84^{ns}

Elevated [CO_2] also increased the pod length by approximately 4 cm (Fig. 5.4). The number of seeds per pod also increased in these plants to approximately 8 on average compared to 5 observed in ambient CO_2-grown plants (Fig. 5.5). This was in agreement with the results reported by Sionit *et al.* (1987) for soybean. However, the seed dry weight was significantly reduced in CO_2-enriched plants. The total seed yield increased by almost 50% but this was offset by significant reduction in seed dry weight in CO_2-enriched plants (Table 5.2). HI did not differ significantly in both CO_2 treatments (Table 5.2; $P > 0.677$).

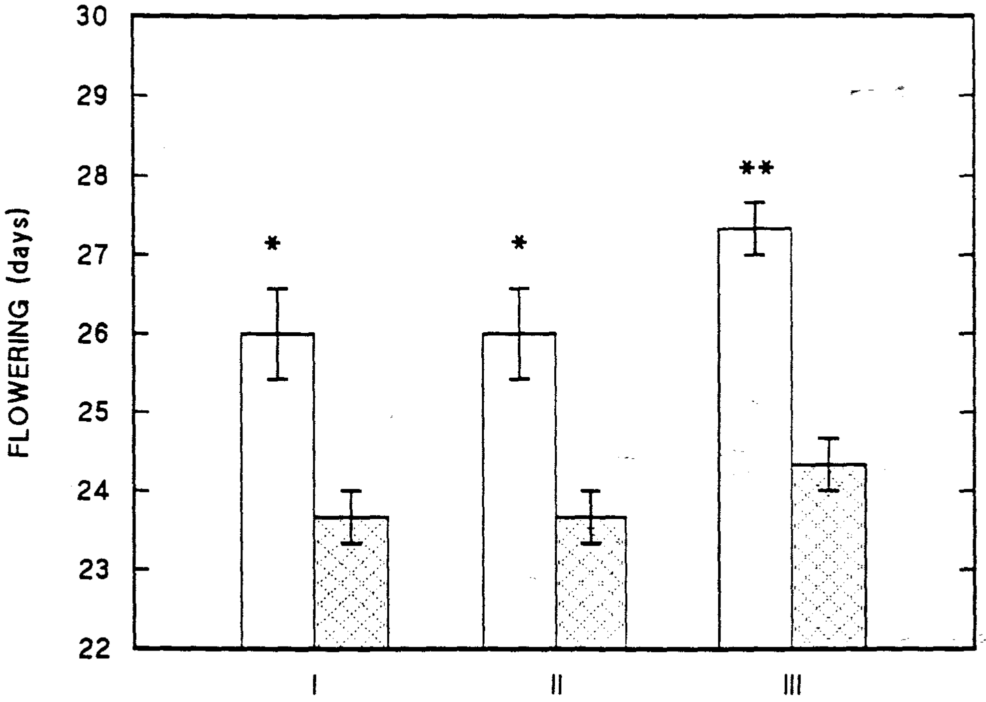

Fig. 5.2. Shows the response of timing of flower initiation in bean plants grown at 360 (open bars) or 700 (hatched bars) μmol mol^{-1} CO_2. (n=12 per each sampling I, II and III; Error bars denote ±SE; $^{-}$P<0.10, *P<0.05, and **P<.0.01 indicate significant differences).

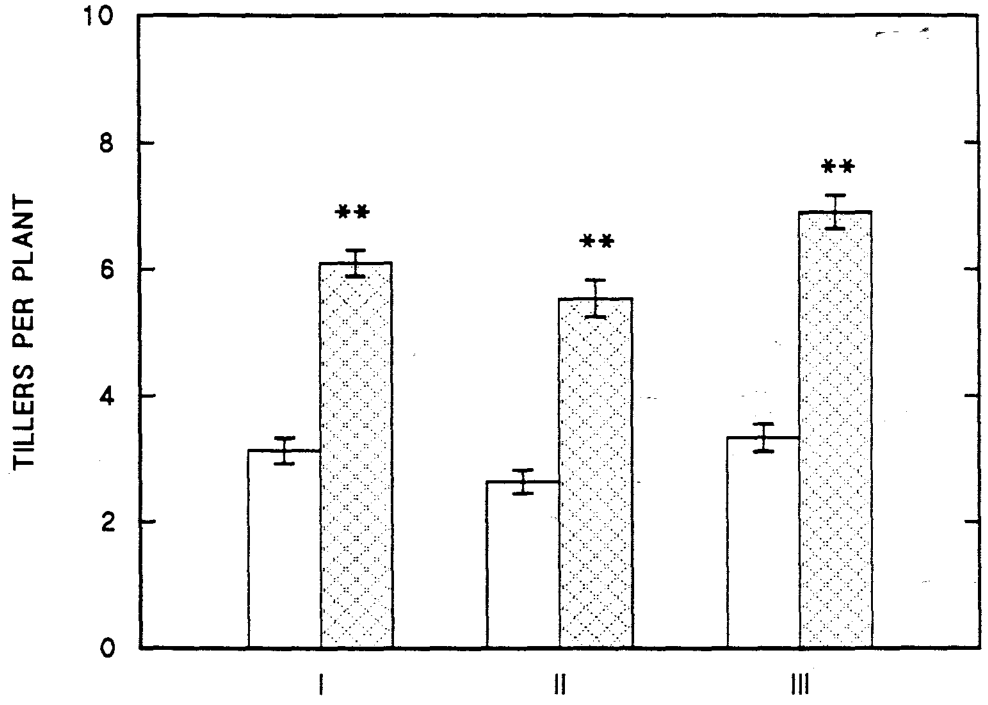

Fig. 5.3. Total number of tillers (lateral branching) per plant in bean plants grown at 360 (open bars) or 700 (hatched bars) μmol mol-1 CO_2. (n=12 per each sampling I, II and III; Error bars denote ±SE; $^{-}P<0.10$, $^{*}P<0.05$, and $^{**}P<.0.01$ indicate significant differences)

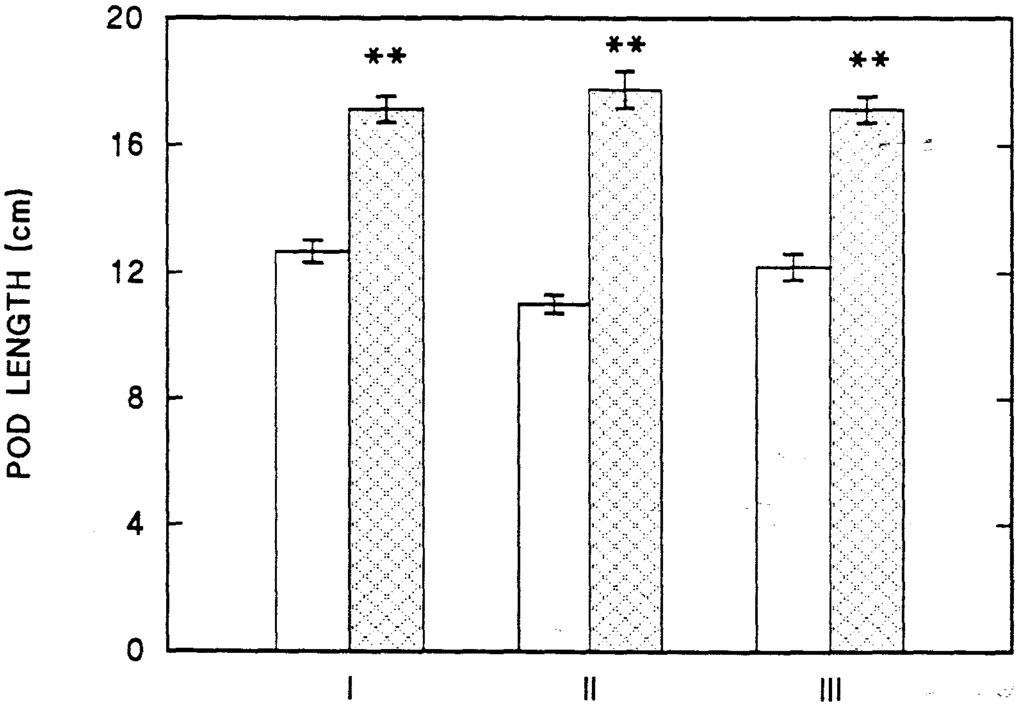

Fig. 5.4. The response of pod elongation in bean plants grown at 360 (open bars) or 700 (hatched bars) μmol mol^{-1} CO$_2$. (n=12 per each sampling I, II and III; Error bars denote ±SE; $^{-}$P<0.10, *P<0.05, and **P<.0.01 indicate significant differences)

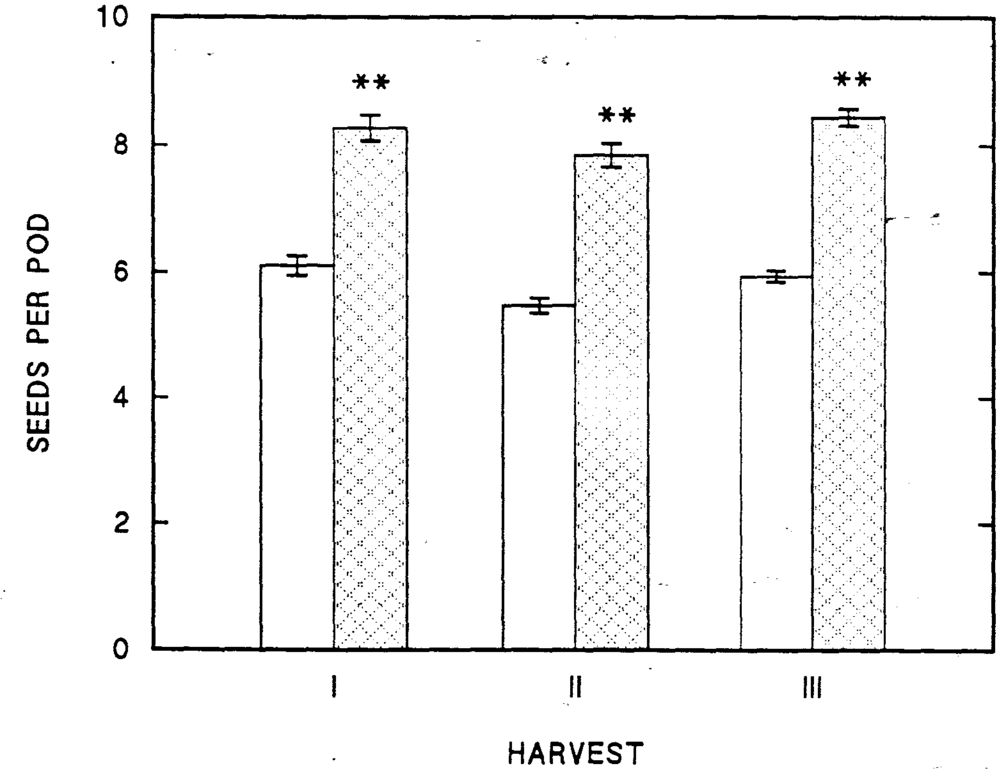

Fig. 5.5. The response of total number of seeds per pod in bean plants grown at 360 (open bars) or 700 (hatched bars) μmol mol^{-1} CO$_2$. (n=12 per each sampling I, II and III; Error bars denote ±SE; $^{-}$P<0.10, *P<0.05, and **P<.0.01 indicate significant differences)

5.3 Discussion

It is obvious that the number of pods that develop in *P. vulgaris* is influenced by the number of flowers that are formed and the percentage of flowers and small pods that are aborted. Factors that affect the ultimate flower number on the plant can be divided into two categories; firstly, factors that have a direct effect including flower induction, differentiation of floral bud development, and flower abortion; and secondly, indirect factors such as branching or tillering which, in turn, influences the number of flowers per plant (Enoch and Zieslin, 1988; Manderscheid and Weigel, 1995). While abscission and/or abortion of flowers and initiated pods is common for *P vulgaris* and other leguminous plants grown under natural growth conditions (Stephenson, 1981; Lee and Bazzaz, 1986; Sage and Webster, 1987), the massive abortion of flowers and initiated pods (75 and 55% on average, respectively Table 5.1) observed in this study in *P. vulgaris* grown under elevated CO_2 must adversely affect the potential for high yields associated with elevated [CO_2] (Kimball, 1983; Allen *et al.*, 1987).

Despite the massive percentage of aborted flowers and small pods in this study, *P. vulgaris* plants exposed to elevated [CO_2] exhibited significantly higher numbers of flowers and pods compared to those grown at ambient CO_2. Most studies of long-term effects of CO_2 exposure have reported significant accumulation of NSC (Chapter 3). A close positive correlation of NSC and flower initiation has been demonstrated. This suggests that NSC, apart from causing major shifts in sink/source ratio, may also be involved in flower initiation and subsequently fruit differentiation, and sometimes abortion (Peña-Valdivia and Ortega-Delgado, 1991; Aloni *et al.*, 1991; Ahmed *et al.*, 1993; Jeuffroy and Chabanet 1994). The subsequent abortion of flowers has been interpreted in several ways by different authors. Some have perceived, and indeed interpreted abortion as an attempt by the plant to dispose of excess NSC accumulated in these structures (Gaines *et al.*, 1974). However, studies on reproductive strategies involving cost and benefit have criticized this approach. For example, while it may be argued that the massive loss of flowers and young fruits is an attempt to dispose of excess NSC, this may not be valid as other nutrients (such N and other minerals) mobilized to flower buds and young pods may be lost in the process (see Bookman, 1983 and references cited therein).

Bookman (1983) demonstrated that in *Asclepias speciosa,* the loss incurred from a mature pod is more costly than the loss of ±128 flowers or 98 young pods, which would equal the cost of one mature pod in terms of N, P, K and Mg nutrients, thus suggesting that the abortion at later stages of fruit development would have a more deleterious impact in terms of nutrient loss than the loss of flowers or small pods. None the less, in the present study, the large percentage of aborted flowers and small pods observed in plants exposed to elevated CO_2 would have a significant and greater effect, than the loss in ambient grown plants which had a lower percentage of abortion.

It has been shown that abortion may be due to competition for maternal resources among reproductive organs (Lee and Bazzaz, 1986). This assumption finds support in the fact that most studies to date on elevated CO_2 and nutrition have reported significant declines in most essential nutrients (Conroy *et al.* 1991) and the similar reductions in nutrients have been observed in *P. vulgaris* (Mjwara *et al.*, 1996; also see Chapter 4). As a result, the plant's capacity to sustain a massive number of flowers up to a fruiting stage is limited. Alternatively, resource competition may be minimal, other factors such as genetic incompatibility between parental genotypes, and hormonal interactions may be more important (Subhadrabandhu *et al.*, 1978; Lee and Bazzaz, 1986; Guinn and Brummett, 1988). Endogenous ABA and ethylene have been implicated in the abortion or abscission of flowers and young fruits (Guinn and Brummett, 1988). Several authors have reported significant accumulation and/or synthesis of essential growth regulators such as endogenous ethylene which is induced by elevated CO_2 concentrations (Woodrow *et al.*, 1988; Woodrow and Grodzinski, 1993). It is not unreasonable to suggest, therefore, that ethylene levels may have increased in the bean plants exposed to elevated CO_2 in this study which was manifested in massive abortion of both flowers and young pods in these plants. Nevertheless, it is not yet clear at this stage whether the changes in flowering are directly affected by resources or altered hormonal signal stimulated by enhanced CO_2 (Ceulemans and Mousseau, 1994). Consequently, additional studies aimed at resolving the interactive effects of elevated CO_2 and changes in endogenous plant growth regulators on phenology are warranted.

Lateral branching or tillering is considered to be a major contributor of additional fruiting sites, and therefore higher yields (Begonia and Aldrich, 1990; Conroy *et al.*, 1994). The significant increase in the number of flowers and pods observed in *P. vulgaris* exposed to elevated CO_2 may, in part, have been caused by the high numbers of tillers observed in plants grown in elevated CO_2 (Fig. 5.3). The increase in pod numbers led to a significant increase in seed numbers per plant in this study (Table 5.2). In addition, pod elongation and number of seeds per pod increased significantly in bean plants exposed to elevated CO_2 (Figs. 5.4 and 5.5, respectively) thus contributing to the high total number of seeds observed per plant. Despite the significant increase in seed yield in plants grown under elevated CO_2, HI did not differ significantly between CO_2 treatments (Table 5.2). HI is defined as the ratio of seed yield to the total above-ground biomass (Donald and Hamblin, 1976). In other words, the relative response of biomass to CO_2 is reflected in the response in yield. However, the benefit of increased seed number may be offset by a reduction in seed weight (Rawson, 1995). This was indeed the case in the experiment as the mass of seeds was reduced in plants grown under elevated CO_2 (Table 5.2). Variable results have been reported on yields under elevated CO_2. Rogers *et al.* (1986) and Baker *et al.* (1992) observed a significant decline in HI in bean and rice, respectively grown under elevated CO_2. Spaeth and Sinclair (1985) and recently Rawson (1995) observed insignificant change in HI in bean plants exposed to elevated CO_2, thus suggesting that HI is largely species-specific (Miglieta *et al.*, 1993).

Seed number and weight are often inversely correlated (Frederick and Hesketh, 1994). The results obtained in this study for plants grown in elevated CO_2 are in agreement. In these plants, seed weight was slightly reduced, yet the number of seeds was significantly increased (Table 5.2). The reverse was true for plants grown under ambient CO_2. Similar findings have been reported by several authors (Wulf and Miller-Alexander, 1985; Sionit *et al.*, 1987). Seed weight or size is often correlated with seed vigor, thus has important implications with respect to the subsequent seedling performance. Larger seeds maintain viability over a long period in dormancy (Woodstock, 1973). They tend to germinate earlier, producing seedlings that are more likely to survive and grow into dominant species often out competing those from smaller fruits sharing the same site (Counts and Lee, 1991; Baker *et al.*, 1994). It appears therefore, that reduced seed weights or sizes of plants grown under elevated CO_2, may adversely affect the competitive advantage in the resulting seedlings.

5.4 Conclusion

The results and the forgoing discussion presented here demonstrate that changes in crop yields may be expected under future elevated CO_2. However, there are many areas raised in the study that need further urgent attention. For example, the question of massive abortion of flowers and small pods in plants grown under elevated CO_2 was the most responsive and direct obvious phenomenon of CO_2-enrichment. This factor will certainly adversely affect the prospect of high yields under future elevated CO_2, as the number of pods that develop on bean plants is influenced by the number of flowers that are formed and the percentage of flowers and small pods that are aborted. There are numerous factors that attempt to explain this massive abortion of flowers and young pods such as competition for resources, NSC accumulation, genetic incompatibility, and hormonal regulation effect. Some of these factors have received some attention but very little has been done on the possible effect of altered hormonal signal stimulated by enhanced [CO_2]. Clearly if the percentage of flower and fruit abortion were to be reduced, the prospects of high yields under elevated CO_2 would be a reality.

CHAPTER 6: INTERACTION OF STOMATAL CONDUCTANCE, WATER USE EFFICIENCY, AND PHOTOSYNTHETIC ACCLIMATION

6.1 Introduction

Understanding stomatal response to elevated CO_2 levels is of major importance because this one ecophysiological process underpins the productivity of all the Earth's terrestrial vegetation by exerting control over the rate of carbon uptake and water use efficiency (Beerling and Chaloner, 1993; Romero-Aranda *et al.*, 1994). Water use efficiency (WUE) has been observed to increase in plants exposed to elevated CO_2 due, in part, to decreased stomatal conductance (g_s) which may in turn be attributed to partial closure of stomata, and/or decline in stomatal density (Jarvis, 1989; Knapp *et al.*, 1994; Reddy *et al.* , 1995; Samarakoon *et al.*, 1995).

However, despite the great attention that has been given to the water use of plants exposed to elevated CO_2, it remains a debatable issue (see reviews by Eamus (1991) and Hinckley and Braatne (1994). Some literature has, for example, highlighted the partial closure of stomata as a cause in the reduction of internal CO_2 concentration (C_i) within the mesophyll cells which inevitably lowers NAR (Van Kraalingen, 1990; Lawlor, 1993). This negative adjustment or 'acclimation' of CO_2 assimilation to enhanced CO_2 thus counteracts the benefits associated with enhanced CO_2 concentration (Jarvis, 1989; Bethke and Drew, 1992; Gunderson and Wullschleger, 1994).

In contrast, similar studies have reported an increase in the C_i despite decreased g_s (Lawlor, 1993), thereby allowing plants grown under elevated CO_2 to operate at high WUE without a corresponding loss of NAR. A further possibility is the simultaneous increase in both g_s and NAR - a situation which will result in high water loss and yet high NAR, particularly when water is not limiting (Ball and Munns, 1992). However, as was pointed out by Jarvis (1989) and recently Gorissen *et al.* (1995) there is very little information to suggest whether these stomatal changes in response to elevated CO_2 are merely short-term transients, or long-term acclimation features. Furthermore, the relationship and the extent to which stomatal factors such as g_s affect photosynthetic acclimation of species exposed to enhanced CO_2 concentrations becomes even more obscure (Chaves and Pereira, 1992; Radoglou *et al.*, 1992; Xu *et al.*, 1994b).

Accordingly, the present study seeks to further our understanding on the extent to which the stomatal components limit NAR and also to investigate the possible interaction between WUE, down-regulation and acclimation of NAR observed in *P.*

vulgaris after exposure to long-term elevated CO_2 concentration (Mjwara *et al.*, 1996; Chapter 3).

During photosynthetic gas exchange, the naturally occurring stable isotopic ratio ($^{13}C/^{12}C$) of carbon dioxide assimilated differs from that of the source air available to plants (Farquhar *et al.*, 1989). There are two primary processes that cause carbon isotope ratios to change during photosynthesis, diffussional fractionation and enzymatic fractionation. Carbon dioxide molecules containing ^{12}C are lighter, and therefore diffuse into the leaf at a faster rate than CO_2 molecules containing the heavier isotope ^{13}C (Lawlor, 1993; Flanagan and Johnsen, 1995). In addition, Rubisco uses $^{12}CO_2$ faster than $^{13}CO_2$ and so discriminates against the heavier isotope (a phenomenon widely referred to as carbon isotope discrimination, (Δ), Farquhar *et al.*, 1982; *Guy et al.*, 1993; Flanagan and Johnsen, 1995).

Carbon isotope discrimination is directly related to the ratio of internal CO_2 (C_i) and ambient CO_2 (C_a) concentrations which in turn is dependent on the leaf conductance (g_s) to water vapour in C_3 species (Farquhar *et al.*, 1982; Farquhar *et al.*, 1989). Therefore, Δ integrates the differential effects of leaf conductance and carboxylation capacity on two stable isotopes of carbon into a measure reflecting C_i/C_a ratio within the leaf tissue. WUE is directly influenced by A/E and E depends on the diffusive conductance to water vapour and vapour pressure difference (VPD) between leaf and air. Consequently, Δ is closely associated with photosynthesis and WUE. Furthermore, current theories predict a negative correlation between WUE and Δ (Farquhar *et al.*, 1982; Ehleringer *et al.*, 1990; Ishmail and Hall, 1992; Condon *et al.*, 1993).

Unlike short-term estimates of WUE from gas exchange measurements, Δ provides time-integrated estimates of the plant intrinsic water use efficiency because of its cumulative nature - i.e. measured from all C accumulated by leaf during its growth and development (Tauer *et al.*, 1992; Hansen and Steig, 1993; Huc *et al.*, 1994). Leaf Δ thus provides an alternative indirect measure of WUE which is claimed to be a largely genetically controlled and heritable trait (Condon *et al.*, 1990; Ishmail and Hall, 1992; Johnson and Asay, 1993). Thus, Δ data obtained from such studies may not only help to elucidate the relationship that exists between stomatal components, WUE, and photosynthetic acclimation to CO_2, but would be an added advantage in clarifying, right at the early stages whether the changes (that control stomatal aperture to diffusion of CO_2 and H_2O vapour) are in fact fundamental features which may be linked to and/or mark a beginning of the regulation of genetic expression to enable plant species to adapt to future elevated [CO_2].

It has long been shown by several workers that leaf gas exchange analysis non-destructively describes primary and secondary responses of photosynthesis to CO_2 enrichment, and can be used to assess biochemical and stomatal mechanisms controlling short-term and long-term responses of carbon fixation to elevated CO_2 (Farquhar and Sharkey, 1982; Sage, 1994). Of importance in this regard is the study of the response of net assimilation (A) to intercellular CO_2 (A/C_i response) which facilitates the study of acclimation because it compares the responsiveness in both CO_2 treatments at the same [CO_2]. The A/C_i response can be interpreted in terms of the biochemical and stomatal processes controlling the long-term responses of photosynthetic capacity to CO_2 enrichment (Long *et al.*, 1993; Sage, 1994).

To understand the relationship that exists between stomatal components and photosynthetic acclimation in *P. vulgaris* exposed to elevated CO_2, I examined the following parameters: NAR, g_s, E, stomatal density, WUE and Δ. Furthermore, theoretical interpretation of A/C_i (generally regarded as one of the vital indices of photosynthetic acclimation, Long *et al.*, 1993; Sage, 1994) was employed to assess acclimation to elevated CO_2.

6.2 Results

6.2.1 Net assimilation rate

NAR in CO_2-enriched bean plants was significantly higher than in plants grown in ambient CO_2 at early stages of growth but subsequently declined to levels comparable to those attained by bean plants grown at ambient CO_2 concentration (Table 6.1). The average difference in NAR between 25 and 35 DAG was less than 2 μmol m^{-2} s^{-1} at this stage between CO_2 treatments, thus showing down regulation of photosynthesis in CO_2-grown plants.

Table 6.1. Comparison of NAR obtained from bean plants measured in their respective growth conditions, ambient (360 μmol mol^{-1}) and elevated (700 μmol mol^{-1} CO_2 concentrations; A_{max} (CO_2-saturated photosynthetic rate values from A/C_i response); and ACE (calculated from the initial A/C_i response curves) at different growth stages.

DAG	[CO_2] μmol m^{-2} s^{-1}	NAR μmol m^{-2} s^{-1}	A_{max} μmol m^{-2} s^{-1}	*ACE* μmol m^{-2} s^{-1} C_i^{-1}
15	360	16.38	25.2	0.046
	700	23.76**	27.3**	0.042*
25	360	19.58	27.6	0.055
	700	21.02*	24.3**	0.036**
35	360	15.43	22.0	0.045
	700	16.04^{ns}	17.1**	0.028**

6.2.2 Stomatal conductance, transpiration rates, and stomatal density

Bean plants grown under elevated CO_2 exhibited a dramatic linear decrease in g_s (Fig. 6.1). A large average difference of approximately 40% was maintained throughout the growth period between the two treatments ($P<0.01$). Similarly, E declined significantly throughout the growth period in CO_2-enriched bean plants (Fig. 6.2,

$P<0.01$). In contrast, plants grown under ambient CO_2 exhibited an initial increase in *E* but declined from 20 DAG though to maturity. Stomatal density (WSD) values did not differ significantly between the two CO_2 treatments (Fig. 6.3, $P>0.05$).

6.2.3 Water use efficiency and leaf isotope discrimination

Water use efficiency increased in both CO_2 treatments. However, CO_2-enriched plants exhibited WUE values that were consistently higher with a maximum of 5.7 compared to 3.5 μmol mol^{-1} observed in ambient CO_2-grown bean plants (Fig. 6.4, $P<0.001$). In addition, WUE was negatively correlated to g_s (Fig. 6.5 with combined $r^2 = 0.9033$, $P=0.024$).

Leaf Δ values declined in both CO_2 treatments with time (Fig. 6.6). However, the values observed in elevated CO_2-grown plants were significantly lower after 15 DAG compared to those of ambient CO_2 plants (Fig. 6.6, $P<0.001$). A strong inverse correlation was found between WUE and Δ, where the slope of the regression was significantly steeper ($P<0.0001$) for plants grown in elevated CO_2 (where $r^2 = 0.7957$) than that of plants growing in ambient CO_2 ($r^2 = 0.7266$, Fig. 6.7)

6.2.4 A/C_i response

The response of A as a function of C_i at three different growth stages 15, 25 and 35 i.e. **A**, **B**, and **C**, respectively is presented in Figs. 6.8**A-C** . The slopes of the plotted A/C_i, show a rapid initial increase in NAR to C_i in all growth stages (Figs. 6.8**A-C**). In all growth stages photosynthetic saturation occurred at approximately 550 μmol mol^{-1} internal CO_2. A significant decrease was observed in the initial slope of A/C_i in bean plants grown at elevated CO_2 with the exception at 15 DAG where the response was significantly higher compared to the response observed in ambient CO_2-grown plants. The *ACE* (calculated from the initial slope of CO_2-response curves) followed the same trend, where NAR and *ACE* declined over the study period for high CO_2-grown plants (Table 6.1; $P<0.0001$). The CO_2 saturated photosynthetic rates (A_{max}) were significantly lower in CO_2-enriched bean plants with the exception at 15 DAG where A_{max} was significantly higher than in ambient CO_2-grown plants (Table 6.1).

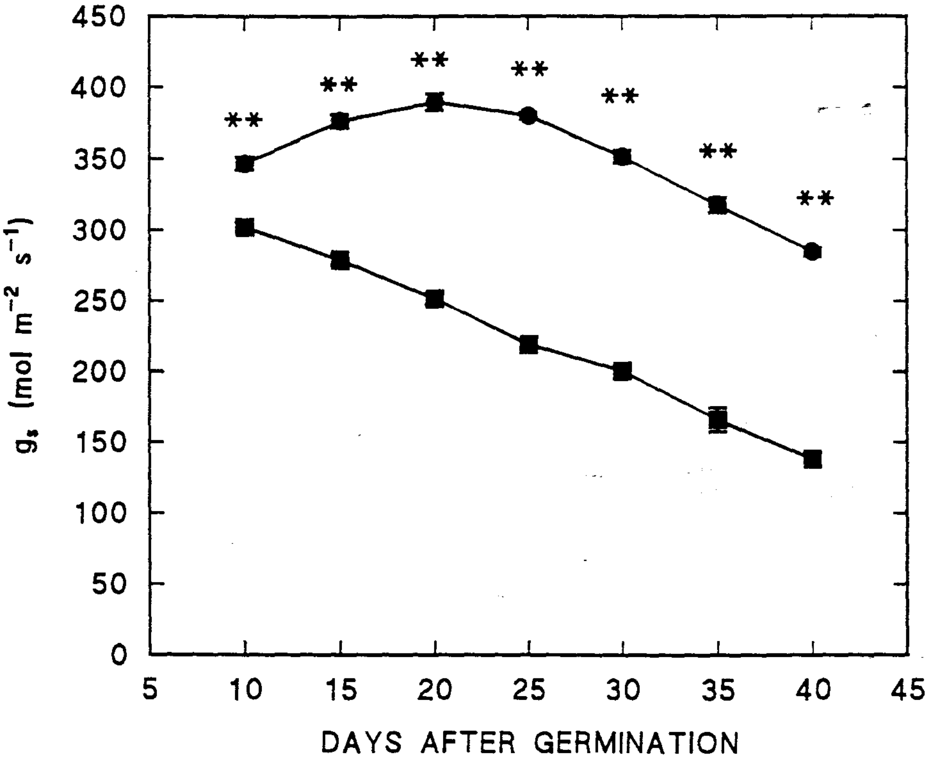

Fig. 6.1 Time course of g_s response measured at incident light of 550 μmol m^{-2} s^{-1} in bean leaves grown at 360 (●) and 700 (■) μmol mol^{-1} CO$_2$ (n=9 per each data point; Error bars denote ± SE; **P<0.01 as tested by Scheffe's multiple range test).

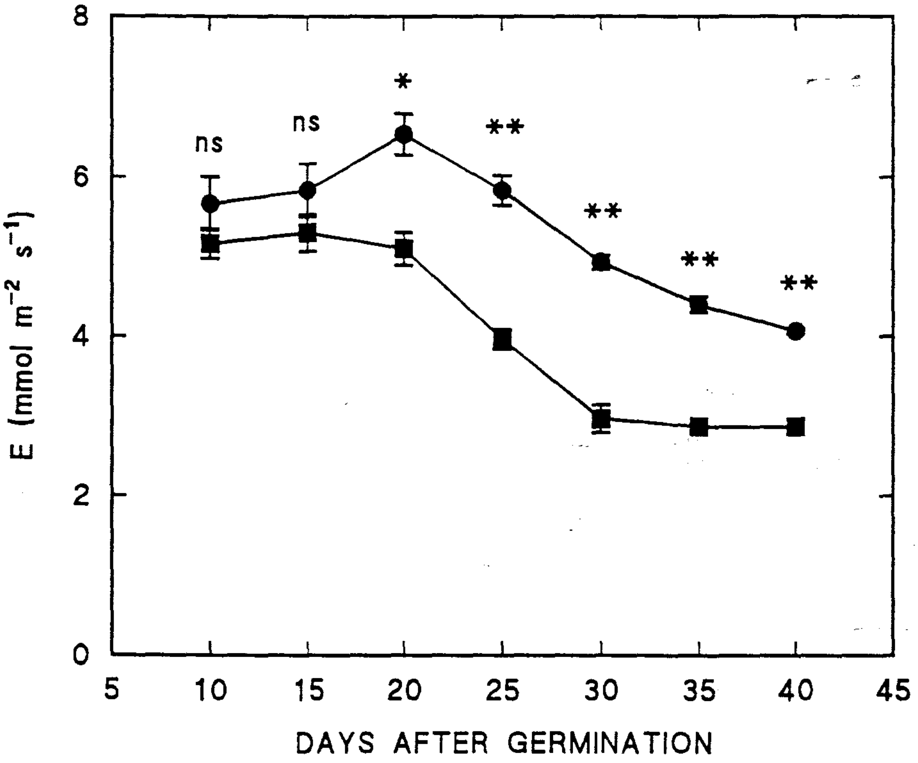

Fig. 6.2 Time course of the effect of CO_2 concentration on *E* of bean plants grown at 360 (●) and 700 (■) μmol mol^{-1} CO_2. (n=9 per each data point; Error bars denote ± SE; **P<0.01 as tested by Scheffe's multiple range test)

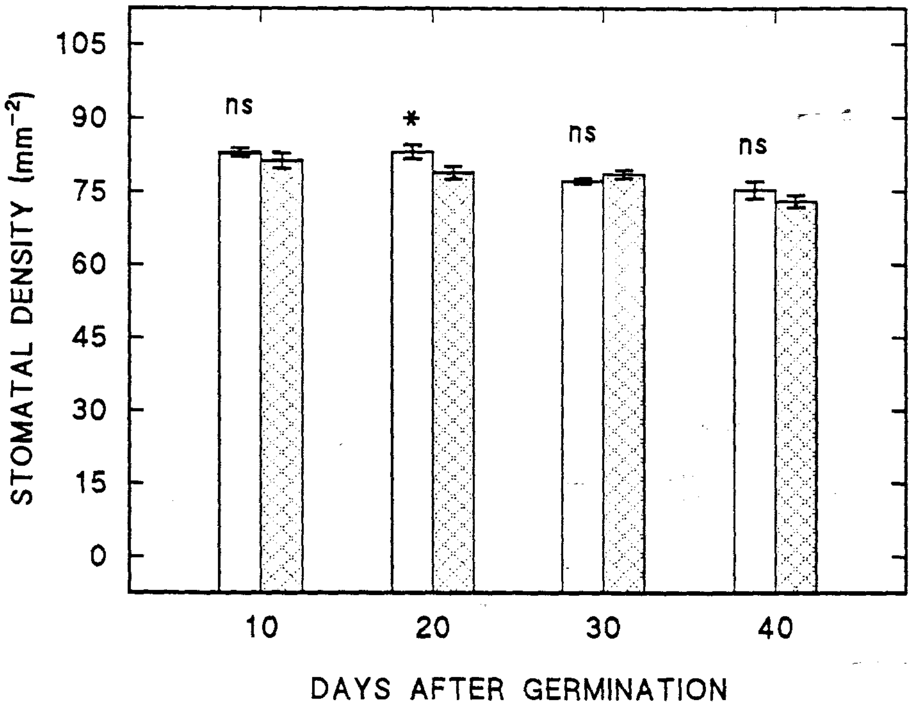

Fig. 6.3 Time course of weighted stomatal density (WSD) response measured in bean leaves grown at 360 (open bars) and 700 (hatched bars) µmol mol^{-1} CO_2 (n=5 per each data point; Error bars denote ±SE; * and ^{ns} indicate significant differences at $P<0.05$ and not significant at $P>0.05$, respectively as tested by Scheffe's multiple range test).

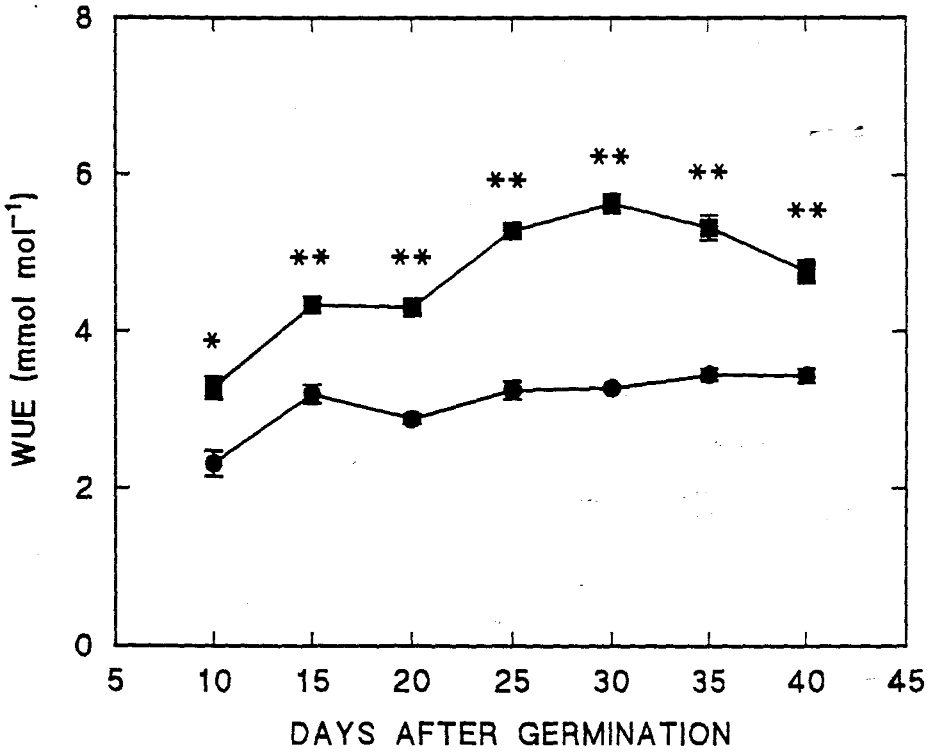

Fig. 6.4 Time course of the effect of CO_2 concentration on WUE of bean plants grown at 360 (●) and 700 (■) μmol mol^{-1} CO_2. (*n*=9 per each data point; Error bars denote ±SE; * and ** indicate significant differences at $P<0.05$ and $P<0.01$, respectively as tested by Scheffe's multiple range test).

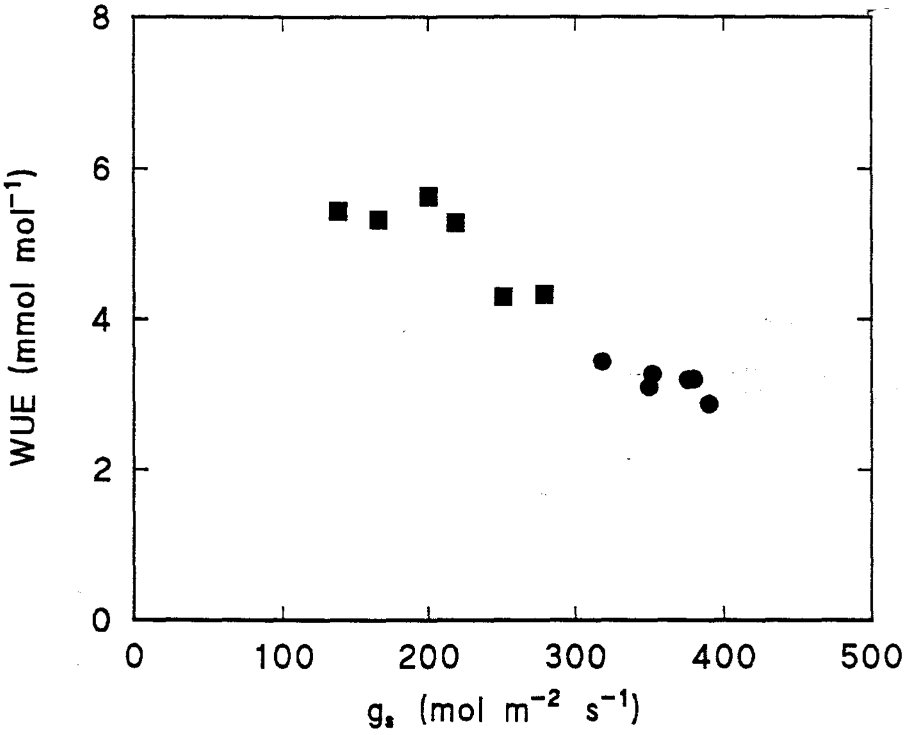

Fig. 6.5 Relationship between WUE and g_s in bean leaves grown at 360 (●) and 700 (■) μmol mol^{-1} CO$_2$. Slopes were significantly different at $P<0.05$ but not at $P<0.01$ (combined $r^2 = 0.9033$).

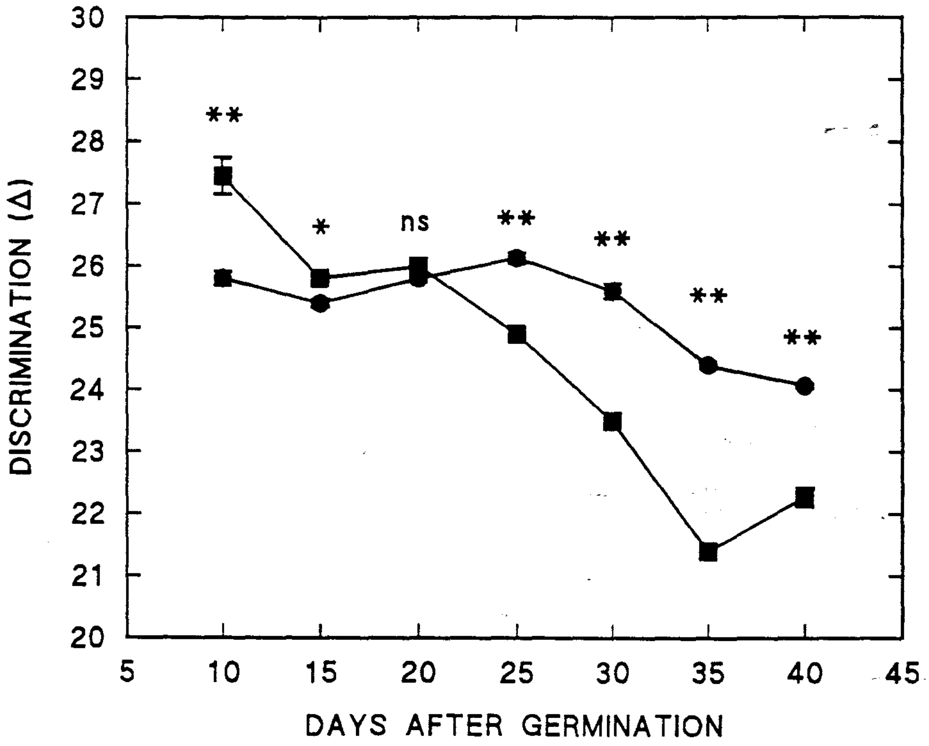

Fig. 6.6 Time course of the effect of CO_2 concentration on Δ in bean leaves grown at 360 (●) and 700 (■) μmol mol^{-1} CO_2. (n=3 per each data point; Error bars denote ±SE; * and ** indicate significant differences at $P<0.05$ and $P<0.01$, respectively as tested by Scheffe's multiple range test).

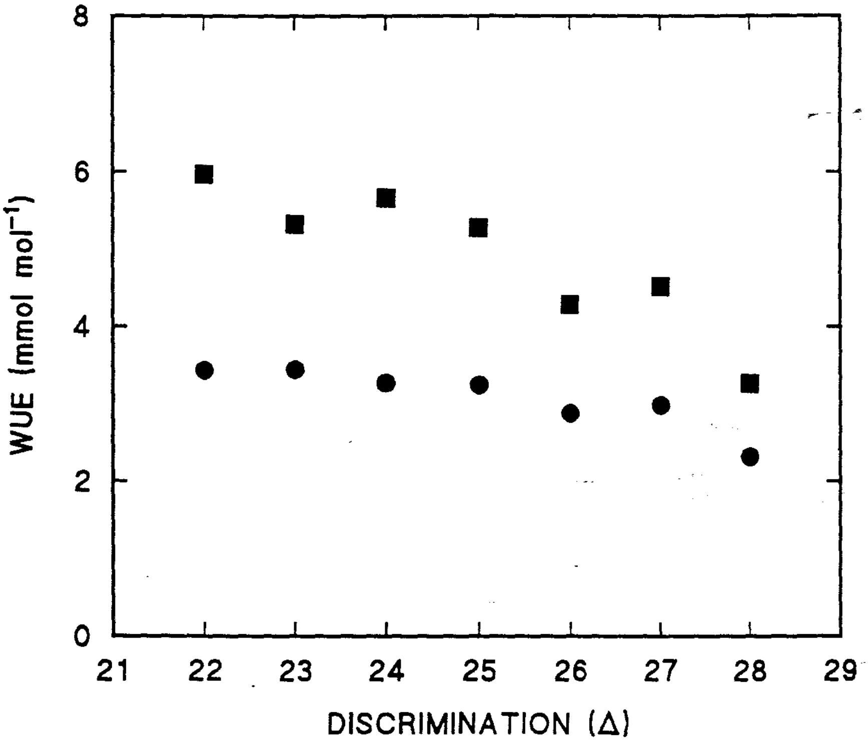

Fig. 6.7 The relationship between Δ and WUE in bean leaves grown at 360 (●) and 700 (■) μmol mol^{-1} CO_2. A negative correlation was found between Δ and WUE, where the slope of the regression was significantly steeper ($P<0.0001$) for plants grown in elevated CO_2 (r^2 =0.7957) than for plants grown under ambient CO_2 (r^2 = 0.7266).

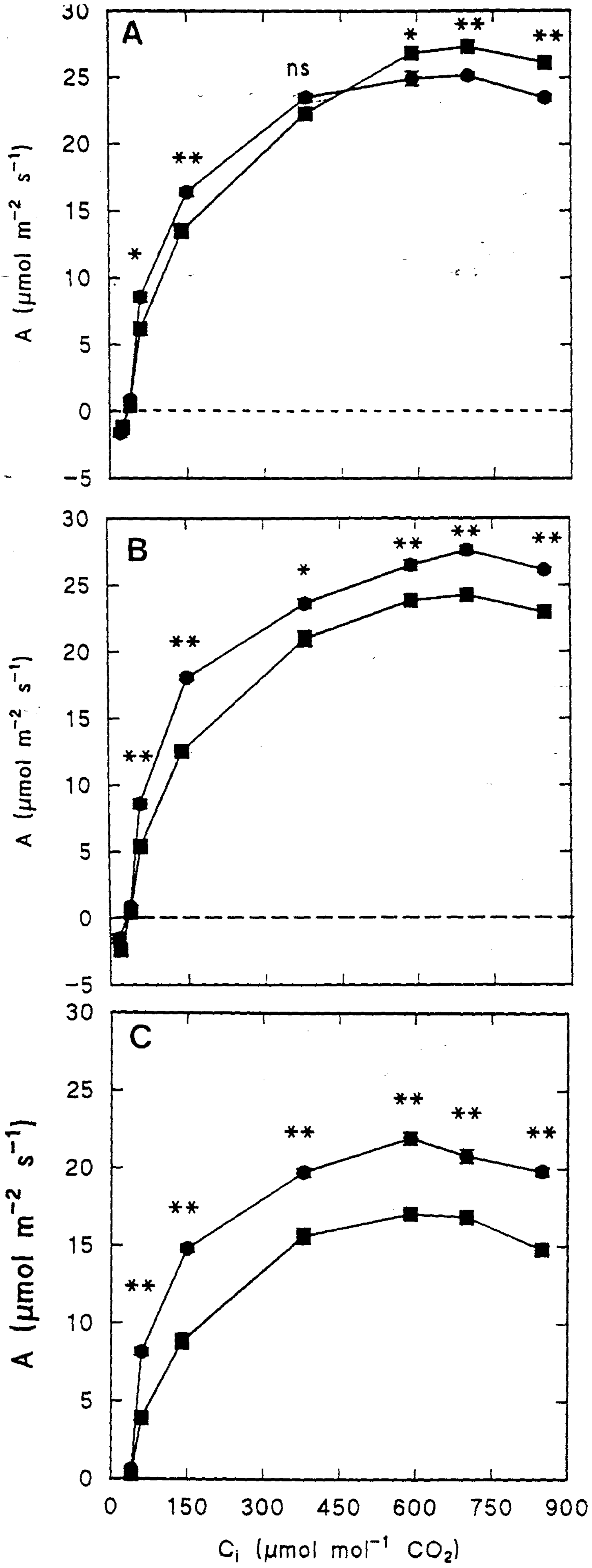

Fig. 6.8 Net assimilation rate as a function of the intercellular CO$_2$ (C_i) in bean leaves grown at 360 (●) and 700 (■) μmol mol^{-1} CO$_2$. Measurements were obtained at three different growth stages 15, 25 and 35 DAG i.e Figs **A**, **B** & **C** respectively. (n=9; Error bars denote ±SE; * and ** indicate significant differences at P< 0.05 and P<0.01 respectively, as tested by Scheffe's multiple range test).

6.3 Discussion

This study seeks to determine the interaction and the extent to which the stomatal components play a role in acclimation or down-regulation of NAR in *P. vulgaris*. Net assimilation rate significantly increased during the initial growth stages in CO_2-enriched plants (see Chapter 3). However, in later growth stages, a significant decline occurred to levels comparable to those observed in ambient CO_2-grown plants (Table 6.1). A similar response was demonstrated previously in a number of plant species (Ehret and Jolliffe, 1985; El Kohen *et al.*, 1993). Various theories and often conflicting arguments have been put forward in an attempt to explain down-regulation or negative adjustment of NAR in plant species exposed to long-term elevated $[CO_2]$. Some researchers have attributed this decline partly to reduction in g_s, which in turn lowers the internal CO_2 supply (Grodzinski, 1992; Lawlor, 1993; Hinckley and Braatne, 1994). This may be the case in this study where g_s declined in bean plants exposed to elevated CO_2 (Fig. 6.1). Such a response has been observed in a wide spectrum of herbaceous plant species including *P. vulgaris* (Nederhoff *et al.*, 1992; Xu *et al.*, 1994b) and woody species (Jarvis, 1989; Berryman *et al.*, 1994; Guehl *et al.*, 1994).

Generally, in plants well adapted to elevated atmospheric $[CO_2]$, g_s declines contemporaneously with transpiration rates (Jarvis, 1989; Bazzaz, 1990; Grodzinski, 1992; Nederhoff, *et al.*, 1992). Indeed, E from high CO_2-grown plants declined proportionately with reduction in g_s (Fig. 6.2), thus the results concur with those reported by these authors. The decline in g_s is often attributed, in part, to partial closure of stomata (Grodzinski, 1992; Lawlor, 1993; Hinckley and Braatne, 1994) or sometimes ascribed to reduction in stomatal density in plants grown in elevated CO_2 (see review by Ceulemans and Mousseau, 1994, and references cited therein). Similarly, there is no consensus on the effect of CO_2 on stomatal density (Beerling and Chaloner, 1993; Knapp *et al.*,1994; Clifford *et al.*, 1995). The findings range from decline (Woodward and Bazzaz, 1988), to no significant differences in stomatal density (Ryle and Stanley, 1992; Guehl *et al.*, 1994), and even significant increases have been recorded (Apel, 1989; Berryman *et al.*, 1994) across a wide range of species. However for *P. vulgaris,* the most general and common tendency reported is that of no significant change under elevated CO_2 (Radoglou *et al.*, 1992; Xu *et al.*, 1994b) with which our results agree (Fig. 6.3). This result contrasted with the work reported by O'Leary and Knecht (1981) in which stomatal density was argued to increase with [CO_2] using a similar bean species.

Peñuelas and Matamala (1990) cautioned that stomatal density is largely affected by the epidermal cells which are often enlarged under elevated CO_2 and such leaf expansion translates into increased leaf areas, with a corresponding decline in stomatal density. Thus the apparent changes in stomatal density may disappear if epidermal cell expansions are considered (Peñuelas and Matamala, 1990). I therefore suggest that the slight (insignificant) decrease in stomatal density observed in CO_2-enriched bean plants can be attributed to expansion of epidermal cells which resulted in substantial increases in leaf areas, which were observed in *P. vulgaris* and reported (Mjwara *et al.*, 1996; also see Chapter 3).

In a similar study, Xu *et al.* (1994b) observed that stomatal density and C_i values in both enriched and ambient CO_2-grown plants (determined under ambient atmospheric CO_2) did not differ significantly. Their findings are in agreement with the results obtained (data not shown); thus providing further evidence that down-regulation or acclimation of NAR may have been caused, to a large extent, by declining g_s and not by stomatal density. However, the observed decline in stomatal density in various species over the last decade may have been caused by other factors such as temperature, which has also been steadily rising with increasing CO_2 concentration (Xu *et al.*, 1994b). Regardless of the causes, the net result of partial stomatal closure was a reduction in E and the resultant enhancement in WUE. This is consistent with a number of reports on a wide range of species exposed to elevated [CO_2] (Cure and Acock, 1986; Bazzaz, 1990; Grodzinski, 1992; Lawlor, 1993). However, other workers cautioned that the water saving benefit associated with partial stomatal closure in plants grown under elevated CO_2 can be counteracted by increased whole plant transpiration since plants tend to have greater leaf area under elevated CO_2 (Morison and Gifford, 1984; Javis, 1989; Wolfe and Erickson, 1993).

As predicted in theory (reviewed by Farquhar *et al.*, 1989), leaf Δ data (Fig. 6.6), especially those of CO_2-enriched plants, showed a negative correlation to WUE (Fig. 6.7). Ehleringer *et al.* (1990) reported a similar correlation in *P. vulgaris*. It has been previously shown that leaf Δ is related to C_i, which in turn, is controlled by g_s. In addition, as E is a function of g_s, a decline in g_s must lead to reduction in E which in turn will improve WUE (Farquhar *et al.*, 1989). However, a continued decline in g_s could in turn, lead to the increase in the relative abundance of $^{13}CO_2$ in the leaf intercellular spaces. Increasing $^{13}CO_2$ may therefore limit the ability of Rubisco to discriminate against $^{13}CO_2$ (Johnson and Asay, 1993). Consequently, leaf Δ decreases as WUE increases. The data which is presented is in line with this observation, as g_s was consistently reduced in high CO_2-grown plants which led to an increase in WUE and reduction in Δ values. In contrast, high Δ values were observed following a significant increase in g_s which led to reduction in WUE in ambient CO_2-grown plants (Fig. 6.6). Because of the large differences in WUE and overall Δ, enhanced CO_2-grown plants exhibited a strong negative correlation between these two parameters compared to that observed in plants exposed to ambient CO_2 (Fig. 6.7).

Jarvis (1989) and recently Gorissen *et al.* (1995) raised concern whether the stomatal changes influenced by elevated CO_2 were indeed permanent features or merely ephemeral artefacts. The high WUE observed in CO_2-grown plants, coupled with low Δ (which is a measure of long-term diffusional and biochemical processes of C_i in the life of the plant tissue), suggests that the changes are not merely transient, but are fundamental features which may mark the beginning or involvement of genetic regulation towards adjustment or adaptation to elevated CO_2. In addition to broad-sense heritability, and consistent variation across genotypes, Δ has become a vital selection criterion in breeding for improved WUE (Condon *et al.*, 1990; Ishmail and Hall, 1992; Huc *et al.*, 1994). Despite much work that has been published involving WUE and leaf Δ, specific research incorporating the effects of enhanced CO_2 remains sparse. Because leaf Δ integrates both biochemical (mainly carboxylation) and physical (CO_2 diffusion) aspects of photosynthetic processes including WUE, it becomes one of the measurements that may enhance our understanding of the interactions that exist between plant species and environmental factors and, in particular, plant performance under the predicted elevated CO_2 conditions in the future.

It is generally accepted that the initial slope of the A/C_i response curve is indicative of plant's photosynthetic capacity and is closely correlated to changes in the amount and activity of Rubisco, while the second slower rise phase, indicates limitations imposed by the rate of regeneration of RuBP and P_i (von Caemmerer and Farquhar, 1981; Sharkey, 1985; Long *et al.*, 1993; Sage, 1994). Photosynthetic down-regulation was evident in *P. vulgaris* exposed to elevated CO_2 concentrations (Fig. 6.8**A-C**), which was clearly shown by the initial slope of A to C_i which was significantly reduced (with the exception at 15 DAG) and confirmed by calculated *ACE* values which were also reduced in CO_2-grown plants (Table 6.1).

The A/C_i response at 15 DAG (Fig. 6.8**A**), indicates that at this stage growth under elevated CO_2 had little effect on Rubisco amount or activity and high A_{max} values realised at high C_i might have been caused by an increase in P_i regeneration capacity. However, at later stages of growth (Figs 6.8**B** and 6.8**C**), continued exposure to elevated CO_2 resulted not only in the decline of Rubisco amount or activity but also the regeneration capacity of RuBP and P_i. This was in agreement with the rationale of Sage *et al.* (1989) in explaining the similar response in *P. vulgaris*. In addition, various studies have demonstrated close agreement between the initial A/C_i curve and the extractable activity of Rubisco (Sage, 1989; Long *et al.*, 1993; Tuba, 1994). *P. vulgaris* also exhibited high A_{max} values which were significantly higher in both treatments, irrespective of whether the plants were previously acclimated to high or ambient CO_2 (Table 6.1). Woodrow and Grodzinski (1993), observed a similar enhancement of NAR in tomato plants, and these authors suggested that the stomata and biochemical reactions within the mesophyll retain the ability to respond to short-term transient exposure to elevated CO_2. This suggests that these changes in photosynthetic machinery are necessary to maintain some form of balance which, according to Long *et al.* (1993), is realised when the capacity for Rubisco carboxylation is matched by its regeneration rate such that the two processes are co-limiting.

6.4 Conclusion

It is clear that with an increase in [CO_2], there is a corresponding decline in g_s, which is largely caused by reduction or partial closure of stomatal pores rather than a decline in stomatal density. Typically, reduction in g_s generally results in reduced E and increasing the A/E ratio and leading to enhanced WUE in plants exposed to elevated CO_2. Because of its close association with a wide range of photosynthetic and environmental parameters, and more importantly, its broad-sense heritability, Δ may prove more useful in our overall understanding of the effects of elevated CO_2 on plant species. One may conclude that the changes observed in the stomatal component are not transient but are fundamental features which may be genetically regulated. This suggests that while elevated CO_2 may play a crucial role in ameliorating drought induced stresses in future predicted drier climates (Goldewijk *et al.*, 1994), A/C_i analysis, demonstrates that the concomitant decline or down regulation in NAR with time in CO_2-enriched plants may have been largely caused by mesophyll limitations (such as the decline in both Rubisco capacity and P_i regeneration). However, the limitations resulting from stomatal functioning can not be ignored, suggesting that photosynthetic acclimation or down-regulation may have been caused by a complex interaction of both stomatal and biochemical regulatory mechanisms.

CHAPTER 7: GENERAL DISCUSSION AND CONCLUSIONS

7.1 General Discussion

This study was undertaken largely to evaluate the photosynthetic and growth responses of *P. vulgaris* to CO_2-enrichment and to characterize the nature of any acclimation. To achieve this, various aspects of plant performance were investigated. Oechel and Strain (1985) proposed a simplified model (Fig. 7.1) of photosynthate source and sink interaction affected by atmospheric CO_2 concentration. The individual components of the diagram were covered in the preceding chapters, but this chapter integrates these components in order to highlight and interpret significant findings as they relate to overall plant performance. As has been reviewed by Woodrow (1994), these aspects of the long-term acclimation of photosynthesis to high CO_2 can be understood by examining the regulation of carbon flux in terms of two major limitations - source and sink capacity- and nitrogen supply. This has proved to be an extremely useful approach in understanding much of the species-dependent variation in responses to elevated CO_2.

7.2 Acclimation to CO_2 increase

Photosynthesis supplies carbon to all structures and tissues within plants (Fig. 7.1). Therefore changes in this primary process through the rise in atmospheric CO_2 will affect all of these processes. For instance, as elaborated in Chapter 3, NAR was initially stimulated by elevated CO_2 but this proved to be a transient response as it was soon followed by a decline to levels similar to those observed in ambient CO_2. Several factors account for this decline.

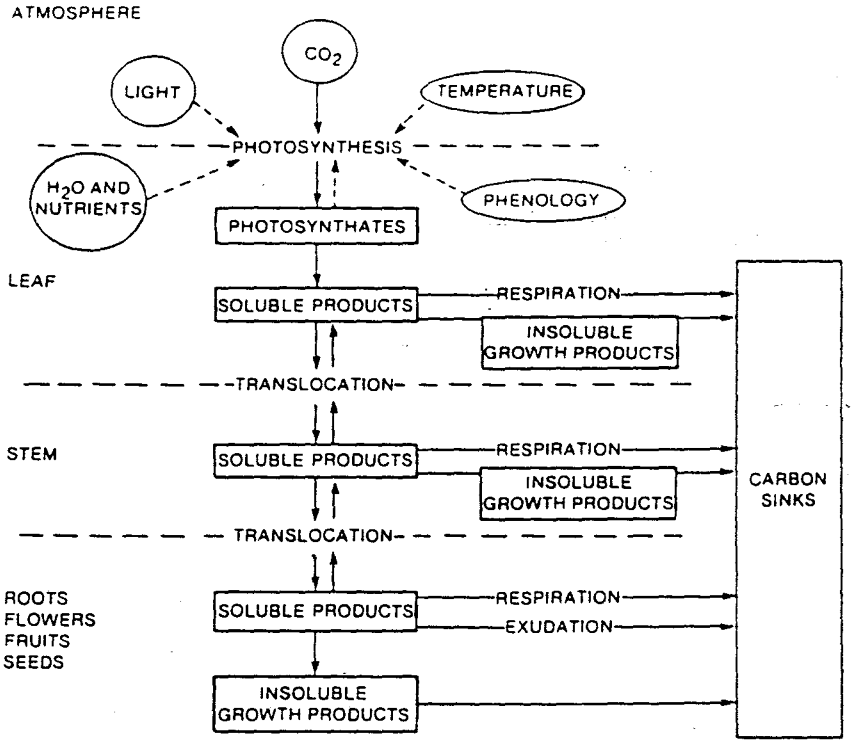

Fig. 7.1 A simplified model of photosynthate, carbon flow and utilisation in plants. The source tissues are primarily leaves and the sink tissues are stems, roots, fruits and seeds (From Oechel and Strain,1985).

Excessive NSC accumulation (particularly starch) induced by elevated CO_2 has been cited as one of the causes that may lead to suppression of NAR (Potter and Breen, 1980; Ehret and Jolliffe, 1985a).

Abnormally enlarged starch grains and their possible physical damage of the chloroplasts have been reported in plants grown at elevated CO_2. In this and other similar studies, excessive accumulation of NSC was reported. However, the observed reductions in NAR may not be entirely be attributed to this factor. Vu *et al.* (1989) and Gucci *et al.* (1991) found no positive correlation between excessive starch accumulation and NAR.

In addition, Vu *et al.* (1989) and Gucci *et al.* (1991) observed extremely large starch grains but no apparent chloroplast damage. In this study, large starch grains were also observed in bean plants grown under elevated CO_2 and similarly, no apparent physical damage to the chloroplast structure was detected (Chapter 4). However, diffusive conductance to CO_2 from the intercellular air spaces to the sites of carboxylation may have been decreased by the excessive NSC accumulation in the chloroplasts (Makino, 1994).

Stitt (1991) proposed that the accumulation of carbohydrates indirectly leads to a decrease in the amounts of proteins and other components of the photosynthetic apparatus. Numerous reports have demonstrated a close relationship between Rubisco activity or amount with NAR (Sage *et al.*, 1989; Besford, 1993; Tuba, 1994). Declining activity and/or amount in long-term elevated CO_2 acclimation studies has frequently been observed and largely interpreted as an attempt to reallocate N from non-limiting nitrogenous compounds to components which limit NAR.

Nitrogen derived from Rubisco, the largest source of nitrogen among the photosynthetic components (Wojcieska, 1994), could be reallocated into electron transport components and/or Pi-regeneration enzymes, which have been observed to limit NAR during long-term exposure to elevated CO_2. Another condition of NAR down-regulation, could be the decline in the photosynthetic capacity per unit leaf area, as a result of increased leaf area and leaf dry weight due to excessive NSC (particularly starch) accumulation under elevated CO_2. This leads to what is termed a dilution effect (Akey and Kimball, 1989). The down-regulation observed in this study may have been caused by this dilution effect. This may well have been the case, as leaf areas and dry weights in this study also increased. However, when the data for foliar N content was corrected for starch, the difference between ambient and high CO_2-grown plants was reduced to insignificant levels, particularly in the initial stages of growth (see Appendix A; Table 8.1 showing corrected and uncorrected N data).

Although the enlarged starch grains do not appear to have caused damage to the chloroplasts, the increase in chlorophyll *a*/*b* ratio (Chapter 3) observed in high CO_2-

grown bean plants, reflects a preferential destruction of chlorophyll *b*, an indicator of thylakoid instability and possible malfunctioning in the light harvesting complex II and the coupling factor CF1 (Walmsley, 1991; Nie *et al.*, 1995). Analysis of the A/C_i curves (Chapter 6) indicated changes in *in vivo* carboxylation capacity (*ACE*) which declined with age in high CO_2-grown plants. This was reflected in the decline in total Rubisco activity particularly with prolonged exposure to elevated CO_2. I conclude that the decline in NAR was probably as a result of a combination of factors such as the excessive accumulation of NSC, decline in Rubisco activity, inability to regenerate both RuBP and Pi, decline in chlorophyll content, and a general decline of nutrient status (Chapter 3).

Reduction in foliar N was accompanied by a concomitant decline in chlorophyll *a* and *b* in elevated CO_2 grown plants. The decrease in leaf chlorophyll may have led to chlorotic leaves which were more prevalent in elevated CO_2-grown plants. This may have contributed to the short leaf longevity observed in these plants. Chlorosis and shortened leaf longevity are largely associated with mineral deficiency as was shown by several authors for plants growing under natural growth conditions (Marschner, 1986) and also for plants grown under elevated CO_2 (Nederhoff and Buitelaar, 1992; Reinert and Ho, 1995). However, several authors have associated chlorophyll loss with senescence (Siffel *et al.*, 1991). Apart from genetic control some growth regulators such as endogenous ethylene have been implicated and found to accelerate senescence (Birecka *et al.*, 1984; Pell and Dann, 1991; Abeles *et al.*, 1992). High levels of naturally produced ethylene have been recorded in growth chamber studies utilising elevated CO_2 conditions (Wheeler *et al.*, 1991; Woodrow and Grodzinski, 1993). Therefore, leaf development and subsequent early senescence in plants grown under elevated CO_2 may have been accelerated by increased ethylene production. In plants grown under elevated CO_2, competition for nutrients by the developing fruit may play a role in abscission, as the massive loss of flowers and pods during the reproductive stages is thought to be, in part, caused by the interaction of ethylene and other growth regulators (Chapter 5). The extent and nature of involvement has yet to be resolved (Ceulemans and Mousseau, 1994).

7.3 Mineral interaction and CO_2

Generally, macro- and micronutrients were reduced in leaves of plants grown under elevated CO_2. Various processes and metabolic functions are directly dependent on these nutrients and their reduction must adversely affect those processes. Nitrogen supply and availability is the most important factor limiting plant growth and crop yield (see Conroy *et al.*, 1994). Leaf nitrogen, in particular, is used for the synthesis of components of the photosynthetic apparatus, such as the Rubisco enzyme and major chlorophyll *a*/*b* binding proteins (Dunsmuir *et al.*, 1983; Wojcieska, 1994). The heavy investment of nitrogen in both of these components of photosynthesis suggests that its shortage will adversely affect carbon fixation and may lead to premature senescence.

One major benefit of elevated CO_2 that has been reported widely in literature is the reduction in g_s which results in lower transpiration rates, and often leads to an increase in WUE (Berryman *et al.*, 1994; Samarakoon *et al.*, 1995). While reduced stomatal aperture may infer the possibility of protection from air pollutants that enter the leaf through this route (see Rogers *et al.*, 1994; and references cited therein), reduction in transpiration may reduce the translocation of some nutrients such as calcium and boron (Nederhoff, 1994). Many authors believe that movement of water in the xylem as a result of transpirational pull will result in the movement of dissolved ions (Kramer, 1983; Marschner, 1986), therefore a reduction in transpiration under elevated CO_2 may adversely affect the movement of these nutrients (Kramer, 1983; Marschner, 1986). This may exacerbate the already declining levels of nutrients induced by elevated CO_2. However, Tanner and Beevers (1990) excluded transpiration pull from playing a major role in translocation of nutrients. They implicated the involvement of other mechanisms which by themselves would be adequate to move nutrients to plant extremities.

7.4 Implication for plant-animal interaction and nutrient cycling

Several researchers have demonstrated previously that changes induced by elevated CO_2 in plant tissues may have profound implications for plant-animal interactions (Lincoln and Couvet, 1989; Thomspson and Drake, 1994). Under elevated CO_2, reduced N concentration has been shown to lower the nutritional value of the plant's biomass to consumers. In contrast the increased production of photosynthates under elevated CO_2 could result in more carbon based secondary compounds (Cipollini *et al.*, 1993). Paul and Ayres (1990) studying disease severity of two common fungal pathogens, powdery mildew and rusts, noticed an increase in the infestation when the host plant was fertilised with nitrogen (Paul and Ayres, 1990). This suggests that such diseases may be less severe in plant species exposed to elevated [CO_2] because they tend to have reduced shoot N (Thompson *et al.*, 1993).

In their study of effects of elevated [CO_2] on plant-fungi and plant-insect interactions, Thompson and Drake (1994) found that there was a reduction in the severity of a pathogenic fungal infection compared with ambient CO_2-grown plants. In a similar study, Akey and Kimball (1989) observed an increased mortality rate of beet armyworm (*S. exigua*) which were reared on plants grown under elevated CO_2. Kucera (1959) and Chapin (1980) pointed out that nutrient deficient plant litter decayed at a much slower rate than litter with a high nutrient content. While Daubenmire and Prusso (1963) did not oppose this *per se*, they pointed to other factors in addition to diminished nutritive quality, *inter alia* the presence of toxic compounds in the dead tissue and changes in anatomical structure of the tissues that may favour or impede expansion of the microbial communities. Anatomical features at the leaf level in plants grown under elevated [CO_2] have been shown to undergo some changes. This has also been observed for other species (Vu *et al.*, 1989; Gucci *et al.*, 1991). Similarly, several authors have demonstrated changes in secondary compounds in plants grown under elevated CO_2 (Lincoln and Couvet, 1986; Zak *et al.*, 1993).

Consequently, it is highly likely that the litter produced from these plants will be distinctly different from that produced from plants grown under ambient CO_2, and may, therefore, be decomposed at different rates and by different microbes. While progress attempting to elucidate these processes has been minimal, some trends and patterns in feeding of insects and microbes on plant tissue exposed to elevated CO_2 are beginning to be made (see review by Ceulemans and Mousseau, 1994). The change and rate of nutrient recycling into the soil from plant biomass rich in carbon, but poor in nutritive quality particularly nitrogen, may result in profound changes in the structure and composition of natural ecosystems. Before such conclusions can be made, however, further investigations are still needed to fully elucidate the complex interaction between the microflora and fauna in relation to nutrient cycling under elevated CO_2 conditions.

7.5 Proposals for future research

Studies conducted using controlled environments are important and are still going to play a major role in understanding concepts of plant performance particularly at the ultrastructural, physiological and molecular levels. They offer unique conditions to study one aspect with all other variables largely eliminated. The results presented in this study hopefully contribute to the pool of knowledge on the effects of CO_2 at the leaf level. It would be of great interest to compare these data with work conducted in multifaceted and diverse natural conditions.

Recent attention in studies of elevated CO_2 has been in the extrapolation of knowledge of leaf-level processes to the canopy, ecosystem, and biosphere levels (Amthor, 1994; Luening *et al.*, 1995). This concept was echoed during the Xth International Photosynthesis Congress, held in Montpellier, France, 1995, where virtually all speakers presenting in the symposium No 29: *Photosynthesis : Global Aspects*, emphasised the urgent need of 'scaling up'. This concept involves, as has been adequately defined by Luening *et al.* (1995), the integration of processes in both space and time. Therefore, a major challenge facing plant scientists is to extend understanding from the scale of leaves to larger canopies and ecosystems. To predict long-term responses of larger canopies to environmental perturbations, such as increased global CO_2, requires systematic integration of a wide range of factors (including natural fluctuations and interactions of parameters such as temperature, water, nutrients, herbivores, pathogens and symbionts and many more) as they occur in the natural ecosystem (Bazzaz *et al.*, 1985).

Progress in this regard will require high-tech experimental techniques to maintain elevated CO_2 concentrations within natural systems for long periods of time. Most systems seeking to achieve this are unfortunately, expensive. As a result, only affluent countries and independent laboratories are largely undertaking these experiments. However, some progress has been made towards obtaining cheaper cost-effective equipment and that is still capable of maintaining a high standard for quality research (see Kimball, 1992). In this valuable article, Kimball (1992) compared the approximate costs of different CO_2-enrichment facilities such as free-air CO_2 enrichment (FACE), open-top chambers (OTC) and sunlit controlled environment chambers (Soil, plant, Atmosphere Research chambers, SPAR). These attempts will hopefully open this exciting area of research to a broader community of interested plant researchers, who will accelerate and enhance our understanding of direct impacts of elevated CO_2 in natural systems. Not until adequate research is undertaken in integrated ecosystems will realistic predictions of plant responses be realised.

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APPENDIX A

Low CO2

Nutrient Status, Phaseoulus vulgaris, effect of starch and NSC

DAG	Uncorrected	Corrected		Uncorrected	Corrected		Uncorrected	Corrected		Uncorrected	Corrected		Uncorrected	Corrected		Uncorrected	Corrected		Uncorrected	Corrected		Uncorrected	Corrected		Uncorrected	Corrected		STARCH
	P	P	%DIFF	Mg	Mg		N	N		Ca	Ca		K	K		Cu	Cu		Fe	Fe		Mn	Mn		Zn	Zn		
10	1	1.05	5.00	0.63	0.67	6.35	6.11	6.46	5.73	5.59	5.59	0.00	1.76	1.86	5.68	13.23	14	5.82	75.33	79.67	5.76	31.02	32.8	5.74	26.33	27.84	5.73	54.43
15	1.07	1.13	5.61	0.71	0.76	7.04	6.97	7.4	6.17	5.28	5.91	11.93	1.93	2.05	6.22	13.5	14.37	6.44	92	97.91	6.42	35.24	37.51	6.44	30	31.93	6.43	60.4
20	1.04	1.11	6.73	0.77	0.83	7.79	6.62	7.1	7.25	5.48	5.61	2.37	1.92	2.06	7.29	15.53	16.7	7.53	100.3	107.71	7.36	40.97	43.95	7.27	30.33	32.55	7.32	68.3
25	0.98	1.05	7.14	0.69	0.74	7.25	6.64	7.1	6.93	5.68	6.13	7.92	1.89	2.04	7.94	13.8	14.9	7.97	104.7	113.03	8.00	45.21	48.22	6.66	29.66	32.01	7.92	74.06
30	0.94	1.04	10.64	0.68	0.75	10.29	6.5	7.2	10.77	5.21	5.77	10.75	1.98	2.19	10.61	13.01	14.43	10.91	110.1	122.11	10.91	47.36	52.52	10.90	27.33	30.31	10.90	98.36
35	0.91	1	9.89	0.65	0.72	10.77	6.74	6.86	1.78	4.74	5.21	9.92	1.84	2.02	9.78	13.5	14.86	10.07	104.7	114.51	9.41	44.09	48.48	9.96	26	28.61	10.04	91.26
40	0.84	0.913	8.69	0.64	0.7	9.38	5.56	5.94	6.83	4.63	5.03	8.64	1.84	2	8.70	12.38	13.47	8.80	96	104.43	8.78	44.34	48.23	8.77	21.33	23.09	8.25	80.76

High CO2

DAG	Uncorrected	Corrected		Uncorrected	Corrected		Uncorrected	Corrected		Uncorrected	Corrected		Uncorrected	Corrected		Uncorrected	Corrected		Uncorrected	Corrected		Uncorrected	Corrected		Uncorrected	Corrected		
	P	P		Mg	Mg		N	N		Ca	Ca		K	K		Cu	Cu		Fe	Fe		Mn	Mn		Zn	Zn		
10	1.03	1.18	14.56	0.64	0.73	14.06	6.48	7.44	14.81	4.73	5.43	14.80	2	2.29	14.50	15.43	17.73	14.91	69.33	79.7	14.96	37	48.23	30.35	28	32.18	14.93	130.13
15	0.9	1.2	33.33	0.71	0.83	16.90	7.62	8.9	16.80	4.56	5.33	16.89	2.16	2.52	16.67	15.08	17.63	16.91	84.66	98.99	16.93	42	42.53	1.26	26	32.18	23.77	144.8
20	0.98	1.09	11.22	0.72	0.88	22.22	7.65	9.42	23.14	4.66	5.74	23.18	2.08	2.56	23.08	15.23	15.07	-1.05	88.66	109.24	23.21	48	49.11	2.31	25.6	30.4	18.75	188.46
25	0.91	1.05	15.38	0.66	0.79	19.70	7.26	8.72	20.11	4.82	5.78	19.92	2.12	2.54	19.81	13.2	15.85	20.08	93	111.71	20.12	53.61	59.14	10.32	24.33	31.54	29.63	167.5
30	0.89	1.05	17.98	0.63	0.74	17.46	6.33	7.47	18.01	4.21	4.97	18.05	2.16	2.54	17.59	13.1	15.46	18.02	87.55	103.09	17.75	54.84	64.74	18.05	21	29.22	39.14	152.93
35	0.85	0.99	16.47	0.6	0.7	16.67	5.54	6.47	16.79	3.9	4.55	16.67	2	2.33	16.50	12.1	14.14	16.86	84.3	98.53	16.88	53.37	62.38	16.88	20	23.38	16.90	144.5
40	0.79	0.91	15.19	0.59	0.68	15.25	5.04	5.84	15.87	3.52	4.07	15.63	1.92	2.22	15.63	11.24	13.02	15.84	70.66	81.89	15.89	53.37	61.85	15.89	17.33	20.08	15.87	137.16

Table 8.1 Compares the macro- and micronutrient expressed gram per total dry weight including NSC (uncorrected) and gram per structural dry weight excluding NSC (corrected) data (see Wong 1990, for explanation of terminology used). The percentage difference is given in bold .

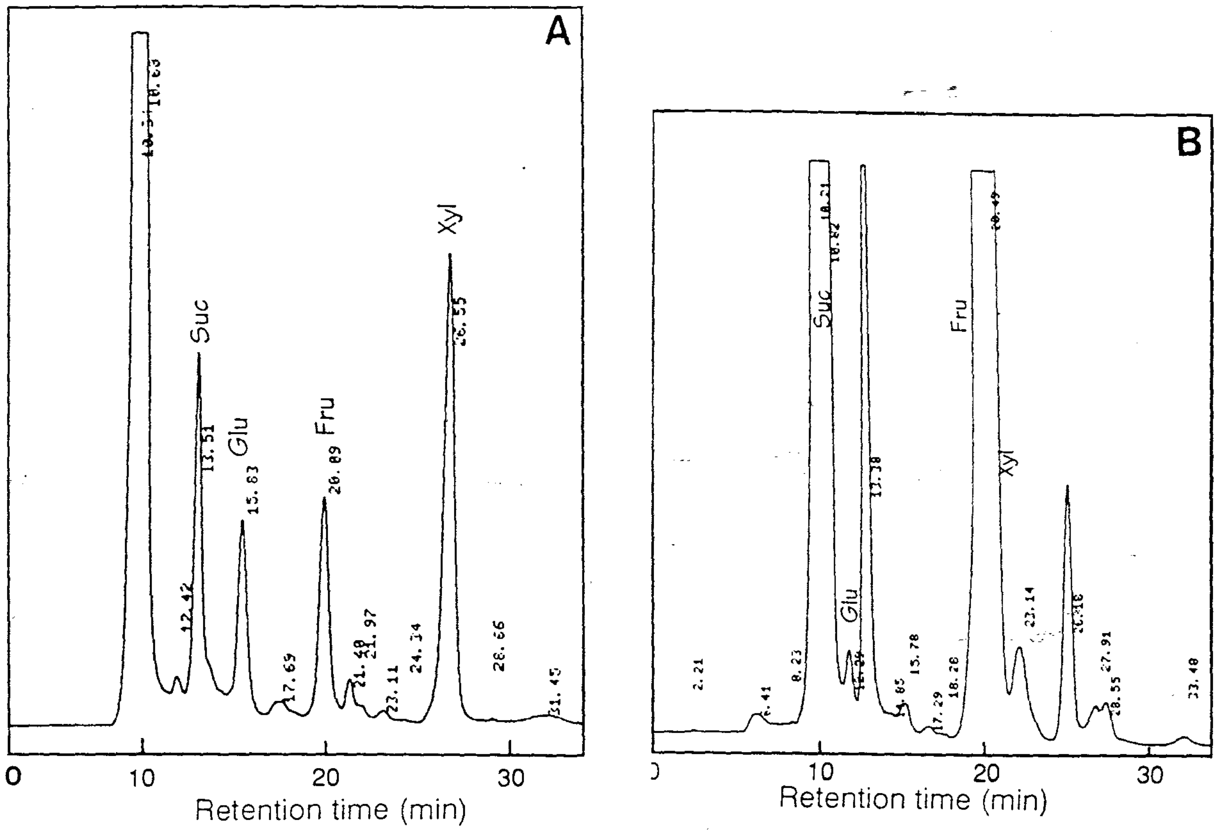

Fig. 8.1 HPLC chromatograms of NSC extracted from leaves of 25 DAG harvested between 13 and 15h 00 of bean plants grown at 360 (**A**) and 700 (**B**) µmol mol^{-1} CO_2. Peaks: Suc, sucrose; Glu, glucose; Fru, fructose and Xyl, xylitol (internal standard). Sugars were identified and quantified on the basis of retention time and peak areas of aunthetic standards.

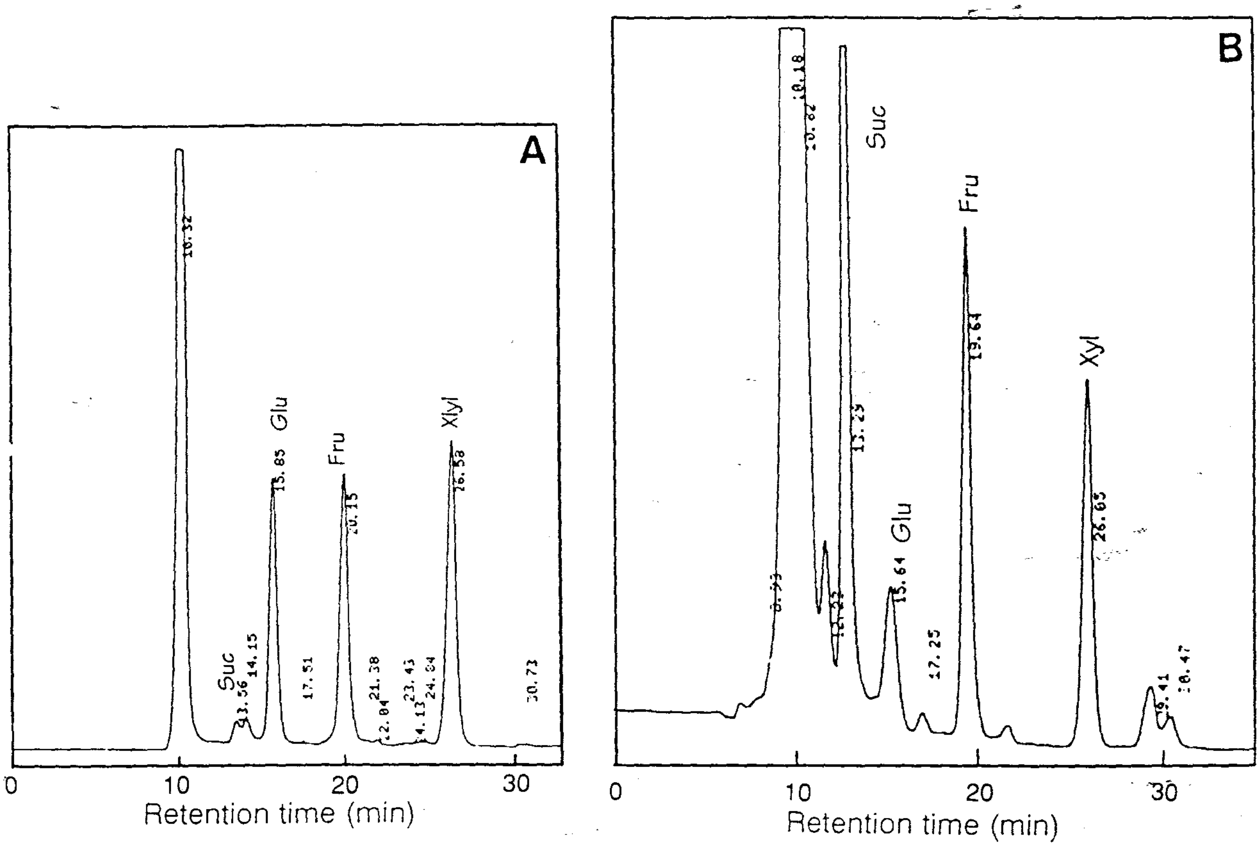

Fig. 8.2 HPLC chromatograms of NSC extracted from leaves of 30 DAG harvested between 13 and 15h 00 of bean plants grown at 360 (**A**) and 700 (**B**) μmol mol^{-1} CO_2. See figure 8.2 for details.

APPENDIX B

Parameter calculation and equations

The basic equations (Farquhar, *et al.* 1980; von Caemmerer and Farquhar, 1981; Field *et al.*, 1989) which were also incorporated in the IRCAL programme (materials and methods), are presented here without details of the theory. Photosynthetic CO_2 assimilation (A, µmol m^{-2} s^{-1}) was calculated from the depletion of CO_2 in the gas stream. According to the above mentioned authors, A depends on the velocity of carboxylation, used. Γ^* is the CO_2 compensation point of photosynthesis in the absence of dark respiration and C is the internal carbon dioxide concentration. Thus the net rate of CO_2 assimilation in the absence of day respiration (R_d) is: V_{cmax}, RUBISCO which is an unstable enzyme capable of carboxylation or oxygenation, hence K_c and K_o which are the Michaelis-Menten constants for CO_2 and O_2 are used. Thus the net rate of CO_2 assimilation in the absence of day respiration (R_d) is:

$$A = V_{c\max}\left[\frac{C_i - \Gamma^*}{C_i + K_c\left(\frac{1+0}{K_o}\right)}\right] - R_d \qquad \text{Equation 8.1}$$

Farquhar, *et al.* (1980) established the dependence of A on intercellular CO_2 using equation 8.1. The resultant equation (Equation 8.3) is directly related to the equation proposed by Ku and Edwards (977):

$$CE = \frac{APS}{CO_2 - \Gamma^*}$$

Equation 8.2

which estimated carboxylation efficiency (*CE*) from the initial slopes of A versus C_i; where *APS* is the apparent rate of photosynthesis.

The dependence of A on the intercellular CO_2 is then:

$$\frac{dA}{dC} = V_{c\max} \left[\frac{\Gamma^* + K_c\left(\frac{1+0}{K_o}\right)}{\left[C + K_c\left(\frac{1+0}{K_o}\right)\right]^2} \right]$$

Equation 8.3

Carbon dioxide compensation point (Γ^*, μmol m^{-2} s^{-1}) has been used for the calculation of *ACE* and its calculation is based on the following equation:

$$\Gamma^* = \frac{\Gamma + K_c\left(\frac{1+0}{K_o}\right)\frac{R_d}{V_{c\max}}}{1 - \frac{R_d}{V_{c\max}}}$$

Equation 8.4

Farquhar and Sharkey (1982) and later Field *et al.* (1989) pointed out that the power of photosynthetic measurement is greatly increased by simultaneous measurement of transpiration (E, mmol m^{-2} s^{-1}) as illustrated in equation 8.5. Once E has been calculated, it is possible to calculate leaf conductance to water vapour, which is the critical parameter for the determination of internal CO_2 concentration (C_i). By rearranging equation 8.5

$$A = g_c(C_a - C_i) - \left(\frac{C_i + C_a}{2}\right)E \qquad \text{Equation 8.5}$$

The resultant equation (8.6, below) is incorporated in the IRCAL software package that was used, adequately estimates C_i as outlined by Field, *et al.* (1989).

$$C_i = \frac{\left(g_{tc} - \frac{E}{2}C_a\right) - A_n}{\left(g_{tc} + \frac{E}{2}\right)} \qquad \text{Equation 8.6}$$

where g_{tc} is the total conductance to CO_2 (mol m^{-2} s^{-1}), C_a is the mole fraction of CO_2 in the ambient air (µmol mol^{-1}) and A_n is net assimilation rate (µmol m^{-2} s^{-1}).