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Physiological aspects of the response to elevated CO2 in lentils (Lens culinaris Medic)

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Physiological Aspects of the Response to Elevated CO_2 in Lentils (*Lens culinaris* Medic)

By

Rima Rabah Nasser

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Dedication

For my beloved Yara and Majed.

For those closest to my heart, my father, my mother, Samir, Rabeeh, Jalal, Jehan and Abeer.

For his soul and who is still alive in my memory, my brother Emad.

Abstract

Rima Rabah Nasser

Physiological Aspects of the Response to Elevated CO_2 in Lentils (*Lens culinaris* Medic)

This study examined the effects of elevated CO_2 and its interaction with drought and nitrogen fertilizer on the growth, production and nodulation of the leguminous crop lentil (*Lens culinaris* Medic) cultivars ILL7979 and ILL6994 (Idlib 3).

Plants were grown under ambient and elevated CO_2 at full and limited irrigation conditions in both open top chambers, which were later proven to be unreliable because of CO_2 leakage, and tightly sealed and ventilated chambers which were reliable. Destructive harvests at anthesis and at maturity were conducted and results from sealed chambers at maturity showed that above ground dry weight was increased by an average of 12% under elevated CO_2, but this increase was not statistically significant. Seed yield was marginally significantly increased by elevated CO_2 ($p= 0.059$) in both fully irrigated and drought stressed plants by an overall average of 19%. Both nodule number and fresh weight were increased by elevated CO_2 by an average of 38% and 45% respectively, but this increase was not statistically significant. All measured parameters in both ambient and elevated CO_2 conditions were significantly reduced by drought. In most instances, the cultivar Idlib 3 showed a better performance than ILL7979. Nitrogen concentration in the seeds and roots was not affected by elevated CO_2, but it

was significantly reduced in the shoots. Similarly, phosphorus concentration was only significantly reduced in the shoots.

The interaction of elevated CO_2 with nitrogen availability was investigated on Idlib 3 using the sealed chambers at five nitrogen levels equivalent to 5, 25, 50, 75 and 100 kg ha^{-1}. Sequential destructive harvests conducted over the growth period showed that, compared to ambient, elevated CO_2 led to a significant increase in LAI after flowering (20–30%), biomass dry weight (35%) and seed yield (53%). Moreover these values increased with increasing levels of nitrogen applied. Although not significantly, nodule number increased under elevated CO_2 and the highest nodule number was observed at the nitrogen level equivalent to 50 kg N ha^{-1} under ambient and 75 kg N ha^{-1} under elevated CO_2. The average increase of nodule number for all treatments under elevated CO_2 was 52%. Examination of total nitrogen and phosphorus concentrations in the dry matter showed that the total uptake was higher, although not significantly, under elevated CO_2 but due to the increases in biomass concentration levels were slightly lower, but not significantly.

The effects of elevated CO_2 on nitrogenase activity in lentils using an acetylene reduction assay on whole plants was undertaken. Technical problems meant that the species under test was switched to white clover and showed that nitrogenase activity was slightly higher under elevated CO_2 than that at ambient, but not significantly. Nitrogen budgeting concluded that improved nitrogen uptake under elevated CO_2 was a consequence of improved N fixation occurring as a result of increased nodule number rather than improved nitrogenase activity.

Author's Declaration

At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other University award.

This study was sponsored by the University of Damascus, Damascus, Syria.

Publications

- Rabah Nasser, R., Fuller, M. P., Jellings, A. J. (2008) Effect of elevated CO_2 and nitrogen levels on lentil growth and nodulation. *Agronomy for Sustainable Development* **28**: 175-180.
- Rabah Nasser, R., Fuller, M. P., Jellings, A. J. (2008) The influence of elevated CO_2 and drought on the growth and nodulation of Lentils (*Lens culinaris* Medic). Aspects of Applied Biology **88**, 103-110.
- Rabah Nasser, R., M. P. Fuller, et al. (2006). The effect of elevated carbon dioxide and nitrogen levels on the growth and nodulation of lentils. *IX Congress of the European Society for Agronomy, Warsaw, Poland, Bibliotheca Fragmenta Agronomica* **11**, 183-184.

Presentations and conferences attended

- Presented a poster at the American Society of Agronomy, Crop Science Society of America, Soil Science Society of America ASA-CSSA-SSSA International Annual Meeting (6-10 November 2005), Salt Lake city, Utah, USA.

- Attended the Royal Society scientific discussion meeting on Food crops in a changing climate (26-27 April 2005).
- Presented a poster in the Association of Applied Biology Postgraduate 2005 Poster Competition at Homerton College, Cambridge on 15 December 2005.
- Planned to attend and present an oral presentation at the IX ESA (European Society for Agronomy) congress 4-7 September 2006, Warszawa, Poland. However, due to urgent circumstances, I was forced to cancel my participation, though; the paper was still published in the book of proceedings.
- Presented a poster in the VC's Research and Innovation Conference, 4th April 2007, Plymouth University, Plymouth, UK.
- Attended the VC's Research and Innovation Conference, April 2008.

Modules attended

- **SFAC 511:** Research and development project management (EndNote, project plan, literature review, abstract, table and figures...).
- **CROP 511:** Impact of climate & change on plants.

Graduate school skills development courses attended

- Learning and teaching for general teaching association (GTA). An Oral presentation of the research project was presented.
- Problem solving techniques.
- Preparing effective poster presentation.
- Presenting your research using Microsoft PowerPoint.
- Problem solving techniques.
- Intermediate PowerPoint.

- Creating web pages using Microsoft Front Page.
- Visio – working with shapes.
- Visio 2002 – Charts and Drawings.
- Developing professional writing skills for PhD.
- Job search technique.
- Introduction to Microsoft Excel.
- Excel Pivot Tables & Macros.
- Creating Web Pages using Share point Design.
- Enterprise' skills.
- Advanced EndNote.
- Risk Management for Research Students.
- Preparing for the viva.

Other activities

- Visited the International Centre for Agriculture Research in the Dry Areas (ICARDA), Syria 2/2/2004).
- Attended the MSc Sustainable Crop Production Field Trip to Brittany, France (29/04/2004to 6/05/2004).
- Attended the School of Biological Science safety training day (07/01/2005).
- Attended the Graduate School Careers focus workshop with UK GRAD SW Hub representative.
- Invited to write a chapter in a Grain legumes book.

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1.Chapter 1

General Introduction

The research presented in this thesis is concerned with the physiological response of the leguminous crop plant Lentil (*Lens culinaris* Medic) to a growing environment enriched in CO_2 which simulates the potential climatic situation in approximately 70-90 years time (2080- 2100). Increasingly, it is important to study the aspect of the effects of elevated CO_2 and other factors of the changing climate because of the growing evidence that these factors will significantly impact on crop production worldwide. The following section gives a brief explanation of climate change and scenarios predicted for the future.

1.1. Explanation of the increase in atmospheric CO_2

It is now commonly accepted that the world's climate is changing and becoming warmer. The natural balance in the atmosphere has been disturbed by a significant increase in carbon dioxide and other anthropogenic gases which strengthen the so-called greenhouse effect, and which is likely to warm the world over the next few decades (Nilsson, 1992; Gribbin, 1992).

The natural greenhouse effect occurs because of the blanket of atmospheric gases surrounding the earth's surface (Houghton, 1991). Incoming solar radiation (mainly in the short wavelength regions) is largely invisible to the atmosphere and passes directly to the earth's surface where it is absorbed and re-emitted at longer wavelengths (in the infrared region). This infrared radiation radiated by the warm surface of the earth is less invisible and is absorbed by certain gases in the atmosphere called greenhouse gases

(CO_2, water vapour, CH_4, O_3, N_2O and chlorofluorocarbons) (Gribbin, 1992). These gases also partly emit infrared radiation, and by this process of absorption and re-radiation, an essential portion of the thermal radiation returns to the earth's surface preventing it from being cooled (Allen, 1998). Consequently, the average temperature of our plant's surface is about 15 °C which is calculated to be 33°C warmer than it would be without these greenhouse-effect gases (Allen, 1991; Wellburn, 1994). The term 'greenhouse effect' is used because of the similarity between the atmosphere's properties and the effect shown by glass in a greenhouse with glass being less transparent to long wave radiation compared to shortwave (Houghton, 1997). Although all the greenhouse gases are important, the most important contributor to the overall warming process is carbon dioxide because of its relative abundance and, whose concentration is undoubtedly increasing in the atmosphere, most probably due to human activities (Wellburn, 1994).

Carbon dioxide is transferred between a number of natural carbon reservoirs (biosphere, oceans, and atmosphere) through a process described as the carbon cycle (Figure 1.1). Note: human and animal respiration was not included in the cycle is because they do not add a net carbon amount to the atmosphere as the amount they exhale do not exceed the carbon they take by eating plants or animals that eat plants (www. gcrio.org/doctorgc/index.php/drweblog/C48/).

In this cycle, about one quarter of the total amount of carbon dioxide in the atmosphere is cycled in and out each year. One half of the exchange occurs with the land biota through the processes of respiration and photosynthesis, and the other half occurs across the ocean's surface through physical and chemical processes (Houghton, 1997). These exchanges between the reservoirs form a steady balance and, therefore, any small

changes in the land and the oceans reservoirs, which are much larger than the atmosphere, could have a great effect on the atmosphere. For example, if just 2% of the carbon dioxide stored in the oceans is released, the amounts of carbon dioxide in the atmosphere will double (Warr and Smith, 1995; Houghton, 1997). According to the data taken from bubbles trapped in ice cores from Antarctica, this delicate balance was stable for several thousands of years before industrialization within about 10 parts per million per volume (ppmv) with a mean of 280 ppmv (Gribbin, 1992; Houghton, 1996; Houghton *et al.*, 1997). Since the industrial revolution (1750-1850), the concentration of carbon dioxide has risen considerably from about 280 ppmv to the current level of 379 ppmv ($\mu mol\ mol^{-1}$) reported in 2005 (IPCC, 2007).

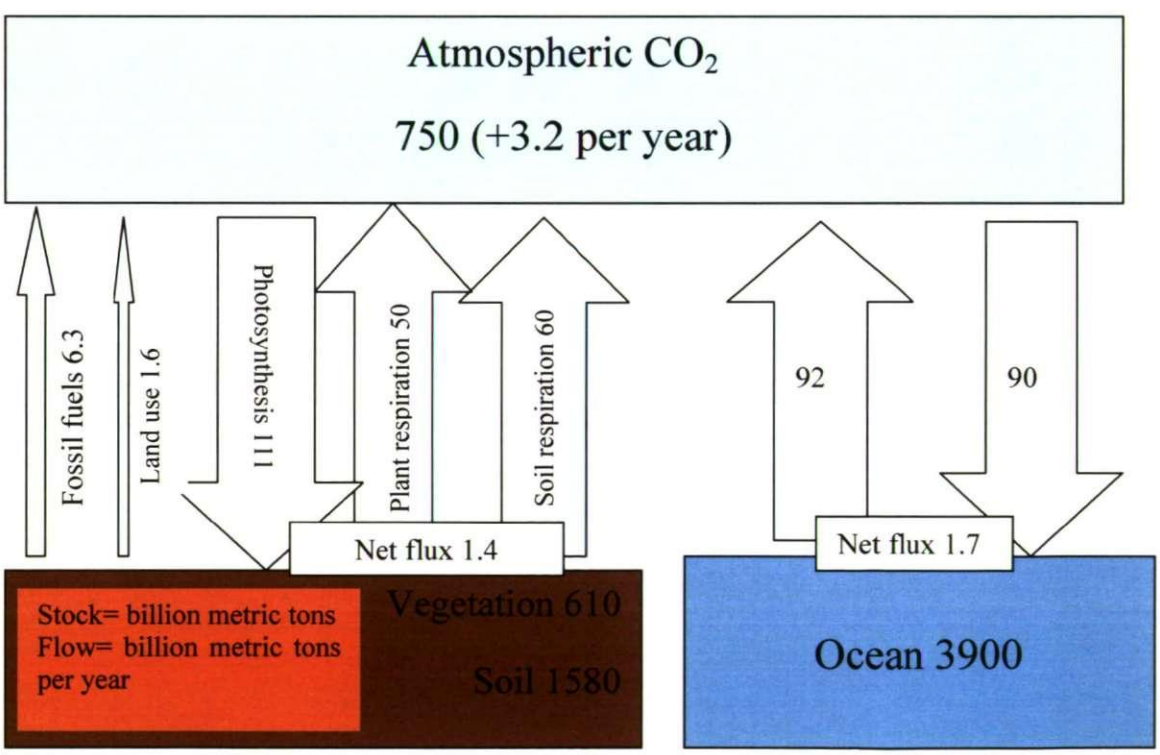


Figure 1.1. The global carbon cycle, recreation of a chart by Kling, (2002).

This remarkable increase in atmospheric carbon dioxide concentration can be attributed to the burning (oxidation) of the major reservoirs of carbon (coal and oil) because of human intervention (Warr and Smith, 1995).

In order to study the changes in the concentration of carbon dioxide in the air, an accurate series of measurements started to be taken in 1958 from Mouna Loa Observatory in Hawaii, which is situated at a distance from industrial pollution and hence was considered to be representative of the well-mixed state of the atmosphere (Gribbin, 1992). Strong evidence can be taken from the continuous records from Mouna Loa which show that the concentration of atmospheric carbon dioxide went up gradually from about 315 parts per million in 1957 to about 360 part per million in the 90s (Gribbin, 1992; Warr and Smith, 1995; Allen, 1998). According to these measurements, there is an increase of about 1.5 ppmv each year in the atmospheric carbon dioxide concentration, which adds an annual amount of about 3.3 gigatonnes to the atmospheric carbon reservoir (Houghton, 1997). The increase in CO_2 levels from 1995 to 2005 was even higher at an average of 1.9 ppm yr^{-1} which resulted in a concentration of 379 ppm (IPCC, 2007) (Figure 1.2). A similar upward trend has been recorded from the monitoring of CO_2 in Australia from 1972 to 1981 (Allen, 1998).

Most of the extra amounts of carbon dioxide in the atmosphere have come from the intensive burning of fossil fuels (coal, oil and gas) since the mid-19 th century (Warr and Smith, 1995). According to Gribbin (1992), by the early 1980's, about 5000 million tonnes (5Gt) of fuel were being burnt each year which means that there was an annual amount of about 20 Gt of carbon dioxide added to the atmosphere from the combustion of fossil fuels. An average of 23.5 $GtCO_2$ emissions from annual fossil fuel use was also reported in the 90's, and these rates increased to 26.4 $GtCO_2$ over the period from 2000

to 2005 (IPCC, 2007). About 95% of fossil fuel burning occurs in the northern hemisphere and, consequently, the concentration of CO_2 there is higher (by about 2ppmv) than the southern hemisphere (Houghton, 1997).

The other main source of CO_2 emissions into the atmosphere, which adds about 1.6 ± 1.0 Gt of carbon (5.9 $GtCO_2$) each year, is land use change and, in particular, the deforestation of tropical rainforests (major carbon sinks) and their replacement by grassland and scrub (Wellburn, 1994; Schimel, 1996; IPCC, 2007). With deforestation, CO_2 is released into the atmosphere in many ways: burning and decay of biomass; oxidation of carbon stored in the biomass of soil; and renewal of forest and soil after deforestation (Drake, 2000).

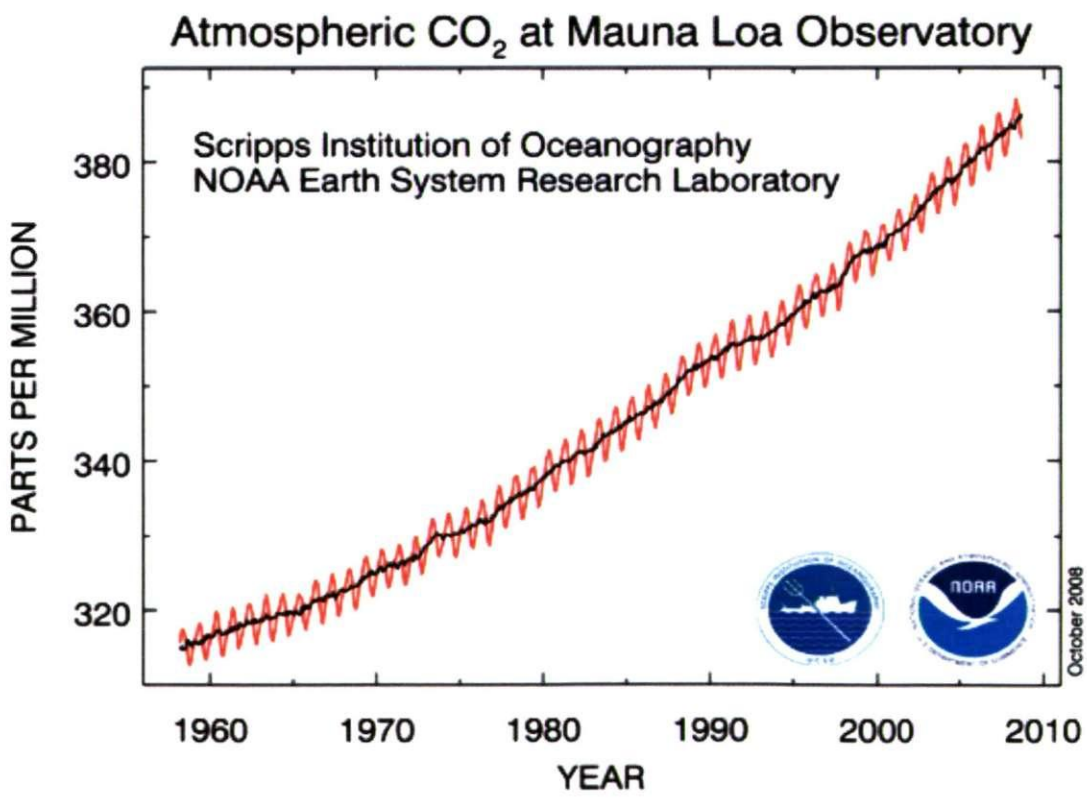


Figure 1.2. Global atmospheric CO_2 levels (ppm) since 1958 as measured from Mauna Loa Observatory in Hawaii. Source Dr. Pieter Tans, NOAA/ESRL (www.esrl.noaa.gov/gmd/cgg/trends).

In addition, about 2-3% of total carbon dioxide emissions into the atmosphere (7-8 Gt) can be directly released from some industrial sources such as cement production (Allen, 1998; Drake, 2000).

About 40- 50% of the net CO_2 emissions into the atmosphere is taken up by the oceans which play a crucial role in the global carbon balance (Allen, 1998). The equilibrium between the concentrations of CO_2 dissolved in the surface water and that in the air above the surface is governed by chemical laws, which state that if atmospheric concentration changes by 10%, the concentration in solution in water changes by only 1%. This change, which occurs rapidly in the upper waters, will help in reducing part of the anthropogenic carbon dioxide added to the atmosphere. However, because absorption at the lower levels takes longer, and the mixing of surface water with that in the lower and deep levels takes hundreds and possibly thousands of years, oceans cannot be considered as immediate sinks for the significant increase in the atmospheric CO_2 (Houghton, 1997).

Together with the fact that carbon dioxide and other greenhouse gas emissions are increasing, significant changes in global climate are more likely to happen in the future (Houghton, 1997; Nilsson, 1992; Crowley, 2000; IPCC, 2007). It is not easy to estimate what might be in store for future years, and prerequisite knowledge about what carbon dioxide changes are likely to be is needed for any estimation (Houghton, 1997). To predict CO_2 changes, it is essential to make predictions or assumptions about population growth, economic growth, energy use, and development of energy sources for the whole world. As any specific assumptions may not be accurate, a variety of different assumptions should be made to acquire some information about a range of possibilities

for the future or the so-called **Scenarios** (Houghton, 1997). The next section will discuss some of these scenarios.

1.1.1. The IPCC Emission Scenarios

The Intergovernmental Panel on Climate Changes (IPCC) produced a series of projections for greenhouse gas emissions up to 2100 and their implications on future climate change. In this section, only carbon dioxide emissions scenarios will be discussed.

The first series of emissions scenarios is known as **IS92a - f**, and the three main scenarios are IS92a, IS92e, and IS92c. The middle case, IS92a, is closest to a 'business-as-usual' scenario in which CO_2 concentration is likely to rise without any procedures taken to regulate emissions (Wellburn, 1994). This scenario assumes moderate economic growth (2.3-3.9% per year), moderate world population (11.3 billion by 2100), and a mix of energy sources (Jepma and Munasinghe, 1998). Working with these assumptions, the levels of carbon dioxide in the atmosphere are anticipated to reach a peak of over 600 ppmv by 2100 (Schimel, 1996; Jepma and Munasinghe, 1998).

In the scenario IS92e, the assumptions made are of rapid economic growth (3.0% to 3.5% per year), moderate population growth (11.3 billion by 2100), high use of fossil fuels, and the probability of nuclear use (Jepma and Munasinghe, 1998). With these assumptions, the predicted levels of carbon dioxide in the atmosphere may exceed 900 ppmv (Schimel et al, 1996).

On the other hand, under scenario IS92c, the levels of carbon dioxide in the atmosphere are estimated to stabilise below 500 ppmv by 2100. This scenario assumes a reduced

economic growth, a limited rate of increase in global population (6.4 billion by 2100), and severe constraints on fossil fuel use (Jepma and Munasighe, 1998). The emissions of CO_2 from anthropogenic sources, in accordance with the figures predicted by the three different IS92 scenarios, range from 5 to 35 Gt each year (Alcamo, 1995).

After the 1992 scenarios were evaluated in 1995, a decision by the IPCC plenary in 1996 was made to develop new sets of scenarios that overcome the weaknesses in IS92 scenarios and to benefit from advances made since 1992 (Nakicenovic *et al.*, 2000). The new set of scenarios is called the **SRES** scenarios which were published in the Special Report on Emissions Scenarios in 2000 and in the IPCC Third Assessment Report (TAR) in 2001. These scenarios covered a wide range of future emissions and these scenarios were developed as a set of four alternative scenario families (A1, A2, B1, and B2), which are subdivided into six main groups (the three scenario families A2, B1, and B2, plus three groups within the A1 family A1FI, A1T, and A1B), and each scenario family includes a coherent narrative part called a "storyline" (IPCC, 2000). The main characteristics of the four SRES storylines and scenario families as described in the IPCC Special Report on Emissions Scenarios (2000) are as follow:

- "The A1 storyline and scenario family describes a future world of very rapid economic growth, low population growth, and the rapid introduction of new and more efficient technologies. Major underlying themes are convergence among regions, capacity building, and increased cultural and social interactions, with a substantial reduction in regional differences in per capita income.
- The A2 storyline and scenario family describes a very heterogeneous world. The underlying theme is self-reliance and preservation of local identities. Fertility patterns across regions converge very slowly, which results in high population

growth. Economic development is primarily regionally oriented and per capita economic growth and technological changes are more fragmented and slower than in other storylines.

- The B1 storyline and scenario family describes a convergent world with the same low population growth as in the A1 storyline, but with rapid changes in economic structures toward a service and information economy, with reductions in material intensity, and the introduction of clean and resource-efficient technologies. The emphasis is on global solutions to economic, social, and environmental sustainability, including improved equity, but without additional climate initiatives.
- The B2 storyline and scenario family describes a world in which the emphasis is on local solutions to economic, social, and environmental sustainability. It is a world with moderate population growth, intermediate levels of economic development, and less rapid and more diverse technological change than in the B1 and A1 storylines. While the scenario is also oriented toward environmental protection and social equity, it focuses on local and regional levels".

The range of CO_2 concentrations projected for the year 2100 according to these scenarios ranges from 540 to 970 ppm (Figure 1.3) (IPCC, 2001). The SRES emission scenarios were also used in the IPCC Fourth Assessment report (AR4) (2007) and from the six main groups, three groups (B1, A1B and A2 demonstrating low, medium and high emission scenarios) were selected for future climate projections (Meehl *et al.*, 2007). For example, according to the coupled climate carbon cycle models, which agree that the efficiency of the earth system (ocean and land) to absorb anthropogenic CO_2 would be reduced by the future climate change, the atmospheric CO_2 concentrations for

A2 emission scenario are expected to be between 730 and 1020 ppm by 2100 which is higher than the standard value of 836 ppm (Meehl *et al.*, 2007).

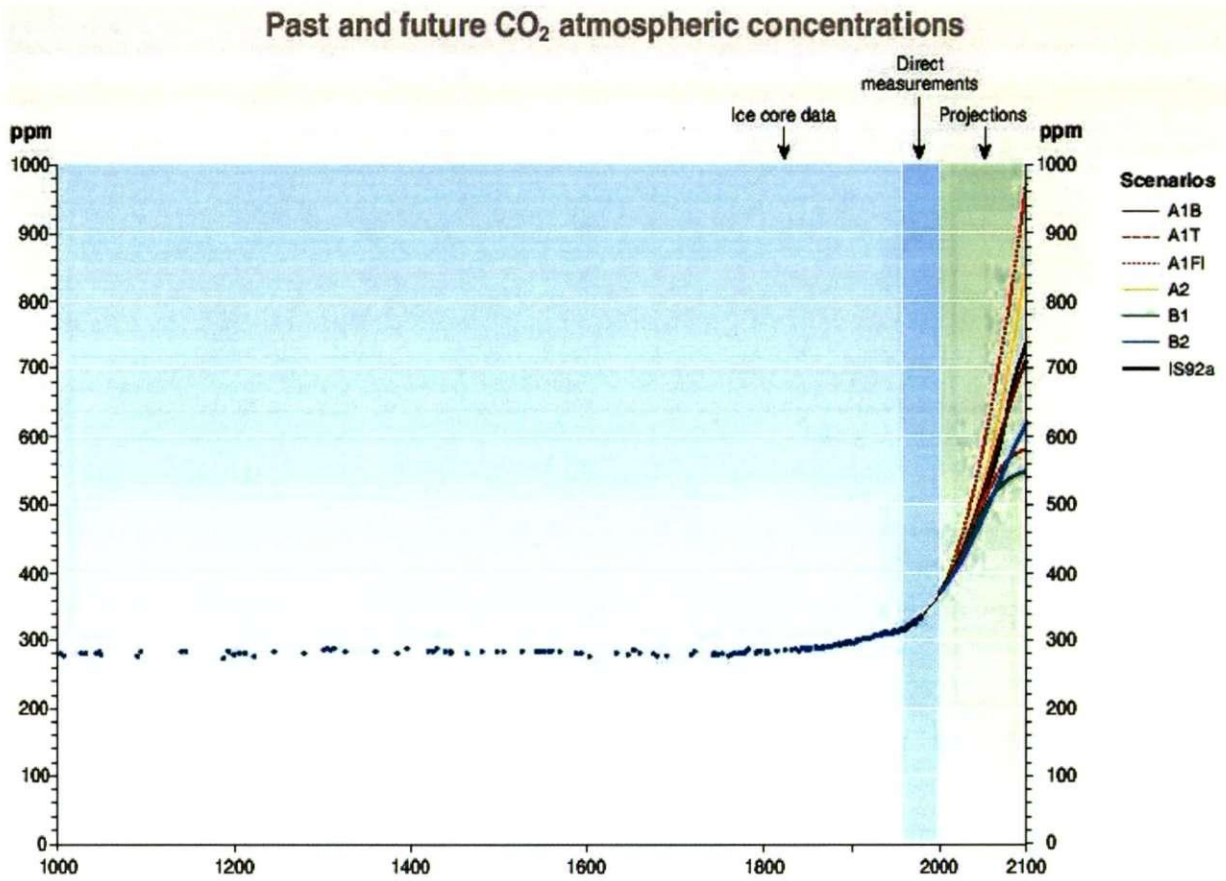


Figure 1.3. Atmospheric CO_2 concentration from year 1000 to year 2000 from ice core data and from direct atmospheric measurements over the past few decades. Projections of CO_2 concentrations for the period 2000 to 2100 are based on the six illustrative SRES scenarios and IS92a. (Source IPCC, 2001)

1.1.2. The GISS scenarios

Three scenarios for future climate change, labelled A, B and C, are being studied by the Goddard Institute for Space Studies (GISS) GCM (Global Climate Model). In this study, the levels of greenhouse gas emissions for the year 1958 are taken as a basis for comparison. Assuming that greenhouse gas emissions will continue to increase at

accelerating growth rates similar to those in 1970s and 1980s, scenario A, conducted for the period 1958-2062, assumes that an equivalent-doubling of CO_2 could be reached by about 2030 (Hansan *et al.*, 1988). Scenario B (1958- 2039), based on the assumption that the annual increase in the greenhouse gas emissions will be approximately stable at present levels, assumes that an equivalent-doubling CO_2 could be achieved by about 2060. Unlike scenarios A and B, scenario C assumes a significant decrease in the emission growth rate between 1990 and 2000 and, therefore, equivalent-doubling of CO_2 is never achieved (Hansan *et al.*, 1988).

1.1.3. The UKCIP02 climate change scenarios

The UKCIP02 scenarios have been prepared by the UK Climate Impacts Programme to describe the possible changes in the climate of the United Kingdom through out the 21st century. Unlike the scenarios published in 1998, these scenarios are based on the new IPCC emission scenarios published in 2000 (SRES emission scenarios). These scenarios describe four future climate change scenarios for the UK and classified as Low, Medium-low, Medium-high, and High referring to their respective global warming rates. According to these scenarios, carbon dioxide concentration in 2080s range between 525 ppm to 810 ppm (Table 1.1).

UKCIP02	2020s	2050s	2080s
Low Emissions	422	489	525
Medium- Low Emission	422	489	525
Medium- High Emissions	435	551	715
High Emissions	437	593	810

Table 1.1. Future global atmospheric CO_2 concentrations (ppm) estimated by the UKCIP02 scenarios (Source Hulme *et al.*, 2002)

Although the different scenarios have drawn slightly different pictures, carbon dioxide levels in the atmosphere are still rising. This increase is associated with an increase in the average global temperature, and in the average sea-level. According to IPCC (2007), by the year 2100 the average temperature is likely to increase from 1.8 °C (likely range 1.1°C to 2.9 °C) to 4.0 °C (likely range is 2.4 °C to 6.4 °C), and an average increase range between 0.18 m to 0.59 m for sea levels.

One of the new features resulting from the changes, is the new look of the belts of vegetation surrounding the globe. In particular, the tundra in the far north, where the soil is frozen for much of the year, will gradually shrink. As a result of the warming of the tundra, which holds 14% of CO_2 stored in all the soils of the world, the decomposition of dead leaves and plants will become faster, releasing greater amounts of carbon dioxide into the atmosphere than previously (Woodward, 1992). Furthermore, the warming trend is also believed to lead to a reduction in the ocean's and land's ability to absorb anthropogenic CO_2, which means a larger fraction of anthropogenic CO_2 would stay airborne in the atmosphere (positive feedback to the global carbon cycle) leading eventually to higher atmospheric CO_2 concentrations (IPCC, 2007).

Since plants interact with the gaseous environment in the uptake and fixation of CO_2 by the process of photosynthesis a consideration of photosynthesis is necessary in order to understand potential interactions.

1.2. Photosynthesis and the role of the enzyme Rubisco

Photosynthesis is defined as a biological process whereby the energy from light is collected and stored before being transformed by a series of events into biochemical energy essential to power life. This process, which can occur in algae, higher plants and certain kind of bacteria, is considered the source of life on earth as it provides food to all other organisms on earth, and provides the starting point of most energy (Hall and Rao, 1992; Blankenship, 2002). The overall summary equation of photosynthesis, which in green plants takes place in chlorophyll containing organelles called chloroplasts, is $6CO_2 + 6H_2O \xrightarrow{\text{Light}} C_6H_{12}O_6 + 6O_2$. This process is carried out over two sets of reactions called the light and dark reactions and while the former takes place in the thylakoid membranes of chloroplasts, the latter occur in the stroma of the chloroplasts (Danks *et al.*, 1983). In the light reactions, chlorophylls, the molecules of green pigment in the plant, absorb the visible radiation in the range of 400 –700 nm, and convert it to ATP (adenosine triphosphate) and NADPH (reduced nicotinamide adenine dinucleotide phosphate) via electron transport chains. These electron transport chains are arranged in a series of two photosystems, PS I and PS II, which are connected to the reaction centres P700 and P680 respectively (P stands for pigment; 700 and 680 are the maximum wavelengths absorbed in nm). The electrons released from excited P700 are used to reduce $NADP^+$ to NADPH (Danks *et al.*, 1983; Taiz and

Zeiger, 2006), and these electrons needed for the reduction of chlorophyll, are removed from water by PS II which are then donated to PS I.

During the electron transfer chain, protons are also transported across the membrane and the inside of the thylakoid lumen. This movement creates a pH difference which means creating energy which is used to make ATP (Blankenship, 2002).

The converted energy, ATP and NADPH, which are gained in the light reactions, are then used in the metabolism of CO_2 and the production of carbohydrates by a series of reactions called the dark reactions (Light independent reactions) (Danks *et al.,* 1983; Blankenship, 2002). The main and primary path of carbon metabolism is called the Calvin cycle which is usually found in all C_3 plants (where the first product of CO_2 fixation is a 3 carbon molecule) like wheat, barley, lentils and others, which form the majority of plant species (90 % of all plant species on earth) (Sage, 2005) and usually grown in temperate zones as crop plants (Hall and Rao, 1992; Blankenship, 2002). There are also some plant species, which are normally adapted to grow in tropical areas where temperature and light intensity are very high, which can fix CO_2 using two pathways: (1) initially, the CO_2 is fixed in the mesophyll chloroplasts by the enzyme phosphoenol pyruvate (PEP) and four carbon acids oxaloacetate and malate are produced and commonly called the C_4 pathway, and these acids are then decarboxylated in the inner bundle sheath cells with the subsequent release of CO_2 (2) the normal Calvin cycle then takes place in the bundle-sheath chloroplasts (Danks *et al.,* 1983; Hatch, 1987; Hall and Rao, 1992). CO_2 released into the bundle sheath cells is 3-10 times more concentrated than that of the ambient in this system (Hatch, 1987; Furbank *et al.,* 1989). Hence, CO_2 fixation rate in C_4 plant species, which form about 4% of all plant species (Ghannoum *et al.,* 2000), occur at a rate 2 to 3 times faster than that of C_3

plant species which only use Calvin cycle and therefore photosynthesis in C4 plants is considered more efficient than that in C_3 plants (Danks *et al.*, 1983). Additionally there is another pathway found in species of Crassulaceae, which are mainly succulent plants growing in the arid regions, called Crassulacean Acid Metabolism (CAM). In this pathway, the plants take in CO_2 at night and convert it to malate, and during the daytime next day, the normal C_3 cycle is active (Hall and Rao, 1987; Larcher, 2003).

The following section will only discuss C_3 carbon metabolism.

1.2.1. The Calvin Cycle

This process can be divided into three phases; carboxylation, reduction and regeneration. In the first step, CO_2 is bound to an acceptor ribulose 1,5 biphosphate (RuBP) and, by RuBP-carboxylase/oxydase (Rubisco), and two molecules of 3-phosphoglceric acid (PGA) are produced as the carboxylation product. In the reduction phase the overall reaction is the reduction of carboxylic acid to aldehyde (Blankenship, 2002). This occurs through a series of reactions in which PGA is reduced to triose phospate at the expense of ATP and NADPH. Twelve molecules of triose phospate are formed, of which two are taken out of the cycle to give rise to glucose, and the others continue to the last step in the cycle, regeneration (Figure 1.4) (Danks *et al.*, 1983; Larcher, 2003). During the regeneration phase of the Calvin cycle, a complex series of reactions occurs, in which triose phosphate is regenerated into ribulose 5-phophate (Ru5P), which in the phosphorylation step of this phase gives RuBP (Blankenship, 2002).

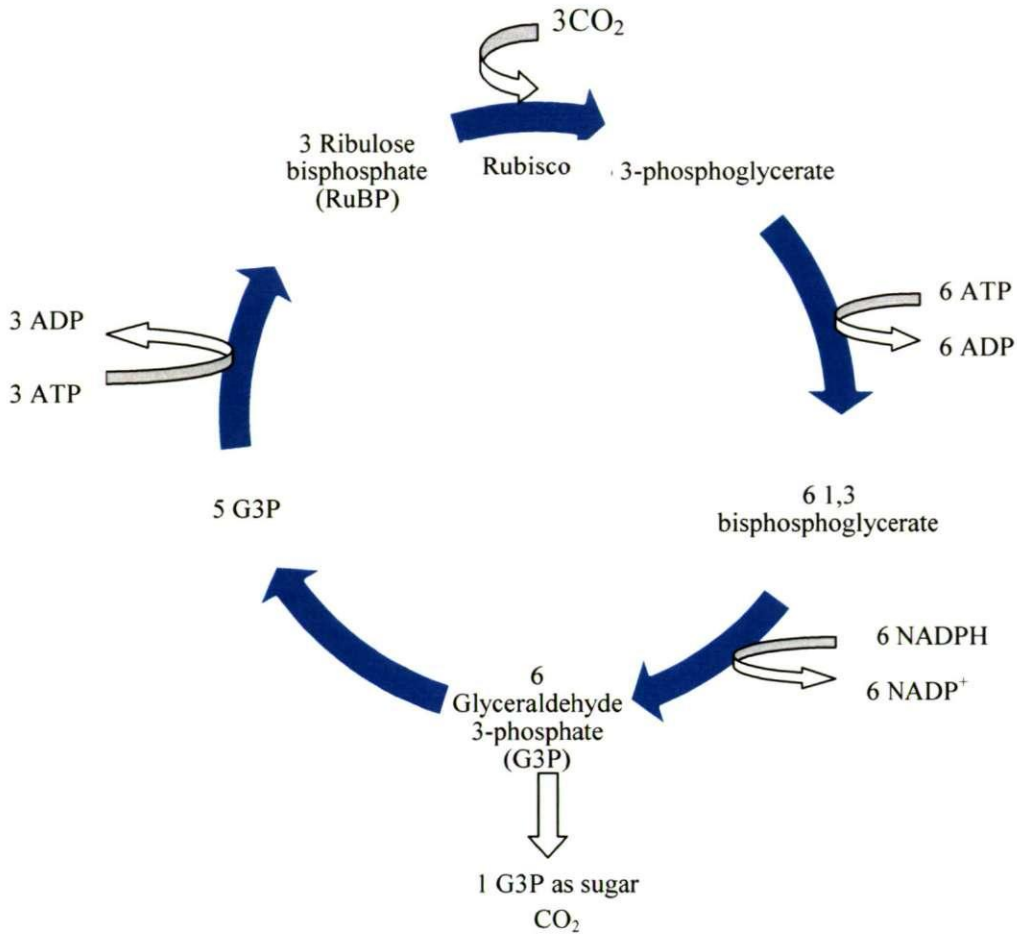


Figure 1.4. The Calvin cycle restructured according to Hall and Rao, (1992).

The fundamental and the crucial role of the enzyme Rubisco in life on earth is clearly demonstrated as it carries out the carboxylation step in carbon fixation (Fuller& Jellings, 2003). This enzyme however, has two distinct functions; carboxylation and oxygenation, with the latter being a significant drain on the plant resources as Rubisco reacts with oxygen instead of carbon dioxide and for about one third of the time oxygenation is carried out instead of carboxylation (Blankenship, 2002). In this activity, 2-phosphoglycolate and PGA are formed. The 2-phosphoglycolate inhibits the Calvin cycle, and it must be metabolized to PGA for the final recovery by the Calvin cycle, and the overall process, including oxygenation and recovery, is called photorespiration in

which O_2 is consumed in light and CO_2 is released (Heldt, 1997; Oliver, 1998; Douce and Heldt, 2000). The function of Rubisco as an acceptor for carboxylation and an acceptor for oxidation is regulated by the supply or abundance of CO_2 and O_2. A high concentration of O_2 favours photorespiration, and by contrast, a high concentration of CO_2 favours photosynthesis (Ball and Passioura, 1995; Larcher, 2003). Although Rubisco has a high preference for CO_2 over O_2, photorespiration is still a significant activity because of the much higher atmospheric concentration of O_2 (21%) compared with 0.035% for CO_2 (Blankenship, 2002). As a consequence, C_3 plants under natural conditions lose about 30% (range: 20 –50%) of photosynthetically acquired CO_2 in the form of photorespiratory CO_2. It has been hypothesised that RuBP carboxylase will be more efficient in conditions of higher CO_2 concentration in the atmosphere surrounding the plant (Larcher, 2003). Therefore, one strategy to increase photosynthesis and crop yield in C_3 plants has been to reduce photorespiration which can be achieved by lowering the O_2: CO_2 ratio in the atmosphere surrounding the plant (Danks *et al.*, 1983).

1.3. Some possible effects of elevated CO_2 on plants growth and productivity

The increase in the levels of carbon dioxide in the atmosphere has a positive effect on the agricultural yields for most crops, which are expected to rise as a result of the increased CO_2 concentration the so-called CO_2 fertilization (Houghton, 1997; White, 2001) and in this area, a great number of experiments have been carried out to study the response to controlled CO_2 conditions. Rogers *et al.* (1994), according to data taken from numerous experiments concluded that on average, a doubling of CO_2 levels leads

to an increase in yield of about 33%. The C_3 plants have been observed as the most responsive to elevated CO_2 (Allen, 1998). The terrestrial ecosystem model (TEM) forecasts that for doubled CO_2 levels with no climate change (i.e. no warming), the global net primary production NPP, which is the net amount of carbon captured by land plants through photosynthesis each year, will show an increase of about 16.3%. This response however, ranges from no increase for some northern and temperate ecosystems to 50% for deserts (Melillo *et al.*, 1993). The next section will discuss in more details some aspects of plant response to CO_2 enrichment conditions.

1.3.1. The effects of elevated CO_2 on photosynthesis in C_3 and C_4 plant species

Atmospheric CO_2 is the main source of carbon for the three photosynthesis pathways in terrestrial plants (C_3, C_4 and CAM), and where C_3 and C_4 are considered as the most common contributors to global primary productivity, CAM photosynthesis is usually considered of a less importance (Sage and Monson, 1999; Ehleringer, 2005). C_4 and CAM photosynthesis both have mechanisms that essentially lead to a higher CO_2 concentrations at the carboxylation site in RuBP (ribulose 1,5 biphosphate), and due to these mechanisms photorespiration is reduced (Ehleringer and Monson, 1993; Ehleringer, 2005). Unlike C_4 and CAM photosynthesis, the C_3 photosynthesis pathway does not have any CO_2 concentrating mechanism and depends only on CO_2 diffusion from the surrounding atmosphere into the site where carbon fixation takes place (Ehleringer, 2005). Therefore, the CO_2 concentration inside the leaves of C_3 plant species is about similar to that in the surrounding ambient. This level is however below the saturation level (about 1000 ppm) (Lindroth and Dearing, 2005) for the fixation

enzyme Rubisco (RuBP-carboxylase/oxydase) (Drake *et al.*, 1996; Lindroth and Dearing, 2005). Since, the carbon assimilation rate, directly affects all growth processes, plant functioning, and plant productivity, it is clear that the response of C_3 and C_4 plant species to the change in atmospheric CO_2 will not be the same (Ward, 2005). Higher atmospheric CO_2 concentration leads to higher levels of CO_2 at the carboxylation site in C_3 plant species and as a result photorespiration activities are reduced and thereby increasing the efficiency of the enzyme Rubisco leading to an overall increase in net photosynthesis (Bowes, 1991). On the other hand, the response to elevated CO_2 in C_4 plant species, which already have almost saturating levels of internal CO_2 that eliminates photorespiration, is generally assumed to be relatively negligible (Cure and Acock, 1986; Henderson *et al.*, 1995; Ghannoum *et al.*, 2000). In many C_3 plant species, the net assimilation rate increases with CO_2 enrichment conditions and for doubling CO_2 levels, an increase of up to 75% has been reported depending on the environmental conditions and the type of plant growth (Shaw *et al.*, 2005). Further stimulation of photosynthesis is also expected at higher temperatures which are predicted to be associated with higher atmospheric CO_2 levels (Sage and Sharky, 1987).

In contrast to the carbon fertilization effect, long term exposure to elevated CO_2 has also been reported to have a negative effect on photosynthesis with what so-called photosynthesis acclimation occurring. Photosynthetic acclimation is usually accompanied by a decrease in photosynthetic capacity (photosynthesis measured at saturating light and CO_2) (Chaves and Pereira, 1992) as a result of decreased carboxylation efficiency mainly due a reduction in the concentration of Rubisco in the leaf tissues (Drake *et al.*, 1996; Stitt and Krapp, 1999). But in most cases, the reduction in photosynthetic capacity did not lead to a reduction in the net photosynthesis, and

several reviews (Cure and Acock, 1986; Arp, 1991; Bowes, 1993; Curtis, 1996) concluded that elevated atmospheric CO_2 enhanced photosynthesis by about 30- 50%. Furthermore, for the plants to benefit from long term exposure to elevated CO_2, and to avoid down-regulation of photosynthesis, sustained strong carbon sinks are essential (Arp, 1991; Stitt, 1991; Medlyn and McMurtrie, 2005). Therefore, when carbon sink capacity to store the additional carbon is limited, under restricted growth conditions such as that at low temperature or low nutrient availability, photosynthetic capacity must decline in response (Drake *et al.*, 1996). For example, in pot experiments, small pots can restrict root growth limiting both, the sink size and the nutrient availability, and thorough consideration for pot size and rooting volume should be taken (Arp, 1991; Rogers *et al.*, 1994; Drake *et al.*, 1996). On the other hand, when growth is not limited such as that when new leaves are emerging or at the time of fruit filling, plants grown under elevated CO_2 may use the full photosynthetic capacity with little or no reduction (Drake *et al.*, 1996).

1.3.2. The effects of elevated CO_2 on stomatal conductance, water use efficiency and transpiration

Stomata are the main means by which plants adopt to their surrounding environment by regulating gas exchange, mainly CO_2 and water vapour, which means balancing photosynthetic performance with water usage (Chaerle *et al.*, 2005). Stomatal closing and opening reduces or increases stomatal conductance which in turn determines the rate at which CO_2 or water is exchanged (Roelfsema and Hedrich, 2005). Stomatal closure reduces the amount of water transpired, but at the same time reduces the

diffusion of CO_2 into the leaves which reduces the intercellular CO_2 concentration and consequently the photosynthetic rate. In this situation water use efficiency (WUE) which is the mass of carbon fixed per mass of water transpired (Shaw *et al.*, 2005), is increased but at the expanse of photosynthetic rate (Ball and Passioura, 1995; Cowan and Farquhar, 1977). CO_2 concentration plays an important role in regulating stomatal conductance, therefore, when atmospheric CO_2 levels increase, the intercellular CO_2 levels will increase in response but at reduced stomatal conductance (Ball and Passioura, 1995; Shaw *et al.*, 2005). On average a 40% reduction in stomatal conductance in C_3 and C_4 herbaceous species under doubling CO_2 concentration was reported (Rogers *et al.*, 1984; Morison, 1985). This response to elevated CO_2 which results in increased water use efficiency is especially important in arid and semi-arid ecosystems as the growth demand for carbon can be met with less water transpired (Shaw *et al.*, 2005).

Both, the increase in net photosynthesis and the reduction in transpiration rate, contribute to the increase in WUE under elevated CO_2, but while photosynthesis is the main contributor in C_3 plant species (90: 10, net photosynthesis: transpiration rate), the reduced transpiration rate is the main contributor in C_4 plant species at a rate of 27:73 for net photosynthesis: transpiration rate (Acock and Allen, 1985). In fact, although partial stomatal closure reduces transpiration rate, this leads to an increase in leaf temperature and eventually leads to an increase in the transpiration rate offsetting in this way the positive effect of stomatal closure (Idso *et al.*, 1993; Kimball *et al.*, 1995; Allen, 1998). Water use efficiency under CO_2 enrichment may also be offset by increases in total leaf area, which is a response frequently reported (Wolfe and Erickson, 1993), and therefore, the water loss per plant from the increased leaf area

under elevated CO_2 can be similar to that under ambient (Morison and Gifford, 1984). However, the positive effect of elevated CO_2 on WUE is still very pronounced. Kimball and Idso (1983) concluded from their assessment of 46 cited observations that transpiration rate decreases under elevated CO_2 by an average of about 34% at a doubling of CO_2 concentrations, and by coupling this with a 33% increase in economic yield, water use efficiency could double in response. Allen, (1998) demonstrated that water use efficiency of soybean plants grown at 800 μmol mol^{-1} CO_2 was more than twofold greater than that of plants grown at 330 μmol mol^{-1} CO_2.

Additionally, elevated CO_2 could also lead to changes in root characteristics especially in plants grown in water limiting conditions where more carbon could be allocated to the roots increasing in this way their ability to capture water. As a consequence, leaf-level WUE could be improved by the balancing of water uptake and canopy transpiration, and this could be a key mechanism in the survival of the plants when water is restricted (Chaves and Pereira, 1992; Shaw *et al.*, 2005). Overall, it is predicted that as a result of the enhanced water use efficiency by elevated CO_2, biomass production in areas where rainfall is low will still increase (Chaves and Pereira, 1992).

1.3.3. The effects of elevated CO_2 on plant productivity and quality

Clearly, the consequent result of the increased photosynthesis under CO_2 enrichment conditions is an increase in biomass production and normally an increase in the economic yield. Plant responses, however, differ between species and are also highly dependent on other environmental factors such as light, temperature, water and nutrient supply, humidity etc, and their interaction with elevated CO_2 (Rogers *et al.*, 1994). For

most crops, an average increase in economic yield of 30% to 40% has been reported under doubling levels of atmospheric CO_2 concentration (Kimball, 1983; Cure & Acock 1986). However, the growth stimulation observed is frequently much smaller than the photosynthesis stimulation, and one of the main causes for the modest increase is that the additional amounts of carbohydrates are not used by plants for growth but accumulated as starch in the chloroplast as a result of the feedback inhibition (Poorter, 1993).

In general, studies in controlled and field environments showed an increase in biomass accumulation in C_3 plant species in response to elevated CO_2 (Poorter, 1993; Drake *et al.*, 1996; Idso and Idso, 1994). Although C_4 plant species are expected to show little or no response to elevated CO_2 (Cure and Acock, 1986; Henderson *et al.*, 1995), results from different studies have been mixed. Whilst some studies showed no effect for elevated CO_2 on the growth of C_4 plants (Carter and Peterson, 1983; Cure and Acok, 1986; Ainsworth and Long, 2005), others have indicated that some C_4 species showed a positive response to elevated CO_2 (Ghannoum *et al.*, 1997; Wand *et al.*, 1999), and an average increase of 22–33% was reported (Poorter 1993; Wand *et al.*, 1999). Moreover, different functional plant groups showed different response when grown under high levels of CO_2 concentrations. For example, Reich *et al.*, (2001), in an experiment on grassland species from several functional groups and their response to elevated CO_2 and nitrogen availability, concluded that elevated CO_2 increased total biomass in forbs, legumes and C_3 grasses by 31%, 18%, and 9% respectively, but on the other hand the biomass production in C_4 grass monoculture was reduced.

Some suggest that the response in legumes, which are nitrogen fixing species, could be greater than non-fixing species mainly because the additional demand for nitrogen

induced by the enhancement of photosynthesis at high CO_2 concentrations could be supplied by the process of nitrogen fixation carried out in the nodules (Poorter, 1993; Soussana & Hartwing, 1996; Clark *et al.*, 1997; Reich *et al.*, 2001). Moreover, nodules also represent a strong sink for carbon, and as mentioned earlier strong carbon sinks are essential to plants to benefit from long term exposure to elevated CO_2, and therefore more carbohydrate could be transported to the nodules (Lewis *et al.*, 1994; Díaz, 1996). However, the suggestion that stronger response is expected in nitrogen fixing species was not supported in the work of Riech *et al.*, (2001).

The response of plant chemistry to elevated CO_2 is of a great importance and has been frequently investigated in numerous experiments (Lindroth and Dearing, 2005). In general, nitrogen and other nutrient concentrations of plants grown in CO_2 enrichment conditions are reduced (Wong 1979; Coleman *et al.*, 1993), but carbon storage compounds (starch) and defensive compounds (tannins) are increased (Lindorth, 1996; Poorter *et al.*, 1997). The response, however, varies between species, genotypes among species, and depends on recourses availability (Lindorth *et al.*, 2001). Poorter *et al*, (1997) in a study of 27 C_3 plant species concluded that there was a reduction in protein levels (average of 18%), total nitrogen (21%), and nitrate concentration (22%) in plants grown at high concentrations of atmospheric CO_2. Rogers *et al.* (1994) indicated that C:N ratio increases under elevated CO_2 which can affect herbivory. However, the effect on the quality of crop products consumed by man was not significant. Furthermore, in their work on soybean and corn at different CO_2 concentrations, they found no significant difference in the percentage of moisture, fat, protein, or crude fibre in the seeds. Bhattacharya (1993) in a study on sweet potato grown under various levels of

CO_2 also found no difference in the quality on the basis of consumer scores of sensory perception.

1.4. The interaction of elevated CO_2 with other environmental factors

1.4.1. The interaction with drought

Drought is always considered to be a major cause for crop productivity loss (Du *et al.*, 1996), and it is anticipated that in addition to the increase in CO_2 levels in the atmosphere, there will be climatic change leading to an increase in temperature and water stress condition in many areas (Tolbert and Zelitch, 1983; Christensen *et al.*, 2007). Therefore, the investigation of the interaction of CO_2 enrichment and drought is of a great importance.

As described earlier, higher concentrations of atmospheric CO_2 lead to increased photosynthetic rate, reduced stomatal conductance and increased water use efficiency (Ball and Passioura, 1995; Shaw *et al.*, 2005), and root growth and spread is also improved, which enables plants grown under water stress conditions to reach and capture more water (Idso and Kimball, 1992; Bhattacharya, 1993: Rogers *et al.*, 1994). For these reasons, it is anticipated that the agricultural yield of plants grown under water limited condition should be enhanced by elevated CO_2 (Morrison, 1993). This view has largely been supported by a great number of experiments (Idso and Idso, 1994; Clark *et al.*, 1999). In sweet potato plants grown under water limited conditions at different levels of CO_2, the yield (storage roots) and root: shoot ratio was greater under the higher

concentrations of CO_2 (Bhattacharya, 1993). Clark *et al.*, (1999) indicated that net photosynthesis in three pasture species; *Trifolium repens* (C_3 legume), *Plantago lanceolata* (C_3), and *Paspalum dilatatu* (C_4), which were grown at CO_2 concentration of 350 or 700 μmol mol^{-1} at ample or restricted water conditions, were increased by elevated CO_2. The increase in *Trifolium repens* was 50% at ample water conditions and when minimum soil moisture values were reached the increase was over 300%. Similarly, Clifford *et al.*, (1993) in their work on stands of Groundnut (*Arachis hypogaea* L.) demonstrated that net photosynthesis, dry matter production and pod yield increased under elevated CO_2 and the percentage increase was greater when water was limited. Manderscheid and Weigel (2007) reported that CO_2 enrichment increased biomass and grain yield in wheat by less than 10% under well watered conditions and by 44% under drought conditions. Similar conclusions were also found in wheat by Kimball *et al,* (1995), in cotton (Kimball and Mauney, 1993), and other plant species. Generally, CO_2 enrichment conditions enhanced growth and production in water stressed plants of several plant species and in many cases the increase was proportionally as large as or even larger than that when water was not limited (Gifford, 1979; Sionit *et al.*, 1980; Kimball *et al.*, 1993). From the growing body of literature, it can be concluded that the negative effects of drought can be mitigated by the expected increasing levels of atmospheric carbon dioxide.

1.4.2. The interaction with nutrient availability

The faster growth rates and increased biomass production at increasing levels of atmospheric CO_2 (Bhattacharya, 1993; Mauney *et al.*, 1993; Rogers *et al.*, 1994) can

consequently lead to a more rapid depletion of soil nutrients, and thereby nutrient limitations, nitrogen in particular can be a major cause in restricting production under CO_2 enrichment conditions over the time (Wong, 1979; Bazzaz, 1990; Newton, 1991; Bhattacharya, 1993). Stitt and Krap (1999) indicated that Photosynthetic acclimation usually comes with a reduction in nitrogen concentration in the plant tissues, will be more pronounced when nitrogen is limited.

Whilst plants response to elevated CO_2 and nutrient availability, particularly nitrogen, has been the aim of numerous studies (Stitt and Krap, 1999), different studies have shown different results. Some have reported a positive growth response to elevated CO_2 over a wide range of nutrient availability (Sionit *et al.*, 1981; Sionit, 1983; Cure *et al.*, 1988; Hocking and Meyer, 1991; Kimball *et al.*, 1993), whilst others have reported a reduction in the growth when nitrogen was limited (Goudriaan and de Ruiter, 1983; Arp *et al.*, 1998).This difference has been attributed to the difference between species, the degree of the nutrient deficiency or the way the fertilizer was added (Pettersson and MacDonald, 1994). The growth enhancement response to CO_2 enrichment was always shown to increase with the increasing levels of nutrients available (Sionit *et al.*, 1981; Patterson and Flint, 1982; Sionit, 1983; Hocking and Meyer, 1991). Kimball *et al.* (1993) indicated that under CO_2 concentration of about 650 μmol mol^{-1} compared to that of 330 μmol mol^{-1}, the seed cotton yield increased by 56 % and 74% in wet and dry conditions respectively when nitrogen fertilizer was added, and when there was no added nitrogen, there was still a positive response to elevated CO_2 and the seed yield increased by 54 % under wet conditions and 52 % under dry conditions. Conroy *et al.*, (1990) reported that although the phosphorus available in the soil was low, there was an increase in phosphorus uptake by pine species grown under high levels of CO_2.

Thus it appears that nitrogen use efficiency and nitrogen uptake are usually increased by elevated CO_2, but not at a very low nitrogen supply (Stitt and Krap, 1999). Kimball *et al,* (1993) indicated that there was a contradictory result between nutrient solution experiments and that of soil experiments. The growth response to elevated CO_2 when nitrogen was limited was greater in soil experiments even at levels considered harmful, which can benefit unfertilized vegetation, especially in third world countries and unmanaged ecosystems.

As mentioned earlier, nitrogen concentration in plant tissues usually declines under elevated CO_2, and this reduction is especially more obvious when nitrogen supply is limited. High nitrogen supply, on the other hand, led only to small or even non-significant reductions in the nitrogen concentration of the leaves of many species (Stitt and Krap, 1999).

Overall, there have been a huge number of experiments on the effect of elevated CO_2 on different crop species from different functional groups. However, until recently, there has not been any research on the leguminous crop lentils in spite of its great importance in the dry areas especially in the Indian Sub-continent and in dry areas of the Middle East. The response of this crop to elevated CO_2 and its interaction with drought and nitrogen availability was the main aim of this research. More details about this significant crop are given in the following section.

1.5. Lentil (*Lens culinaris* Medic)

Lens is a Latin word that describes exactly the shape of the seed of a cultivated legume now known as *Lens culinaris* which is a name that was given by the German botanist Medikus in 1787 (Cubero, 1981).

Lentil (*Lens culinaris* Medic) is classified in the genus Lens, which is in the sub family Papilionaceae and the family Leguminosae (Muehlbauer *et al.*, 1985). The primary gene pool of *Lens culinarise* comprises ssp. *culinaris* and its wild progenitor ssp. *orentalis* (Ladizinsky, 1993). There are three wild species in the secondary gene pool: *L .ervoides* (Brign.) Grand, *L. nigricans* (M.Bieb.) Gordron and *L. odemensis* Ladizinsky (Muehlbauer *et al.*, 1995).

The primary product of the cultivated lentil is the seed, which is a valuable human food product containing a high amount of protein (22- 34.5%), carbohydrate (65%) and other minerals and vitamins (Muehlbauer *et al.*, 1985; Yadav *et al.*, 2007), and in many countries, lentils are used as a meat substitute (Duke, 1981). However, while the seeds have large concentrations of lyceine, they are deficient in sulphur-containing amino acids, methionine, and cysteine. Therefore, a combination of cereal grains, which are rich in sulphur-containing amino acids and poor in lyceine, and lentils provides a very nutritionally well-balanced diet (Abu-Shakra and Tannous, 1981; Muehlbauer *et al.*, 1995). The seeds are mostly eaten as dhal in soups, and the flour can be mixed with cereal flour and used in cakes, breads and some baby food (Kay, 1979; Duke, 1981; Muehlbauer *et al.*, 1995). There are also some dishes in which the lentils are mixed with cereals (Muehlbauer *et al.*, 1995), and in some parts of India, the whole seeds are eaten

salted and fried (Kay, 1979). Young pods can also be used as green vegetables, and the seeds can be a source of starch for textile and printing industries (Duke, 1981).

Additionally, lentil residues can be used as a livestock feed as they have high average of protein and crude fibre (Kay, 1979). Furthermore, in the Indian subcontinent, lentil have been grown as a green manure in one year rotation with cereals, especially rice (Saxena, 1981).

Furthermore, lentils, as all legumes, have the capability of fixing atmospheric nitrogen through the symbiotic relationship with *rhizobia* bacteria (Islam, 1981). The process of nitrogen fixation in legumes is essential for both maintaining soil fertility and natural and agroecosystems productivity (Azam and Farooq, 2003). Lentils are usually nodulated by the *Rhizobium leguminosarum* bacteria group, and in general, using artificial *Rhizobium* inoculation with effective strains can increase both nodulation and yield in field grown crops of lentils. The magnitude of this response however, varies between cultivars, the *Rhizobium* strains used and with the soil type (Islam, 1981; Rennie and Dubetz, 1986). In fact, in well nodulated lentils, symbiotic nitrogen fixation could provide the plants with up to 92% of the nitrogen required for the growth (van Kessel, 1994). Additionally, after removing the grain yield, lentils can provide the soil with about 59 kg ha^{-1} of nitrogen, which improve soil nitrogen status (van Kessel, 1994). Because of the nitrogen fixation characteristic, legumes can be grown with minimum inputs of fertilisers which give them a significant role in agriculture (Bohlool *et al.*, 1994). Furthermore, lentil can be grown in rotations with cereals helping in breaking disease and insect pest cycles that accumulate in monoculture systems, contributing in this way to the sustainability of cereal-dominated cropping systems (Sarker *et al.*, 2002).

1.5.1. Origin and Distribution

L. culinaris (Medic) originated in the Near East and Asia Minor (Zohary 1972; Williams *et al.*, 1974; Ladzinsky 1979, 1993). Since its domestication, lentils have become one of the most important food crops in the semi-arid regions of the world, especially in the Indian sub-continent and in the dry areas of the Middle East.

Lentils are also sown in the USA, Bangladesh, Afghanistan, Pakistan, Morocco, Ethiopia, Egypt, Mexico, Chile, Peru, Argentina, Colombia and Spain (Kay, 1979: Muehlbauer *et al.*, 1985). Lentils are one of the few crops which can be grown in marginal agricultural areas without fertilisation, irrigation, herbicides or pesticides. In the major producer regions, there has been a significant shift towards cereals, with legumes being relegated (Muehlbauer *et al.*, 1995).

1.5.2. Plant Description

Plants of *L. culinaris* ssp. are annual bushy herbs, erect or semi-erect, divided into many branches with thin stems (Duke, 1981).

The plant height ranges from 15 to 75 cm depending on the genotype and the environmental conditions, but usually the plant height for the majority of genotypes ranges from 25 to 30 cm. The plant has a tap root with fibrous lateral roots (Saxena and Hawtin, 1981). Three rooting patterns may occur: (i) a highly branched shallow root system with a large number of tap root nodules; (ii) a slender deep- tap root; (iii) an intermediate type (Kay 1979). In the surface or upper layers of the soil, the tap root and the lateral roots have a great number of indeterminate nodules in various forms from round to elongated (Saxena and Hawtin, 1981).

The stem which is usually thin and weak is square and ribbed. From the main stem, the primary branches arise and secondary branches arise from these. The number of primary and secondary branches is affected by the genotype, the stand density and the environmental conditions (Wilson and Teare, 1972). The leaves are compound, pinnate and usually ending in a tendril or bristle with 4-7 pairs of alternate or opposite leaflets 1-2 mm long, which are oval and have sessile small stipules (Duke, 1981).

The flowers are solitary or multiple on peduncles which originate from the nodes of the plant. One to three (and rarely four), flowers can found in each peduncle (Muehlbauer *et al.*, 1985). A typical papilionaceous structure of legume flowers can be found in lentils. They are small, 4 to 8mm long, white, pale purple or purple blue and usually self-fertilised, but cross-pollination can occur sometimes (Kay, 1979). Each flower has a calyx of five equal sepals, and the corolla comprises two wings and two lower petals which lie within the wings, and the keel is formed when the lower petals are united in their lower margin. The stamens are diadelphous (9+1) with the upper stamen free and the ovary contains 1or 2 ovules (Muehlbauer *et al.*, 1980; Summerfield *et al.*, 1982).

The pods are oblong, laterally compressed, 6 to 20mm long with one or two (and rarely three) seeds (Saxena and Hawtin, 1981: Summerfield *et al.*, 1985). Seeds have a lens shape and are smooth, green, greenish-brown or light red with black speckles with their size ranging from 2-9 mm in diameter. The weight of 100 seeds varies from 2-8 g. The cotyledons are red orange, yellow or green, bleaching to yellow (Duke, 1981) (Plate 1.1).

1.5.3. The production of lentils

Lentil production has increased considerably since the 1980's (Oram and Agcaoili, 1994). The world production in 2005 exceeded 4 million metric tonnes with over 4 million hectare harvested (McNeil *et al.*, 2007). Over 90% of the global production takes place in North America, the Indian subcontinent, Turkey and other countries like Australia, Iran, Syria and China (McNeil *et al.*, 2007). According to the FAO, the largest producer in the years 2006 and 2007 was India followed by Canada and Turkey. The highest average yield in the same years however, was reported in China (2205.8 and 2400 kg. ha^{-1} respectively), whilst the mean yield in the largest producer, India, was only 629.14 and 949.10 $kg.ha^{-1}$ respectively (Table 1.2).

The major world exporters are Canada, which in 2005 exported 576,000 t, Turkey (118,000 t), Australia (108,000 t) and the United States of America (160,000 t). The major importers in 2004 were Bangladesh (110,000 t), Sri Lanka (93,000 t), Egypt (89,000 t) and Colombia (63,000 t) (McNeil *et al.*, 2007).

Plate 1.1. Lentil plant with its flowers and pods.

Lentil production in the years 2006 and 2007				
	Quantity (tonnes)		**Average yield (kg.ha^{-1})**	
	2006	**2007**	**2006**	**2007**
India	950000	1400000	629.14	949.10
Canada	692800	669700	1249.41	1258.30
Turkey	622684	580260	1415.51	1184.20
United States of America	235000	154584	1426.75	1294.8
Syrian Arab Republic	165000	165000	1137.93	1137.93
Nepal	157963	164694	832.69	870.50
China	150000	180000	2205.88	2400.00
Bangladesh	120000	119000	921.66	820.60
Iran	113225	115000	502.02	511.10

Table 1.2. The quantities and the average yields of lentils in the major producers in the years 2006 and 2007 reported by FAOSTAT. (Source. FAOSTAT. PRODSTAT, November, 2008) (http://faostat.fao.org/site/567/DesktopDefault.aspx?PageID=567).

1.5.4. Culture Conditions

1.5.4.1. Temperature: Lentils are well adapted to cool temperatures, and the crop is usually sown soon after the autumnal rains and grown in winter in the Mediterranean regions as well as in Pakistan and northern India. In extremely cold countries such as USA, Turkey, Canada, Chile and Argentina, lentils are seeded in spring, as the crop is incapable of surviving very cold winters (Hawtin *et al.*, 1980; Muehlbauer *et al.*, 1985).

Optimum temperatures for germination in lentils are reported to be 18-21°C, with a minimum of 15 °C, and a maximum of 27°C (Duke, 1981). The seeds however, can germinate over a wide range of temperatures in light or in darkness, and the optimum temperature varies between genotypes, age and size of the seeds. Germination in small seeded cultivars is usually faster than the larger ones at temperatures of 15 - 25 °C (Summerfield, 1981).

Warmer temperatures are required for vegetative development (Muehlbauer *et al.*, 1995), and temperatures around 24 °C are optimum for yields, but this varies among cultivars (Kay, 1979). For example, optimum temperature range for the cultivar "Large Blond" is 19-29 °C, and that for "Ancia" is 21-25 °C (Duke, 1981). Also, some lentil genotypes are responsive to cold vernalization which can lead to earlier flowering (Summerfield, 1981).

1.5.4.2. Moisture: About 95% of the lentils produced worldwide is from rain fed areas (McNeil *et al.*, 2007), and therefore, the amount and the distribution of the rainfall are major factors in determining the crop productivity (Summerfield, 1981). Kay (1979) indicated that the crop requirements of annual rainfall are about 750 mm, with dry conditions, before harvest. In West Asia and North Africa, the crop is grown in winter

(sown in Dec- Jan and harvested in May) with a yearly average rainfall of 300-400 mm, which occur mainly during the vegetative growth period. However, the rainfall is reduced considerably from March to May which coincides with the reproductive stage of lentils and this significantly reduces the yield (Silim *et al.*, 1993; Erskine *et al.*, 1994; McKenzie et al., 2007). In general, lentils have a moderate tolerance to drought but the degree of tolerance varies among cultivars. Small seeded cultivars showed greater tolerance to drought mainly because they mature earlier than larger seeded cultivars, and in this way they avoid the stress (Muehlbauer *et al.*, 1995). Lentils are very sensitive to water logging and usually the damage caused by water logging can be greater than that of drought (Duke, 1981; Summerfield, 1981). In general lentil yield increases considerably with irrigation, but special consideration should be taken not to overdo the irrigation because of the crop high sensitivity to water logging (McKenzie *et al.*, 2007).

1.5.4.3. Soil: Lentils can be grown in many types of soils ranging from light loams and alluvial soil to black cotton soils, and they grow best on clay soils (Duke, 1981). The crop is frequently grown in marginal lands with poor fertility, low rain fall, and with pest and weed competition (Summerfield, 1981; McKenzie *et al.*, 2007). Lentils are very sensitive to soil acidity and a minimum pH value of 5.65 is required for maximum yield and the yield is considerably reduced below this value (Mahler and McDole, 1987). In addition, nodulation can be restricted in soils with high or low pH because of a direct effect on the survival of the *Rhizobium* bacteria (Islam, 1981). Furthermore, lentils' response to soil salinity differs between the growth stages, and while germination and seedling emergence is not affected by NaCl for values up to 7.9 dS m^{-1}, the total dry matter was reduced by 48% and seed yield by 92% when salinity increased

from 1.3 to 5.3 dS m^{-1} (Ayoub, 1977). Additionally, lentil plants are considered to have a moderate tolerance to alkaline soils (Kay, 1979).

1.5.4.4. Day length: In general, lentils are long-day plants and flowering occurs sooner in longer (16-24 h) compared to shorter (6-12 h) photoperiods, but some cultivars are day-neutral (Kay, 1979; Summerfield *et al.*, 1985).

1.5.5. Planting Procedures

In order to achieve the best yields, seeds with greater than 90% germination and treated with seed protectants, are recommended. When planting in the field for the first time or after a long time without lentils, seeds should also be inoculated with the proper strain of *Rizobium leguminosarum* (Muehlbauer *et al.*, 1995).

The land should be ploughed and harrowed and herbicide (Muehlbauer *et al.*, 1995). The seed may be broadcast or planted in rows 25- 30 cm apart to a depth of 1.25-6.25 cm depending on the seed size (small seeds shallower) and the available soil moisture (Kay 1979). The crop emergence can be faster when planting on the south-and east-facing slopes as they get warmer during the early stage of the growing season (Muehlbauer *et al.*, 1985). Seeding rates vary according to genotypes, for example 15 kg ha^{-1} for microsperma types (seeds of 2-6 mm in diameter) was used in northern India, and 115 kg ha^{-1} for macrosperma types (6-9 mm in diameter) was used for irrigated crops in Egypt. A seeding rate of 125 kg ha^{-1} was used for some cultivars in northern Syria (Oweis *et al.*, 2004), and a seeding rate of 65-80 kg ha^{-1} is recommended for "Brewer", the most common cultivar grown in the Palous region in the USA (Hawtin *et al.*, 1980).

Some fertilisers can be applied to achieve optimum growth and yield, and the rates recommended are:

Molybedenum : added as a seed dressing at 35 g ha^{-1} .

Sulphur: 17 to 22 kg ha^{-1} applied to other crops grown in rotation with lentils.

Phosphorous: 44-66 kg ha^{-1} should be applied to soils containing a phosphorus concentration of 4ppm or less. (Muehlbauer *et al.*, 1995).

Potassium: 22 kg ha^{-1} of K_2O on sandy or severely eroded soils is recommended to improve both yield and cooking qualities (Wassimi *et al.*, 1978).

Nitrogen: seldom applied to the well nodulated lentils, but when seeded early into cool and wet soils, a small starter of 10-25 kg ha^{-1} inorganic nitrogen will be advantageous before the significant symbiotic dinitrogin fixation begins (Saxena, 1981).

Weed control, mechanical or chemical, is important to improve the production of lentils as they are weak competitors (Muehlbauer *et al.*, 1995).

Lentil crops are harvested within 80-110 days with early cultivars, and within 125-130 days with late cultivars (Duke, 1981). The harvesting should not be delayed since lower pods shatter easily resulting in seed losses (Kay, 1979). The crop is usually harvested by hand in most middle-eastern countries, and as a consequence, the high cost of labour is a major factor in the decrease of lentil production in these areas, except Turkey (Khyrallah 1981; Haddad and Arabiat 1985).

Drought and heat stress are major environmental factors which limit lentil yields particularly in the Middle East region (Erskine, 1985).

Other factors limiting lentil yields are insects, diseases and weeds which need to be controlled (Muehlbauer *et al.*, 1985).

The main insects which attack and damage lentils during development and storage are: seed corn maggots, leaf weevils, thrips, lygus bugs, bruchid beetles and lepidopteran pod borers.

The major diseases which cause serious problems for lentils are: root rot and wilts, rust, ascochyta blight, seed borne fungi and viruses (Muehlbauer *et al.*, 1995). From the weeds, Broomrape is a problem, especially in the Mediterranean and the Near East (Muehlbauer *et al.*, 1985).

Research, particularly by ICARDA (International Centre for Agricultural Research in the Dry Areas) is trying to produce cultivars which tolerate stress conditions and which resist major diseases and pests. It is also seeking to improve cultivars suitable for mechanical harvesting (Muehlbauer *et al.*, 1985).

1.6. Methods used in CO_2 enrichment experiments

Different systems have been used to study the effects of CO_2 enrichment conditions on plants and these mainly include:

- Controlled environment in closed systems (growth rooms, greenhouses).
- Open top chambers.
- Free air carbon dioxide enrichment systems (FACE).

Other methods such as the use of genetically manipulated plants for perturbing the C/N balance of plants, and the use of natural CO_2 vents have also been used but on relatively

limited scale (Schulze and Mooney, 1993). Each of the methods has advantages and disadvantages, and usually the objectives of the study, its scale and the details required are the main derive in determining the methodology to be used (Schulze and Mooney, 1993). This section will briefly describe the use, advantages and disadvantages of the main 3 methods used in CO_2 enrichment studies.

1.6.1. Controlled environment in closed systems

Growing plants within controlled environments allow researchers to study the effects of each environmental factor (CO_2, temperature, water input, nutrients, photoperiod etc) individually and in combination with other environmental factors. From such studies, the understanding of the mechanism of interactions can be inferred and models for predicting the effects of climate change on plants can be developed. These models however, have to be regularly tested against data from field-like studies and refined accordingly (Gifford and Rawson, 1993). Systems used in CO_2 controlled conditions studies vary from highly accurate computer controlled chambers, which allow for different combinations and seasonal and diurnal variation of the different environmental components (Payer *et al.*, 1993), and automated CO_2- controlled, long term greenhouse (CO_2LT), to other relatively less accurate systems such as semi-controlled green houses, portable temperature gradient tunnels (Gifford and Rawson, 1993), solar domes (Cotrufo and Ineson, 1996), and other systems designed to study the above and below-ground gas exchange and carbon balance of the vegetation and called the so-called Wageningen Rhizolab (van de Geijn *et al.*, 1993).

The main advantages of this system are the precise and constant control over all or certain factors while varying others; they are location, time and season independent (Schulze and Moony, 1993).

On the other hand, disadvantages include that enclosed controlled systems are not often used for in-situ studies because of the high cost and complexity which limit the number of replicates and treatments in factorial experiments (Leadley and Drake, 1993); size restrictions also limit the number of replicates in the controlled facilities; in most chambers light intensity is usually low and there is a difficulty to control CO_2 concentration due to localized pollution or when experimenters enter the facility (Schulze and Moony, 1993). Controlled environments also have a high demand for energy and so they are more costly to use in remote areas (Leadley and Drake, 1993).

1.6.2. Open top chambers

Open top chambers (OTCs) are the most common method used in CO_2 enrichment studies, especially in long term field studies (Leadley and Drake, 1993). Most of OTCs, which are mainly developed to reduce the difference in light and temperature between enclosed chambers and the field, are cylindrical in shape without a top, and they are usually placed over an area of field vegetation (Jones *et al.*, 1993). However, square chambers were also used (Kimball *et al.*, 1983), and they are cheaper to construct but they may not as strong to withstand a stormy weather as the round type chambers (Rogers and Dahlman, 1993). In CO_2 enriched experiments, a fan blows air mixed with

pure CO_2 at a controlled rate to maintain a target concentration for CO_2 into the lower part of the chamber and the air will be expelled out of the open top (Jones *et al.*, 1993).

The main advantages of using OTCs are that the cost of construction, operation and maintenance are relatively low and this allows for more chambers to be installed and used in well designed experiments (Schulze and Moony, 1993). Also they can be converted to enclosed chambers and gas exchange measurements can be taken, and the impact of the chamber on the microenvironment (the chamber effect) on plant growth is relatively small (Leadley and Drake, 1993).

The major disadvantages of open top chambers are that the CO_2 concentrations are more variable than enclosed systems and in fact OTCs neither represent controlled environments nor natural environments (Leadley and Drake, 1993; Schulze and Moony, 1993); irradiance is generally reduced by about 15% and temperatures inside the chambers tend to be higher than outside by up to 2°C (Leadley and Drake, 1993). Furthermore, the enclosure and the forced air cause internal gradients, and watering systems and the uneven distribution of rain water can be a problem. It is also difficult for OTCs to be used for studies on large trees (Lee and Barton, 1993; Schulze and Moony, 1993).

1.6.3. Free air carbon dioxide enrichment system (FACE)

Free air carbon exposure systems (FACE) have been developed in order to study the effects of elevated CO_2 on vegetation and natural ecosystems, and the exchange of carbon between the biosphere and atmosphere in the open air where the natural

environmental variables are not disturbed (Hendrey *et al.*, 1993; Hendrey *et al.*, 1999). In principle, the FACE technology consists an array of vertical or horizontal vent pipes which release the CO_2 enriched air or pure CO_2 gas at the edge of the vegetation plot (Ainsworth and Long, 2005). The system was successfully used in different crops such as wheat (Wall and Kimball, 1993), cotton (Hendrey *et al.*, 1993), and small trees plantations (McLeod, 1993; Kimball *et al.*, 2007).

The major advantages of the FACE system in CO_2 enrichment studies are that the system overcomes the disadvantages of controlled and open top chambers and the so called the chamber effect (McLeod, 1993). They also allow for large areas to be studied without interfering with the natural environmental conditions where the direct and indirect effects of elevated CO_2 on different plant species and the indirect effects on soil can be observed thereby providing provide the only possibility system for studying the ecosystem response to elevated CO_2 (Schulze and Moony, 1993).

Major disadvantages of the FACE system include the high cost of construction, operation, and manpower required. For example, the costs for the construction and operation of the Liphook open-air fumigation system for young forest trees based on 1990 prices are £200,000 for equipment and installation and another £140,000 per annum for electricity and staff salaries (McLeod *et al.*, 1992). With such costs therefore, it is difficult to perform enough replicates in each experiment (Hendrey *et al.*, 1993; Schulze and Moony, 1993). Furthermore, because the open- air plots are usually islands of elevated concentration within an area of lower concentration the so called "island effect" develops, which has physical and biological disadvantages. The transpiration rate of the surrounding vegetation at ambient level could be higher than that at elevated CO_2 plot which may influence the airflow and humidity within the FACE plot. This

"island effect" may also concentrate mobile organisms which might not be the true representative of the global effect of CO_2 enrichment (McLeod, 1993).

Choosing any of the previous methods will be mainly dependent on the aim and the scale of the study and in general if constant climatic and soil conditions are required, growth rooms are the most appropriate, and if the growth and productivity of a single species is the aim of the study, then open top chambers could be the ultimate choice. Open top chambers can also be the choice if the community interaction in low height vegetation to be studied. However, for studies at the ecosystem level where large area can be treated and different interactions without any alteration to the environmental conditions can be observed, the FACE system is the only alternative (Schulze and Moony, 1993).

1.7. Aims of the study

The main aims of the study are:

- Investigate the effect of atmospheric CO_2 enrichment on the growth and nodulation of water stressed and non-stressed lentils.
- Investigate the effects of elevated atmospheric CO_2 and different levels of nitrogen fertilizer on the growth and nodulation of lentils.
- Investigate the effect of elevated CO_2 on nitrogenase activity in lentils using acetylene reduction assay on intact plants.

The objectives are explained later in each relevant chapter.

2. Chapter 2

General Materials and Methods

Materials and methods used for each experiment are explained in the appropriate chapter and a general description for materials and experimental designs used are given in this chapter.

2.1. Plant materials

Following communication with the International Centre for Agricultural Research in the Dry Areas (ICARDA), Aleppo, Syria, two cultivars of lentils, ILL7979 and ILL6994, were recommended and provided for use in drought trials. The cultivar ILL7979 is a dehydration tolerant line (personal communication Ashutosh Sarker, Lentil Breeder at ICARDA). This cultivar is still under experimentation in Syria, and it has also been used by the Centre of Legumes in Mediterranean Agriculture (CLIMA) in farmer's trails in Nepal (CLIMA, 2006).

The cultivar ILL6994 is registered in Syria as Idlib3 and it is a breeding line developed at ICARDA from a cross between the female parent ILL 99, a Moroccan landrace, and the male parent ILL 5588 which is an elite line developed through pure line selection from a Jordanian landrace population. The line was developed following a bulk-pedigree method and was included in the international testing program as FLIP 90-25L (El-Ashkar *et al.*, 2004). It avoids terminal drought stress by early maturation. This cultivar is also proven to be a wilt resistant cultivar. Idlibe 3 is of an erect habit and is suitable for mechanical harvesting (El-Ashkar *et al.*, 2004). Average yields of about 1010 kg ha^{-1} and 1296 kg ha^{-1} have been reported in the period from 1992/93 to 1994/95 and the period from 1995/96 to 1997/98 respectively.

Germination tests showed that germination potential exceeded 90% in both cultivars.

2.2. Controlled CO_2 chamber designs

Two types of growth chambers (open top and enclosed chambers) were constructed and used throughout the investigation. The original open top chambers proved to be rather unreliable and CO_2 leakage between chambers was detected. Therefore, a new design of tightly enclosed ventilated chambers was adopted and new chambers were constructed for the rest of the experiments. Both types of chambers were constructed at the University of Plymouth by technicians Peter Russell and Ashley Noyce.

2.2.1. Open top chambers

These growth chambers were 1 m x 1 m x 2 m (width × length × height) compartments built in a large 4m × 4m phytotron, and each compartment contained a side that could be opened as a door. In the CO_2 enriched compartments, a CO_2 supplementation pipe (perforated polythene) was connected to the chambers, and the supplementation was achieved using cylinders of compressed CO_2 (BOC gases) coupled to a EUROTHERM IRGA type 808 controller dosing device (EUROTHERM, Ltd) which constantly coupled the air in the chamber and pulsed CO_2 from the bottled gas to a set point of 750 μmol mol^{-1} measured within the chamber.

2.2.2. Enclosed chambers

These chambers were 60 cm × 60 cm × 80 cm (width × length × height) constructed from Lexan Excell D Polycarbonate sheets (Gilbert Curry Industrial Plastics Co Ltd) (Plate 2.1) and located in a glasshouse.). At the rear side of each chamber, two pipes were connected, one pipe delivered the air into the chamber, and the other one carried

the air out in order to maintain air circulation inside each chamber. In the ambient chambers, the air was brought from outside the glasshouse where one or two main pipes (depending on the number of chambers) drew the air from outside and then using T shaped pipes, air was distributed to each chamber. For CO_2 enrichment, cylinders of compressed CO_2 (BOC gases) were coupled to an EUROTHERM IRGA type 808 controller dosing device (EUROTHERM, Ltd) which pulsed CO_2 from bottled gas to a set point of 750 μmol mol $^{-1}$ measured in a mixing chamber containing baffles to ensure a thorough mixing of the incoming air with the CO_2 stream. The air mixture was then distributed to the "elevated" chambers through pipes connected to the main pipe coming out of the mixing chamber. (Note; in the nitrogen interaction experiment, the mixing chamber was not used). Pipes coming out of the elevated chambers were re-connected to a single pipe again and vented to the outside using a constantly running extractor fan (computer cooling fan) to draw the air out. Another similar extractor fan was used to draw the air out of the ambient chambers. The pipes used were Superflex PU R Anti-abrasive which operate between -40 °C and + 90 °C (Teignflex, Heathfield Industrial Estate, Newton Abbot, UK).

To ensure maximum insulation and prevent any leakage between chambers, a long self-adhesive 9 mm wide draught strip was used around the doors fixed to the front of each chamber.

2.3. Pots description

Two types of pots were used throughout the investigation. Ten litre square pots (22.5 cm width × 22.5 cm length × 23 cm height) were used in the open top chambers, while in the enclosed chambers the pots were constructed from cylindrical polypropylene

drainage pipe 30 cm high × 10 cm in diameter. A net material (mesh diameter approx 1 mm) was fitted to the bottom of each pot to ensure retention of the growth medium whilst providing free drainage of the pot. Each pot was then placed in a graduated clear plastic beaker so that the level of the drainage water could be monitored and watering adjusted accordingly).

2.4. Data loggers and data handling

Telaire ™ monitors were used to measure CO_2, temperature and relative humidity inside the chambers at 15 minute intervals, and data logged to Hobo™ dataloggers. These monitors were calibrated before use in each experiment, and periodically checked during the experiments. Data were offloaded from the dataloggers every 1-2 weeks using Boxcar™ software, and then the required data exported to a Microsoft Excel file. The complete data over the whole growth period for each logger (1 per chamber) were transferred to one Excel sheet where the daily average for each of the measured parameters was calculated. The calculated averages of all chambers for each parameter were then assembled and presented in one chart. A typical 3 month experiment generated 9000 items of data for each chamber for each of the 3 parameters.

Plate 2.1. The enclosed growth chambers at the end of an experiment. The mixing chamber is on the left of the picture and a Telaire monitors visible in the first chamber.

2.5. Growth media

Two types of growth media were used to grow the plants in the different experiments.

2.5.1. John Innes No. 2 loam based compost was used in the first experiment which investigated the interaction of elevated CO_2 and drought on the growth and development of lentils (chapter 3). Using this medium, it was difficult to separate and investigate the root system and the nodules attached to it. Therefore a pilot experiment

was conducted to choose a medium to facilitate easy root separation for root nodule observation.

2.5.2. A pilot experiment to choose a suitable rooting medium to facilitate root-nodule recovery and assessment

In this experiment the seeds of Idlib 3 were sown in three different media, perlite, sand, and sand mixed with compost (1:1).The seeds were sown in cylindrical pots 10 cm diameter x 30 cm height. One pot of each medium was sown with seeds inoculated with a Rhizobium strain *R. leguminosarum* (ILAR2D) (Soya UK Ltd) (the inoculum was mixed with seeds immediately before sowing), and a second pot with seeds without inoculation. Two seeds were sown in each pot and thinned later to one plant per pot. Thus the experimental design was 3 x 2 factorial and was replicated 3 times in randomised blocks. The experiment was set-up in an unheated glasshouse located on the roof of Davy building at Plymouth Campus. The plants were irrigated using a standard hydroponic' "Optimum™" growth solution (Growth Technology Ltd), which provided the plants with a complete nutrient profile. The plants emerged 7 days after sowing and flowered after 38 - 44 days. At 48 days from sowing, the first replicate was harvested and measurements of nodule number per plant were taken. At 55 days, the other two replicates were harvested and the same measurements taken. Data were analysed using Minitab 13.1, Analysis of Variance of Balanced ANOVA.

Visual observation of the plants during growth showed clearly that the plants growing in the perlite were bigger with greater leaf area and the worst were those growing in sand/compost medium. The weak growth in sand/compost mixture was attributed to a water logging problem although the plants were not extensively watered, the hydraulic

conductivity of this mixture soaked up water moving from the other pots on the same tray. Of the three media used, the perlite medium allowed the easiest root separation with a minimal root loss compared to the sand and sand compost mixture media (Plate 2.2).

Root nodule number per plant was over three times higher in the rhizobium inoculated plants in the perlite medium compared to all other treatments. The next best medium was the sand and the worst was the sand/compost.

This rank order of the media was the same with non-inoculated treatments where the Perlite treatment was able to show the same level of root nodulation as the inoculated sand treatment. The source of the rhizobium for the nodulation on the roots of non-inoculated plants was not investigated but could have originated from naturally occurring rhizobia or from cross-contamination from the inoculated pots. Since both the perlite and the sand used were sterilised before sowing, then cross-contamination is the most likely explanation.

Plate 2.2. Photograph to illustrate the extent of root system recovery from the 3 media (Left – Perlite, Centre – Sand, Right – Sand/Compost).

Expanded perlite (hereafter referred to as perlite) is a mineral manufactured from a silicaeous volcanic rock and is an amorphous glass formed when volcanic lava cools very quickly trapping small amounts (2-5%) of water, and when crushed and heated to about 1000 °C, the trapped water vaporises and puffs out the softened granules up to 30 times their original volume to form a white glass foam (Wilson *et al.*, 1984). Perlite is physically stable and chemically inert, and the granules can hold large amounts of readily available water, but at the same time, because it is free draining, it is well aerated (Wilson *et al.*, 1984; Day, 1991). Perlite can provide an optimum growth medium, and in particular in hydroponic culture it improves rooting enhancing vigorous growth, mainly because of its optimum balance of air and water which largely reduces or prevents water logging. It also, minimizes any root damage when transferring plants or when making a root check (Day, 1991; Anon, 2002). Furthermore, because of its white colour, it can reflect light up under the plants' leaves which can also enhance

growth (Anon, 2003). Using perlite in this experiment has provided an optimum medium for lentils' root and foliage growth, and this was clearly reflected on root nodulation.

2.6. Hydroponic solutions

Two types of hydroponics solutions' were used in experiments where perlite was used.

2.6.1. A Standard hydroponics' growth solution ("Optimum Grow twin pack" Growth Technology Ltd, Unit 66, Taunton Trading Estate, Norton Fitzwarren, Taunton, Somerset TA2 6RX) was used in the second drought interaction experiment.

2.6.2. A complete Hoagland's solution minus nitrogen was used in the nitrogen interaction experiment. Additions of different levels of nitrogen were achieved by adding different amounts of ammonium nitrate to the solution where it was required. The following recipe was used in producing the complete Hoagland's solution minus nitrogen:

Hoagland Solution minus nitrogen (per litre of nutrient solution):
10 ml of 0.05 M monocalcium phosphate
200 ml of 0.01 M calcium sulphate dihydrate
5 ml of 0.5 M potassium sulphate
2 ml of 1 M magnesium sulphate
1 ml of micronutrient stock solution
1 to 5 ml of iron chelate stock solution

Micronutrient stock solution per litre:
2.86 g boric acid
1.81 g manganese chloride - 4 hydrate
0.22 g zinc sulphate - 7 hydrate
0.08 g copper sulphate - 5 hydrate
0.02 g 85% molybdic acid

When diluted 1:1000 the micronutrient stock solution provides the following:

Boron 0.5 mg L^{-1}
Manganese 0.5 mg L^{-1}
Zinc 0.05 mg L^{-1}
Copper 0.02 mg L^{-1}
Molybdenum 0.01 mg L^{-1}

(Hershey, 1994: Hershey, 1995).

In the experiments where hydroponic solutions were used, regular measurements of electrical conductivity (EC) of the water solution in the pots were taken every 3-4 days to ensure that no hazardous levels of salt accumulation occured, and to maintain the levels of conductivity at a similar level over the whole growth period. The measurements indicated when the addition of extra nutrient solution or fresh water needed to be added to the pots. Similarly the pH levels of the solutions were also monitored regularly, and in general, the values ranged between 5.84 and 7.19, but at one stage (03/06/2006) in the second drought interaction experiment, the pH levels dropped to about 4.5 and was corrected using potassium hydroxide.

2.7. Growth measurements taken

2.7.1. Leaf area

A Delta-T Image Analysis System- type (DIAS^{TM}) (Plate 2.3) was used in leaf area measurements. The device contains a camera which takes images of the leaves and measures their area after calibration. The leaves were flattened and placed under the focal plane of the camera and measurements taken and stored electronically.

The area of a sub sample of 5 to 7 leaves from each plant was measured using the leaf area meter, and then these leaves were dried and weighed and the specific leaf area

calculated (SLA = area/dry wt), The remainder of plant leaves were also dried and weighed and the leaf area of the plant was calculated as follows: Plant leaf area = the whole plant leaves (sub sample + remainder) dry weight × SLA. The leaf area index was then calculated as: whole plant leaf area/the surface area of the growing pot.

Plate 2.3. Leaf area meter (Delta-T Image Analysis System- type (DIAS^{TM})).

2.7.2. Dry weight

All plant materials were oven dried to constant weight for 48 hours in a Gallenkamp forced air drying oven and then weighed using an electronic 3 decimal place top-pan balance. The samples were then ground using a small hammer grinding mill in preparation for nitrogen and phosphorus analysis.

2.7.3. Electrical conductivity

Electrical conductivity is a measure of a material's ability to conduct an electric current and measurements of electrical conductivity in solutions indicate the amounts of dissolved polar solids in the solution and the higher these amounts, the higher the conductivity (Erikson, 2002).

An electrical conductivity meter type CMD8500 (Plate 2.4) was used in taking measurements of electrical conductivity of water solution samples collected from pots where plants were fed with hydroponics solutions.

Before taking any measurements, a calibration against a standard solution was undertaken and care was taken to ensure the measuring probe was immersed into the stirred test solution and well rinsed in distilled water in between subsequent measurements.

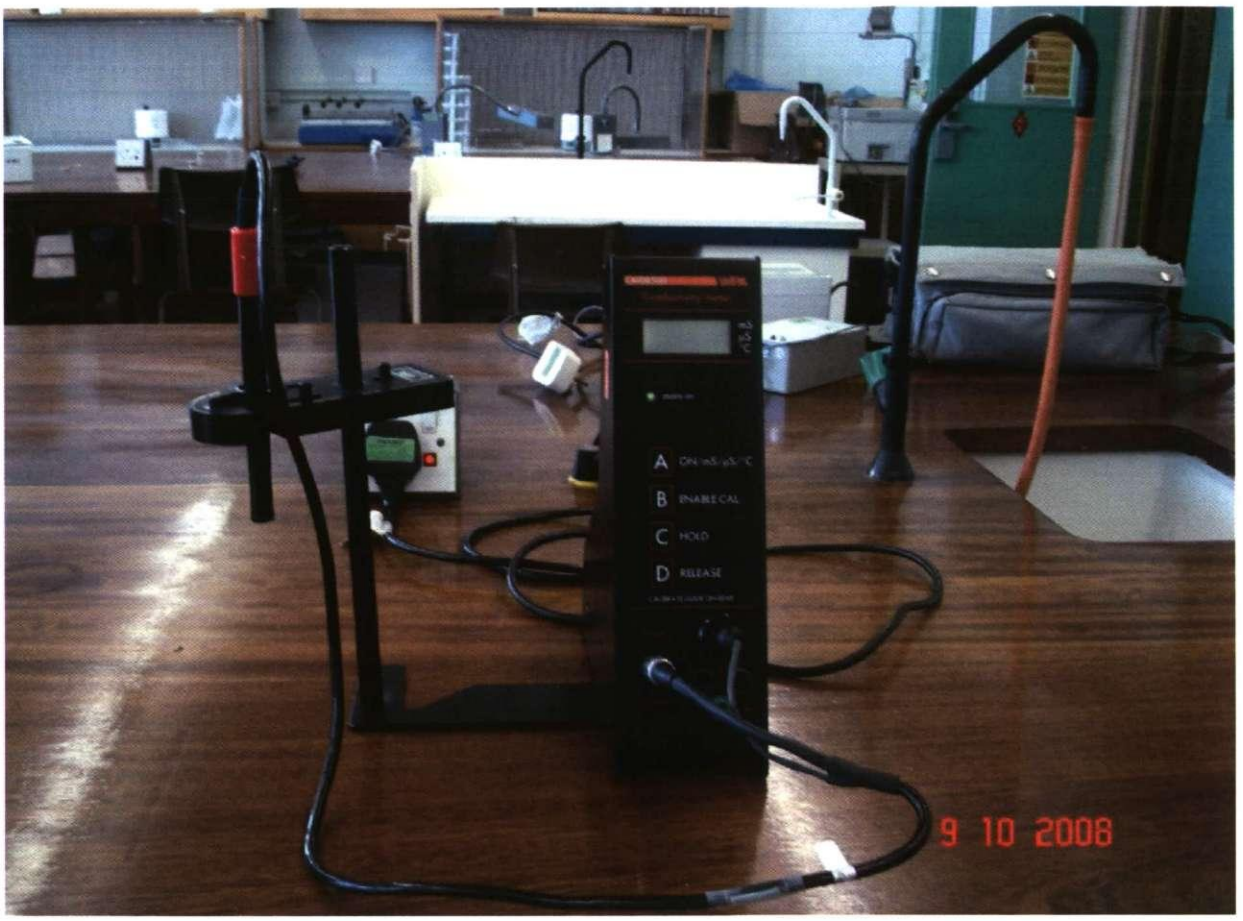


Plate 2.4. Electrical conductivity meter used in the analysis.

2.7.4. pH

The pH of the hydroponic solution affects the rate of absorption and solubility of nutrients in the solution, and maintaining pH levels within the limits for crop production is essential (Day, 1991). pH measurements in the solutions were taken using a pH meter (Denver Instrument Company, USA) (Plate 2.5). In the process, the electrode was immersed in the solution sample and gently stirring until a stable value was reached. Before the first measurement and periodically between measurements, standardization using standard buffer solutions for pH values of 4 and 7 was undertaken.

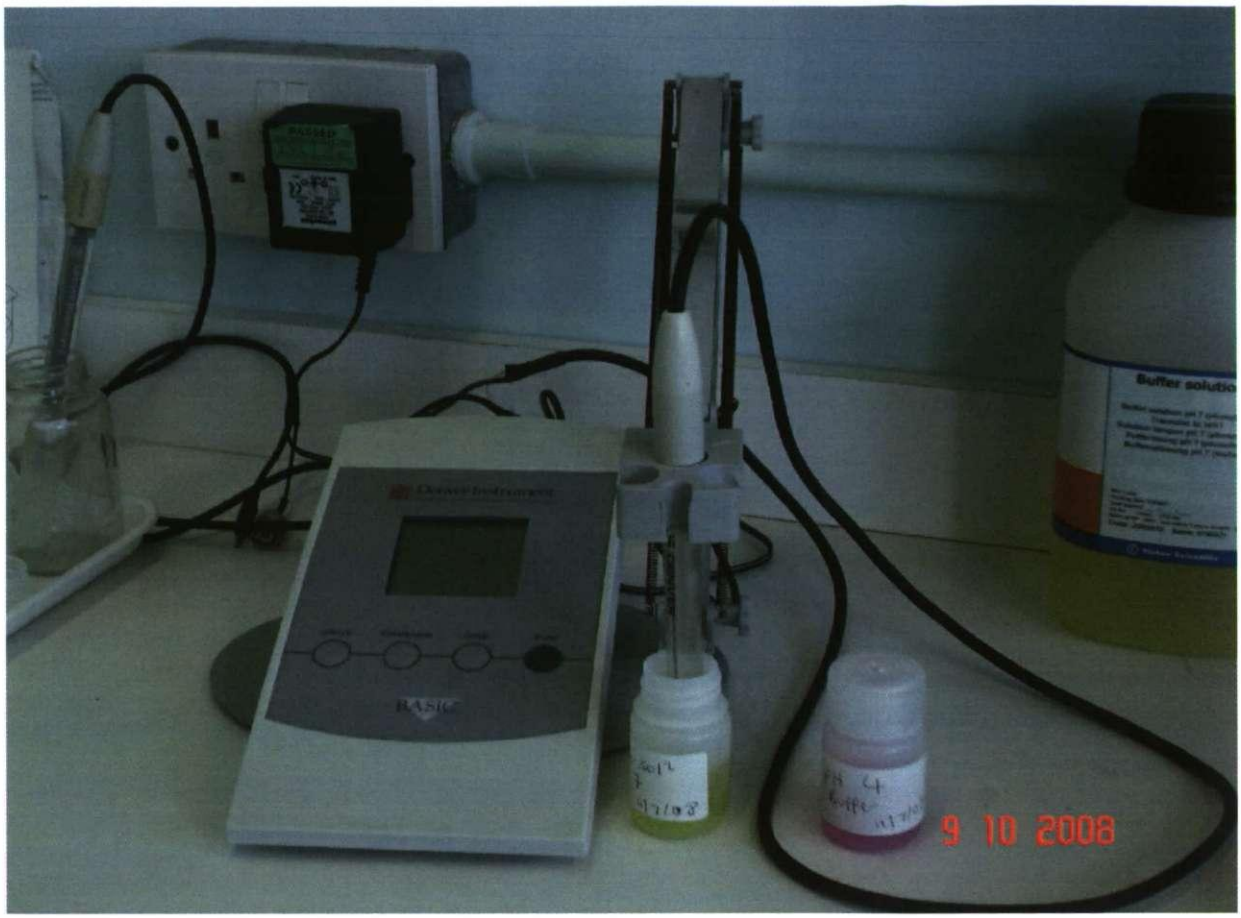


Plate 2.5. pH meter used in the analysis.

2.7.5. Soil moisture

In the first drought interaction experiment, John Innes No. 2 loam based compost was used as a growth medium and the moisture content of the compost was measured using a Theta-probe^{TM} (Delta-T Devices). The regime of irrigation was set for fully irrigated pots to be given water to re-establish 90% of available water content when soil water availability reached 70% of the available water capacity (AWC – calculated gravimetrically following a full saturation of the soil and then allowing pots to drain until drain flow stopped (Kaddour and Fuller, 2004), and for the drought stress treatment to be given water to re-establish 70% of AWC when the soil water availability

reached 45% AWC. Theta probe measurements were taken every 1-2 days and when the minimum levels of AWC for each treatment was reached the water was added to the pots, and after about half an hour these levels were re-checked to ensure the desired levels of AWC for each treatment was achieved and if required more water was added and levels checked again.

2.7.6. Total Kjeldahl nitrogen in plant materials

Kjeldhal analysis was used to determine total nitrogen in the dried plant materials. The basis of the method is that the digestion of the sample with concentrated sulphuric acid in the presence of sodium sulphate and copper catalyst converts nitrogen compounds to ammonium sulphate (Anon, 1988). The ammonium content of the digest solution is then determined using a flow injection analyser and the nitrogen concentrations are calculated and can be reconverted to crude protein if desired.

The following steps were followed in conducting the analysis:

After weighing each sample of the dried plant materials, one sodium sulphate and copper catalyst tablet was added to each sample in the digest tube, and then in the fume cupboard, 10ml of sulphuric acid was added. Then the tubes were put in a digestion block for 3-4 hours where the tubes were heated gradually up to 370 °C and left at this temperature to reflux for 30 minutes. When the tubes were cooled, the digestion solutions were transferred under the fume cupboard to 50 ml volumetric flasks and thoroughly mixed with distilled water to a 50 ml volume. The samples which were still warm at the time were left to cool down for a while, and then the reduced volume is topped up again with distilled water to the 50 ml mark and samples were transferred to

50 ml sealable plastic tubes which were kept in the fridge to be analysed later when the flow injection analyser was available.

Bran and Luebbe Autoanalyser 3 (flow injection analyser) was used to analyse the samples for nitrogen concentration. Values were given in mg L^{-1}, and nitrogen content as g $100g^{-1}$ in the sample dry material was calculated as follows:

N content ($g100g^{-1}$) = (50 × N concentration (mg L^{-1}) /sample dry weight (g))/10000

The principle of the Autoanalyser operation is that the instrument operates a method known as continuous air-segmented flow analysis. An autosampler is filled with samples, standards and quality controls and the order of analysis is programmed into the computer. A peristaltic pump continuously pumps all the reagents and the samples from the autosampler into the chemical manifolds. In the manifolds the samples and reagents are mixed and treated according to the method protocols. On leaving the chemical manifolds the samples are passed through a colorimeter and their absorbance is measured at a specific wavelength. The computer then compares this to the calibration curve and calculates the concentration of the analyte in the sample.

2.7.7. Phosphorus content in plant materials

The phosphorus content was determined according to Murphy and Riley (1962). The same digested solutions used for total nitrogen analysis were also used for phosphorus measurements, and a flow injection analyser (Bran and Luebbe Autoanalyser 3) was used to analyse the phosphorus content in the samples. Similar to that for nitrogen, the values were given in mg L^{-1} and phosphorus content as g.$100g^{-1}$ was calculated in the same way used for nitrogen.

2.7.8. Nitrogen content in the residual water

The nitrogen content in the residual water was analysed according to Greenberg *et al,* (1992). Bran and Luebbe Autoanalyser 3 was also used to analyse the samples. The principle of this method is that the Nitrate is reduced to nitrite by hydrazine in alkaline solution, with a copper catalyst. This is then reacted with sulphanilamide and NEDD to form a pink compound. Phosphoric acid is added at the final stage to reduce the pH, thus avoiding precipitation of calcium and magnesium hydroxide. The addition of zinc to the reducing agent suppresses the complexing of copper by organic material. A dialyser for the high range eliminates interference from coloured samples and suspended solids. The absorbance of the final reacted sample is then measured at 550nm.

2.7.9. Phosphorus content in the residual water

Phosphorus content in the residual water was analysed according to Murphy and Riley (1962), using Bran and Luebbe Autoanalyser 3. The basic description for the method is that Orthophosphate reacts with molybdate and ascorbic acid to form a blue complex. Antimony potassium tartrate is added as a catalyst. The absorbance of the final reacted sample is then measured at 660nm.

2.7.10. Ethylene

A gas chromatograph was used when conducting the acetylene reduction assay for measurements of the gas ethylene generated when noduated roots were incubated with 5 or 10% acetylene.

In general, a gas chromatograph is used to separate chemicals in a complex sample. The samples are injected onto the head of the chromatographic column, and then samples are transported through the column by the flow of inert, gaseous mobile phase. The column itself contains a liquid stationary phase which is adsorbed onto the surface of an inert solid. The purpose of the stationary phase in the column is to separate the different components, causing each one to exit the column at a different time (different retention times). The retention time can be altered by changing the carrier gas flow rate and the temperature. The carrier gas must be chemically inert and most frequently used gases include nitrogen, helium, argon, and carbon dioxide depending on the type of the detector. The outlet stream from the column is monitored using a detector. Different types of detectors which can give different types of selectivity can be used (Anon, 2008). In the current experiment, the gas chromatograph used was a Perkin Elmer 8500 fitted with flame ionization detector (FID) which is sensitive to a wide range of components (Plate 2.6). The column used in the gas chromatograph was an 80/100 porapak Q, 6ft stainless steel, and the oven temperature was 75°C and the injection temperature was 120 °C. The carrier gas was nitrogen with a flow rate of 30 ml min^{-1}.

Plate 2.6. Perkin Elmer 8500 gas chromatograph used in the analysis.

2.8. Statistical analysis

Data from different experiments were statically analysed using Minitab 13.1 software and two types of analysis were mainly used.

2.8.1. Analysis of Variance (Split-Plot Design). In most of the experiments it was impossible, for construction reasons, to completely randomise the CO_2 treatments across the other treatments studied. The experimental design was therefore nested and was akin to a split plot design used in many field experiments. CO_2 (ambient and elevated) formed the Main plot level with the other treatments completely randomised within the Main-plots similar to Sub-plots. In Minitab this analysis is achieved by

performing a GLM with replicates (blocks) as a "random factor" and the interaction between replicates as CO_2 considered as the main plot error. The level of significance of the CO_2 treatments is compared to the main plot error and the rest of the treatments and interactions compared with the sub-plot error (see appendix A).

This type of analysis was used for analysis of the second drought experiment and the nitrogen interaction experiment.

7.8.2. In the first drought experiment (Chapter 3) for logistical reasons the experiment was unbalanced (5 ambient CO_2 chambers and 3 elevated CO_2 chambers) and this necessitated further statistical investigation. Initially the data was analysed in an unbalanced GLM Anova (Analysis of variance for General Liner Model) and subsequently as a two-factor crossed design (including interaction) with CO_2 and drought as the factors and repeated with cultivar and drought as the factors and the results compared with the results of the GLM. The results of the analyses did not alter any of the significance levels for any of the factors or interactions and therefore only the GLM results are presented in the text (Appendix B).

All data were always tested for normality using the Anderson-Darling normality test, and when data was not normal, the residual plot tested for normality (personal communication Dr. Paul Hewson, University of Plymouth). Some data was log transformed to normalise the data used in the analysis. Wherever log transformed data was analysed, back-transformed means were always used for graphical presentations.

3. Chapter 3

The Interaction of Elevated CO_2 and Drought on the Growth and Nodulation of Lentils

3.1. Introduction

Drought is a major factor in reducing crop productivity worldwide and losses caused by water stress can exceed those caused by all other factors combined (Kramer, 1980). The immediate response of plants to water shortage at leaf level is to reduce evapotranspiration by stomatal closure (Kaiser, 1987; Fuller and Jellings, 2003), which leads to a reduction in net CO_2 uptake and photosynthesis. In fact, drought can reduce the net CO_2 uptake due to both stomatal closure and a decline in the photosynthetic mechanism (Cornic *et al.*, 1987). Increasing external CO_2 has the potential to overcome the effect of stomatal closure and improve photosynthesis (Kaiser, 1987). Since CO_2 concentration is an important factor in regulating the opening of the stomata (Allen, 1998), it is anticipated that the increasing levels of atmospheric CO_2, which could reach levels of 815 μmol mol^{-1} by the year 2100 under high emissions of CO_2 (Hulme *et al.*, 2002), could reduce stomatal conductance and improve water use efficiency (the ratio of CO_2 uptake to H_2O transpired) as a consequence. Generally, doubling CO_2 levels, could reduce leaf stomatal conductance for CO_2 and water vapour by about 40%, and that leads to an improvement in water use efficiency by reducing the amount of water transpired and delaying the onset of drought (Rogers *et al.*, 1984; Samarakoon and Gifford, 1996), and by increasing the net assimilation rate (Allen, 1998). Generally, a positive effect of elevated CO_2 on growth under water limiting conditions has been reported. Ben *et al.*, (1987) demonstrated that at high CO_2 concentrations, the apparent quantum yield of O_2 evolution of sunflower was not affected by a considerable drought constraint. Kasier (1987) has collected data obtained by measuring photosynthetic capacity at high CO_2 concentration during leaf drying, and according to this data, the

photosynthetic capacity was more resistant to drought conditions, mainly due to overcoming of stomatal limitation. The rising level of atmospheric CO_2 is also expected to increase the length, density and the spread of the roots in drying soils which in turn improves water capture within the rooting zone (Rogers *et al.,* 1994; Chaudhri *et al.,* 1990; Idso and Kimball, 1992Rogers *et al.,* 1999: Wechsung *et al.,* 1999;). It is reported that when water was limiting under elevated CO_2 conditions, there was no loss in the relative enhancement of biomass and economic yield (Gifford, 1979; Sionit *et al.,* 1980), but even more, it could be greater than that under well watered conditions (Idso and Idso, 1994). For example, at 550 μmol mol $^{-1}$ of CO_2, the grain yield in wheat produced per unit of water lost was 17% and 32% under ample and drought conditions respectively (Kimball *et al.,* 1995). The anticipated positive effects of high levels of atmospheric carbon dioxide on crop production will be accentuated in the regions where crop production is limited by severe water shortage and extreme heat such as in the eastern Mediterranean (Kaddour and Fuller, 2004).

Lentils (*Lens culinaris* Medic), is one of the most important food crops in the semi-arid regions of the world, especially in the Indian Sub-continent and in dry areas of the Middle East (Muehlbauer *et al.,* 1995). The seeds, the main product of lentils, are highly nutritious (22- 34.5% protein, 65% carbohydrate) and the residues can be used as a high protein source for livestock (Muehlbauer *et al.,* 1985). Although lentil is considered as a moderately drought tolerant crop (Kay, 1979), the production of lentils worldwide is substantially reduced by drought and heat, particularly in the Middle East region (Erskine 1985; Yadva *et al.,* 2007). In the Mediterranean region, lentil is a winter grown crop, and during the reproductive stage which coincides with the end of the rainy season, the crop usually suffers from terminal drought and yield is considerably reduced

(Oweis *et al.*, 2004). In an experiment conducted over four seasons in ICARDA station in northern Syria, Oweis *et al.*, (2004) indicated that when limited irrigation was applied, the yield increased by about 77% compared to that when rain fed only (1.81 and 1.04 tha^{-1} under supplemented irrigation and rain fed conditions respectively). Therefore, investigating the effect of increasing atmospheric levels of CO_2 and water availability on lentil production is of great importance since no work has been conducted on these aspects of lentils before. In addition, lentil as a legume is a nitrogen fixing species, and investigating the effects of elevated CO_2 on the nodulation of lentils is equally important. Generally, nodulation and nitrogen fixation in legumes increases under high levels of atmospheric carbon dioxide (Díaz, 1996; Zanetti *et al.*, 1996), but whether it is the case in lentils is also part of the investigation reported in this chapter.

3.2. Aim

To investigate the effect of atmospheric CO_2 enrichment on the growth and nodulation of water stressed and non-stressed lentils.

3.3. Objectives

3.3.1. Objective 1

Investigate the effect of elevated CO_2 on some production parameters (LAI, above and below ground biomass, pod number and seed yield, and harvest index) in lentils and whether these parameters will be increased, unchanged or reduced at high levels of atmospheric CO_2 at limited and/or full irrigation conditions.

3.3.2. Objective 2

Test if the response to elevated CO_2 in the two lentil cultivars studied could be different, and whether the effect of elevated CO_2 on these cultivars is related to the irrigation conditions or not.

3.3.3. Objective 3

Investigate the effects of atmospheric elevated CO_2 on the process of symbiotic nitrogen by studying nodule number, nodule weight, and the nitrogen budget and examine whether these effects are different between the two cultivars and/or the two water treatments.

3.3.4. Objective 4

Test whether atmospheric elevated CO_2 has an effect on seeds quality concerning total nitrogen including protein concentration, and phosphorus concentration and whether the effect is different between the two cultivars and/or water treatments.

3.4. Materials and methods

3.4.1. Plant materials

Two cultivars of lentils, ILL7979, which is still pre-registration in Syria, and, ILL6995, which is registered in Syria and known as Idlib 3, were used, and were provided by the International Centre for Agricultural Research in the Dry Areas (ICARDA), Aleppo,

Syria. Germination tests revealed that germination potential exceeded 90% in both cultivars.

3.4.2. Experiment 1 (Open-top CO_2 chambers)

In this experiment, the two cultivars were grown under ambient and elevated CO_2 under drought and full irrigation conditions. The plants were sown (19/07/2004) into 10 litres square pots containing John Innes No. 2 loam based compost. Nine seeds were sown in each pot (equivalent to approximately 180 plant m^{-2}). Pots were placed in 1 m x 1 m-growth compartments in two large 4m × 4m CO_2 phytotrons located at Seale-Hayne Field Station in the University of Plymouth. Three of the eight compartments were supplemented with CO_2, and the other five were kept at ambient concentration. CO_2 supplementation was achieved using cylinders of compressed CO_2 - IRGA (BOC gases) coupled to an EUROTHERM controller which constantly coupled the air in the chamber and pulsed CO_2 from the bottled gas to a set point of 750 μmol mol^{-1}. According to the **SRES** scenarios published in the Special Report on Emissions Scenarios in 2000 and in the IPCC Third Assessment Report (TAR) in 2001, the range of CO_2 concentrations projected for the year 2100 ranges from 540 to 970 ppm. Therefore a middle of the range concentration of 750 ppm was chosen to represent the elevated CO_2 concentration used in the experiments. Controlling CO_2 in plant growth chambers is known to be very difficult, and it was the case in the current investigation, and actual CO_2 levels were sometimes higher or lower than planned but this was always still within the projected CO_2 levels range.

Telaire monitors were used to monitor CO_2 and temperature every 15 minutes, and data logged using Hobo™ dataloggers. From these data, daily and weekly average CO_2 (Figure 3.1 and 3.2) and temperature (Figure 3.3, 3.4) were calculated.

Eight pots of each cultivar were placed in each compartment, and water stress treatment was superimposed on half of the pots whilst the second half was fully irrigated (4 pots for each treatment) and all treatments were fully randomised in each compartment. The moisture content of the compost was monitored using a Theta-probe™ (Delta-T Devices) as previously described by Kaddor and Fuller (2004). The fully irrigated pots were given water to re-establish 90% of available water content when soil water availability reached 70% of the available water capacity (AWC). The drought stress treatment was given water to re-establish 70% of AWC when the soil water availability reached 45% AWC. This was achieved by watering the two treatments every three to four days with different amounts of water, which were also changing over the development of the lentils. During the early stages, pots in the drought treatment were irrigated with about 150 ml, and the fully irrigated ones with about 300 ml, and as the plants grew and used greater amounts of water, these amounts were increased gradually to 250 and 450 ml in drought and fully irrigated treatments respectively. Watering was terminated when all flowers had set seed (approx 30 days before harvest), in order to facilitate ripening (mimicking conditions).

Photoperiod was maintained at 10 hours a day in the first month, and then increased gradually by 1 hour every three weeks until a final photoperiod of 13 hours was to coincide with the normal photoperiod during the growing season in Syria. Illumination of the chambers was provided by Phillips SONHT400 grow lamps giving a photon flux density of 180-280 μmols m^{-2} s^{-1}. Temperature varied diurnally between 12 °C and 31 °C

with a mean of 22 °C. Records of temperature in chambers revealed that there were some chamber to chamber differences. These data were integrated over time to provide a thermal time value per chamber for each experiment and when compared these showed no significant variation.

Half of the pots in each replicate (8 pots from each chamber; 64 pots from all chambers) were harvested at flowering (the first appearance of open flowers with visibly dehisced anthers in 50% of the plants in the treatment), and measurements of leaf area, fresh weight, and dry weight (after 48 h at 80 °C in Memmert ULM 600 ovens) recorded. The cultivar IL7979 flowered 49 days after sowing (08/09/2004) and two days earlier than the cultivar Idlib 3 (10/09/2004) and the first harvest was conducted over two days on 10 and 11/09/2004. The second half of the pots (64 pots) was harvested at the end of seed filling. The droughted plants were harvested two weeks earlier (on 25/10/2004) than the fully irrigated plants (09/11/2004), there were no noticeable differences in the maturation of the two cultivars or the two CO_2 treatments. The following measurements were taken: fresh weight of the plants; fresh weight of seeds; number of pods per plant; dry weight of plants and seeds.

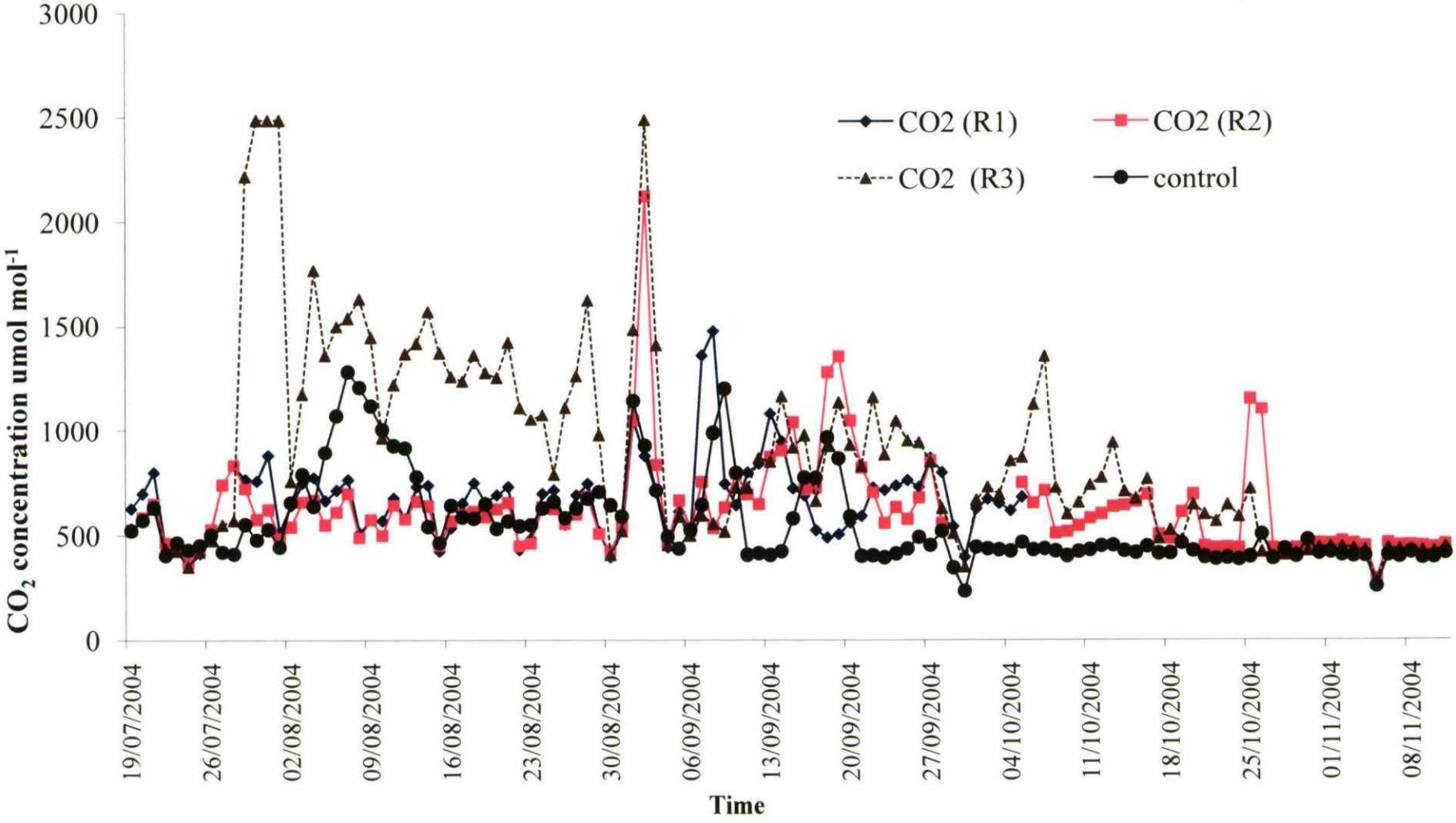


Figure 3.1. Daily average CO_2 concentration over the growth period of lentils in open- top chambers (exp 1.)

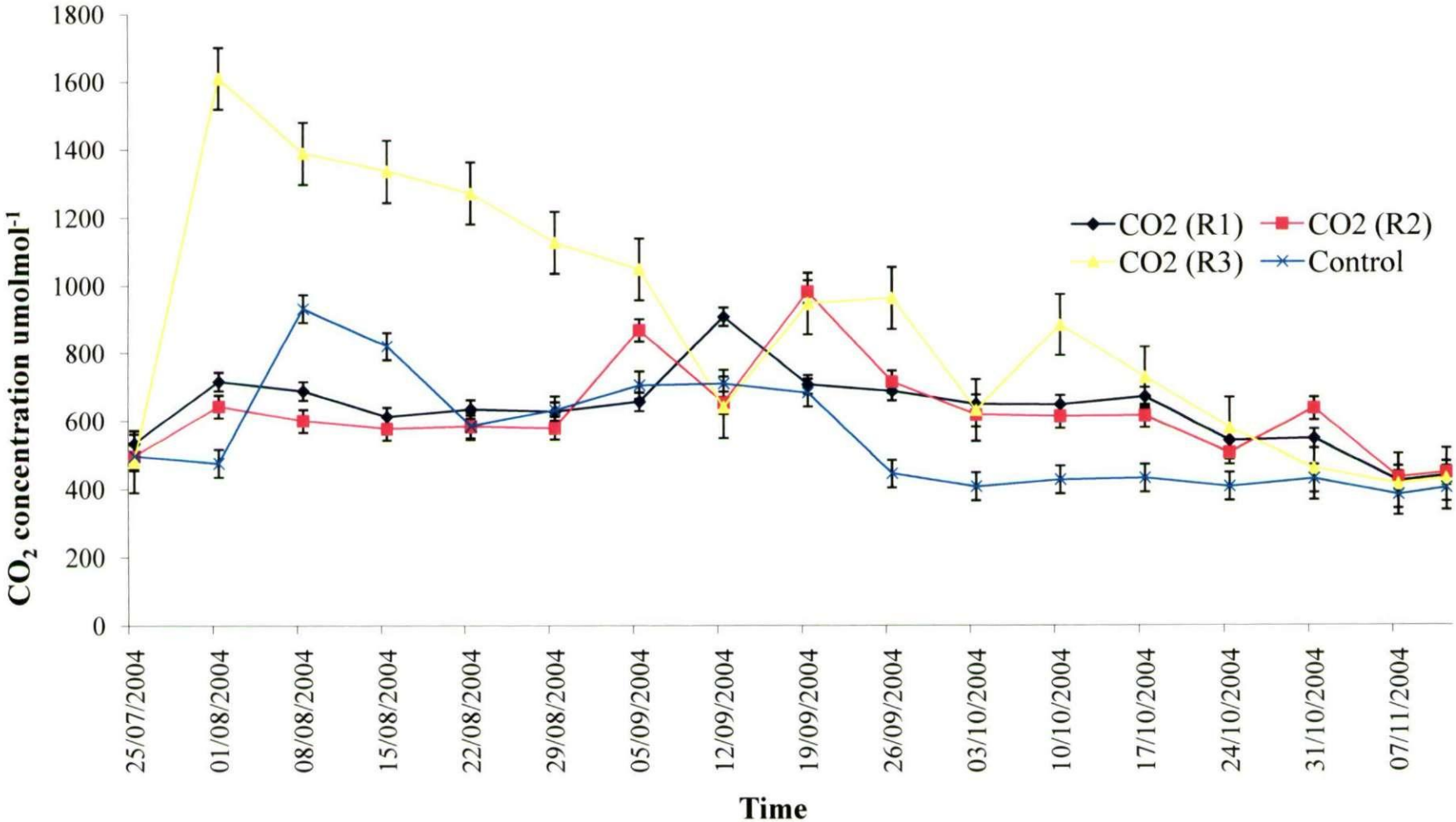


Figure 3.2. Weekly average CO_2 concentration over the growth period of lentils in open-top chambers (vertical bars are +/- 1 se) (exp 1).

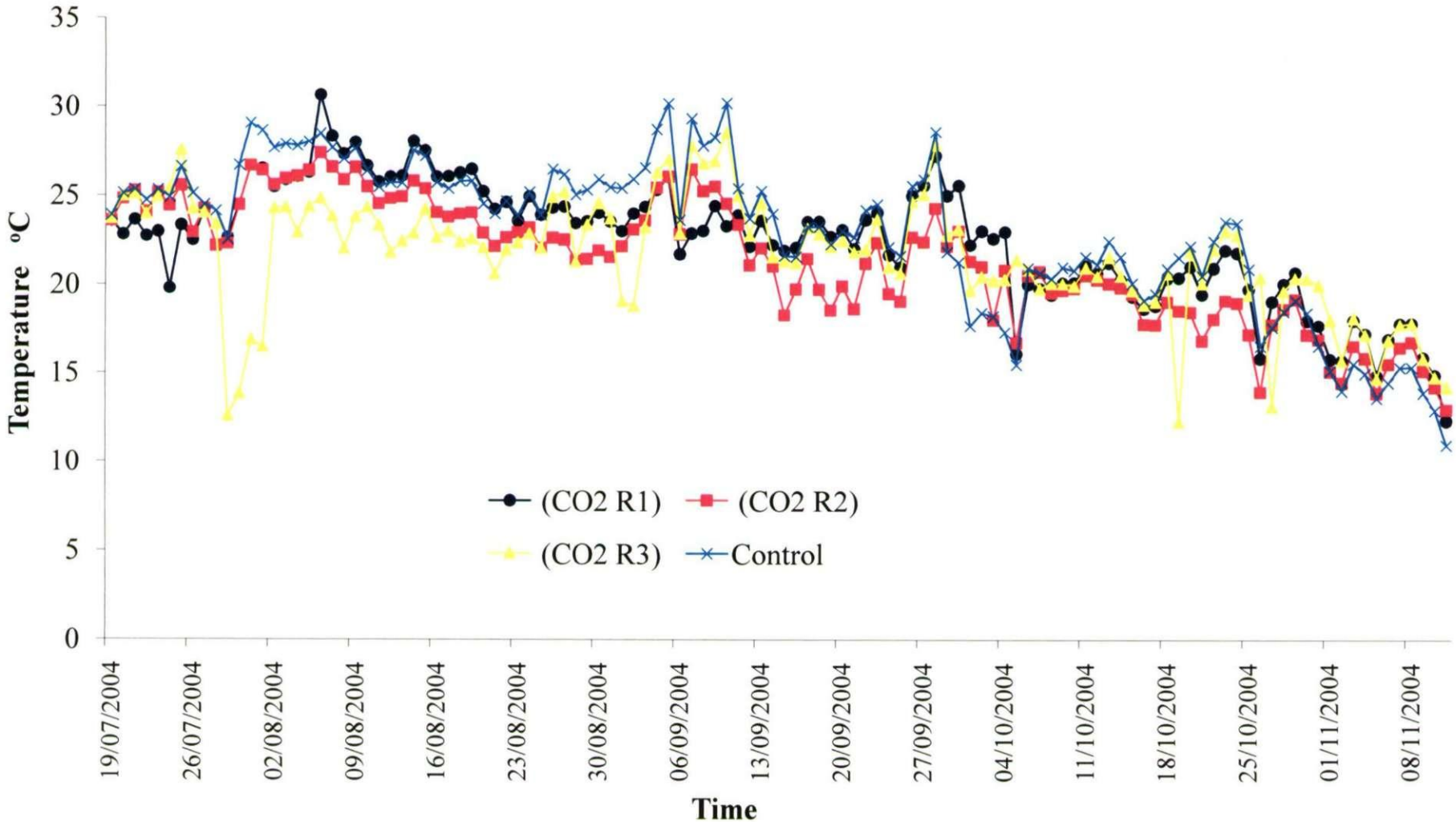


Figure 3.3. Daily average temperature over the growth period of lentils in open- top chambers (exp 1).

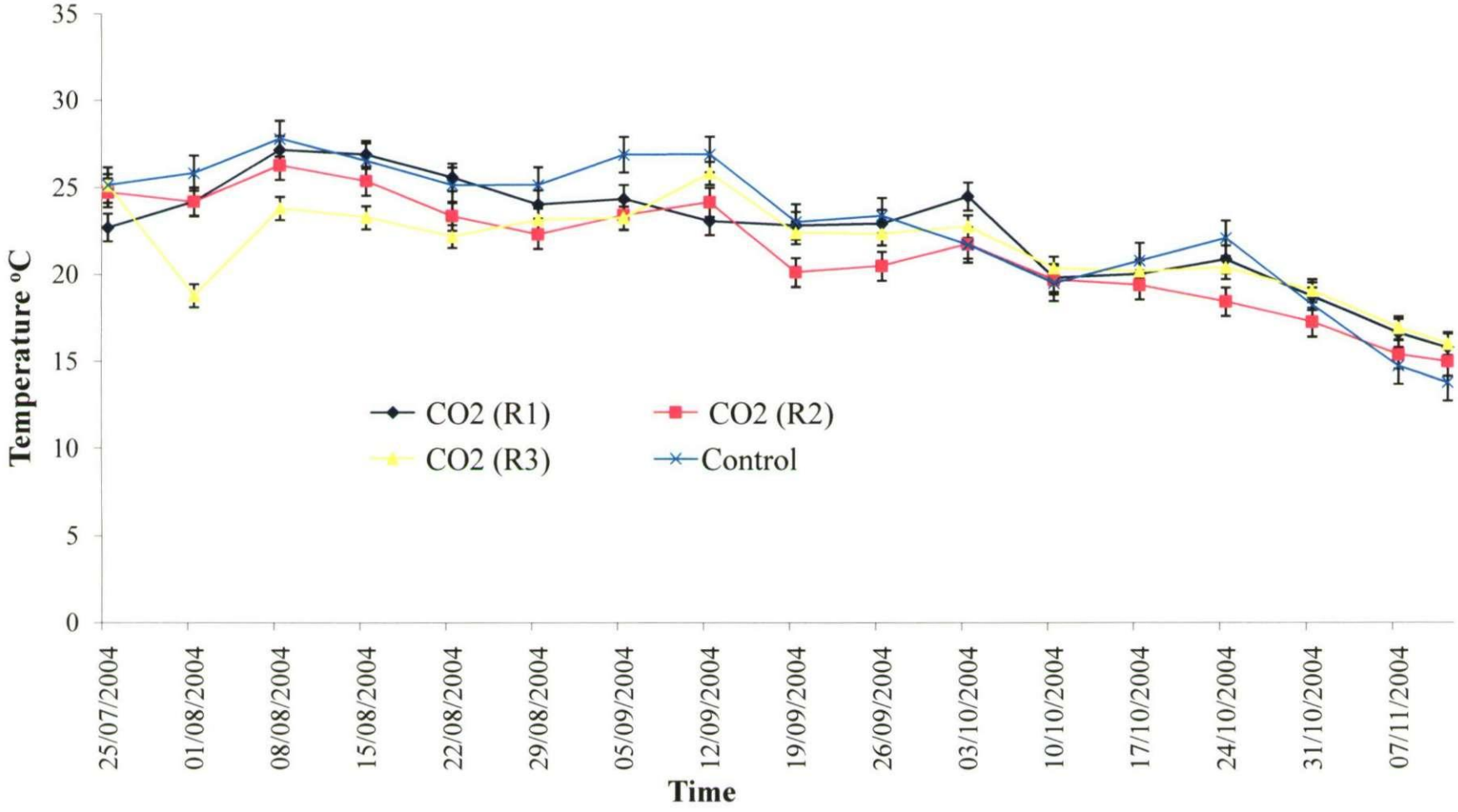


Figure 3.4. Weekly average temperature over the growth period of lentils in open-top chambers (vertical bars are +/- 1 se) (exp 1).

3.4.3. Experiment 2 (Closed chambers)

Plants were grown under ambient (327 μmol mol^{-1}) and elevated (835 μmol mol^{-1}) CO_2 under drought and full irrigated conditions. The plants were grown in eight tightly sealed ventilated chambers (60×60×80 cm) placed in a greenhouse located in Skardon garden at the University of Plymouth (plate 3.1). Half of the chambers were supplied with elevated CO_2 and the others with ambient air. Each chamber contained 24 pots, half sown with Idlib3, and the other half with the ILL7979. Half of the pots of each cultivar were fully irrigated and drought treatment (50% of the full irrigation amounts) was applied on the remainder. The chambers were distributed with 4 chambers on each side of the green house. A decision was made to put equal number of replicates of ambient and elevated CO_2 at each side of the greenhouse (two ambient and two elevated chambers at each side of the greenhouse) and to randomly assign which were used for CO_2 and which for ambient. Since the drought and cultivar treatments were confined to individual pots it was possible to completely randomly allocate their position within each growth chamber.

The seeds were inoculated with *Rhizobium leguminosarum* bacteria immediately before sowing on 23/03/06, and the growth medium used was medium-grade horticultural perlite which facilitated easy root system recovery. Three seeds were sown in each pot (constructed from cylindrical polypropylene pipe 30 cm high × 10 cm in diameter), and thinned to two plants after establishment.

Carbon dioxide supplementation was achieved using cylinders of compressed CO_2 (BOC gases) coupled to an IRGA Eurotherm™ controller which constantly coupled the air in the chamber and pulsed CO_2 from the bottled gas to a set point of 750 μmol mol^{-1}. Telaire TM monitors were used to measure CO_2 and temperature at 15 minutes intervals, and data logged to Hobo™ dataloggers. From these data, daily average CO_2 (Figure 3.5), temperature (Figure

3.6) and relative humidity (RH) (Figure 3.7) were calculated. Analysis of the daily average CO_2 between the elevated CO_2 chambers showed that there were significant differences between the chambers, however, when analysing the production parameters, no Chamber effect was found suggesting that these differences were within the experimental "noise" and when integrated over time did not have significant effects on production.

Daily average temperatures between all chambers (ambient + elevated) were not significantly different. There were small but significant differences in daily average RH humidity between chambers. It is possible that these differences could have affected the results to some extent but this was not investigated further here but it would be worthy of further investigation in the future.

The plants were irrigated using a standard hydroponic' growth solution ("Optimum" Growth Technology Ltd) every 14-18 days (100-150 ml of full irrigation amounts), and similar amounts of tap water supplied in between according to demand. The watering regime for the fully irrigated plants was determined by maintaining at least 1 to 5 mm of drainage fluid in the drainage beaker. The drought treatments plants received half the amount of water received by the full irrigated plants and whenever a hydroponics solution was used for watering, the droughted plants received half the amount of the hydroponics solution but the same amount of nutrients as the solution was double concentrated.

Two destructive harvests, at anthesis and at maturity, were conducted as half the pots (96 pots) were harvested at each harvest. The first harvest was conducted on two separate days (49 pots at each day) which were on 14 and 19/05/06 for the cultivars IL7979 and Idlib 3 respectively as the former flowered on 13/05/06 while the latter flowered five days later on 18/05/06. The second harvest was conducted over a three week period on the following dates 22/07/06, 31/07/06, and 07/08/06 with over 70% of the droughted plants being harvested at the first two harvest points. More than 60% of all plants harvested at the first two harvest

points were from elevated CO_2 treatments.. Measurements of leaf area using a Delta-T Image Analysis System- type (DIAS^{TM}), LAI, above and below ground dry weight (after 48 h at 80 °C in a Gallenkamp 250 °C drying oven), nodule number, and nodule fresh weight were recorded in the first harvest. Above and below ground dry weight, pod number and seed yield were recorded in the final harvest. At the end of the experiment, the pots were soaked in distilled water overnight, drained and washed with distilled water and samples of the solutions were collected and later analysed for N and P (using a flow injection analyser). Because the pots were placed in drainage beakers, any residual drainage water at the end of the experiment was collected and analysed, and every care was taken to make sure that any residual nutrients were analysed. The only potential loss of nutrients from the system was as a result of gaseous loss and this has not been measured or estimated.

Dried plant material was subsequently analysed for total nitrogen by Kjeldhal analysis according to Cerdá *et al.*, (1997) using digestion block instead of a microwave. Phosphorus was analysed according to Murphy and Riley (1962) using Bran and Luebbe Autoanalyser 3 (flow injection analyser). Nodule number per plant was calculated by counting the active nodules which were identified by their pink, red and sometimes light brown colour showing leghmoglobin activity (Somasegaran and Hoben, 1994).

Data were analysed using Minitab 13.1, Analysis of Variance for a split plot design (see Cahpter 2, General Materials and Methods.)

Note. All data were tested for normality using Anderson-Darling normality test. When data was not normal, the residual plot tested and it was normal (personal communication Dr. Paul Hewson, University of Plymouth) and for some data the log data (dry weight at anthesis, nodule fresh weight, nodule number) was normal and used in the analysis. All the figures are drawn from the actual data.

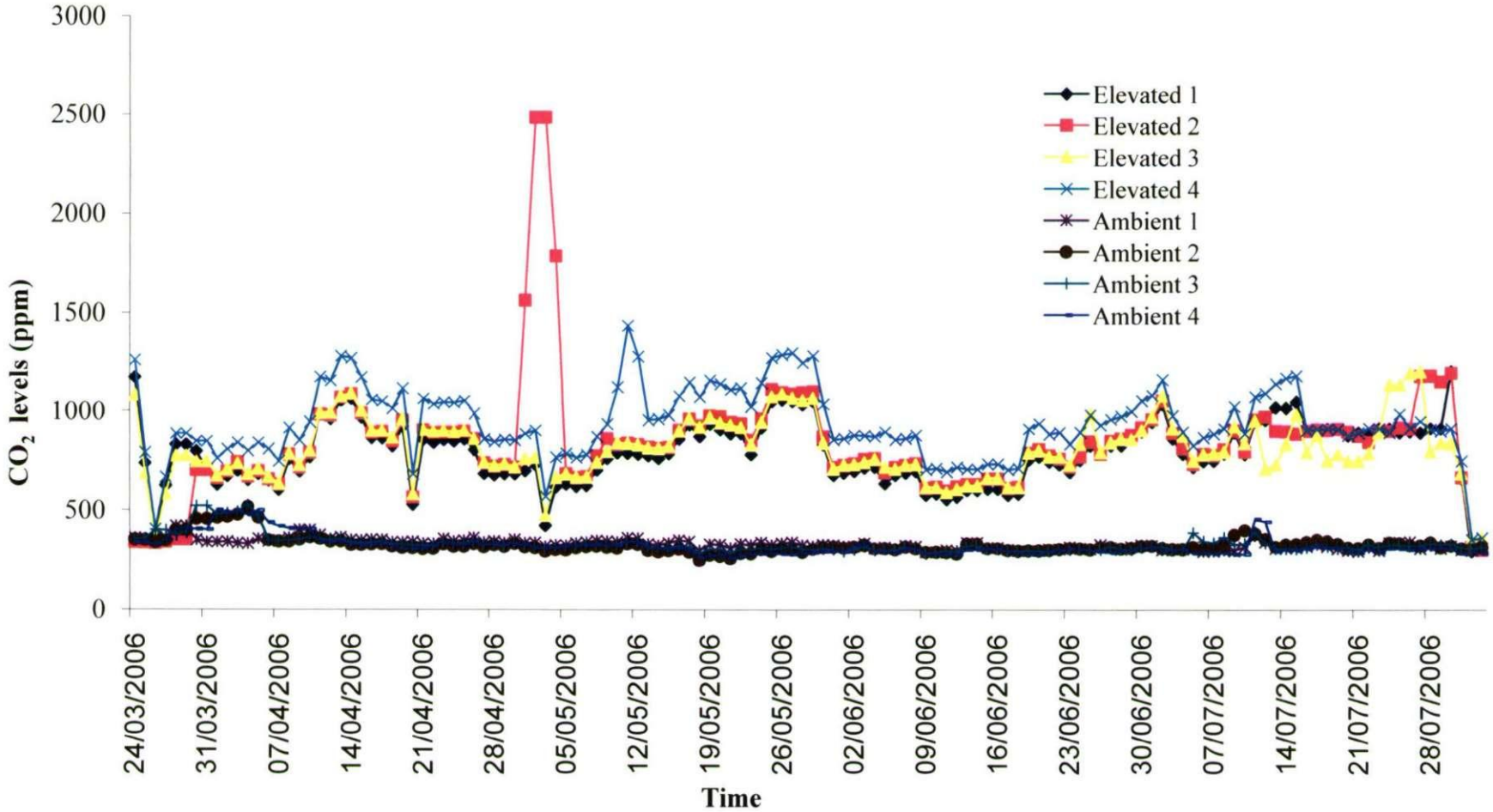


Figure 3.5. Daily average CO_2 concentration over the growth period of lentils in the closed growth chambers (exp 2).

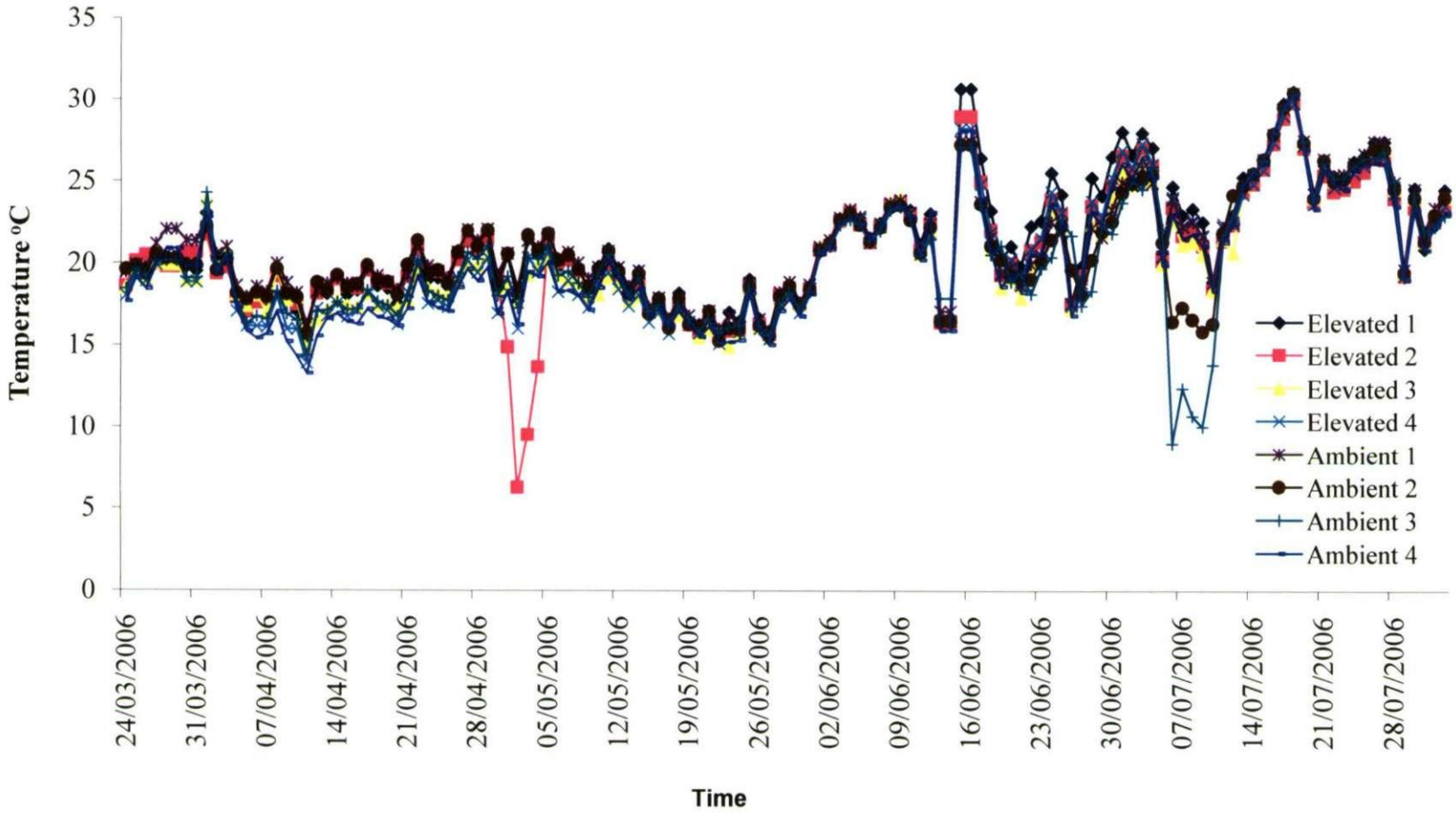


Figure 3.6. Daily average temperature over the growth period of lentils in the closed growth chambers (exp 2).

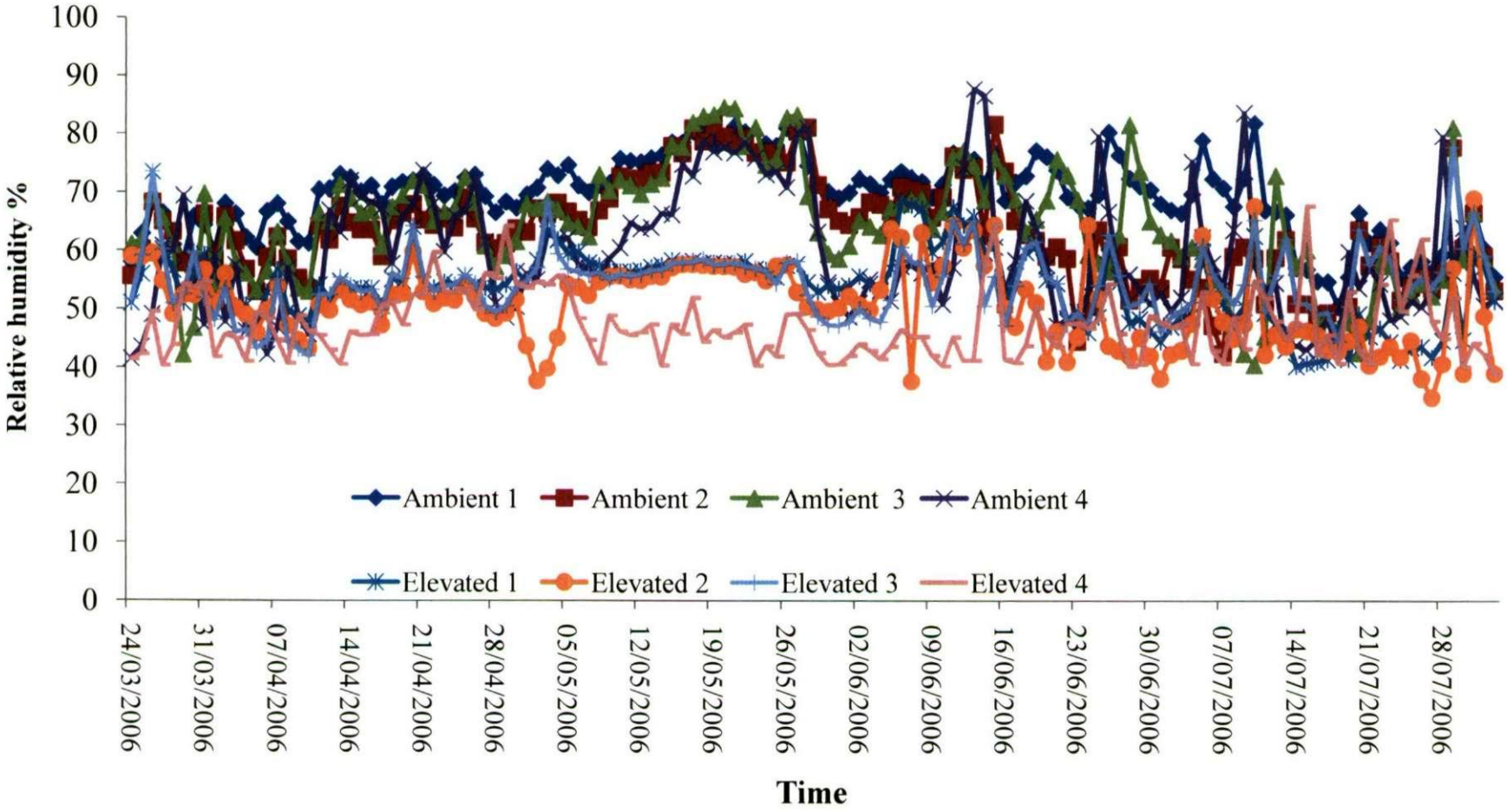


Figure 3.7. Daily average relative humidity over the growth period of lentils in the closed growth chambers (exp 2).

Plate 3.1. The closed chambers used in the second experiment.

3.5. Results

A summary of the results from both experiments are presented in the table below (Table 3.1), and each of the results was discussed later in the text.

	P value for the main and interaction effects in the first drought exp							
Parameter	**CO_2**	**Drought**	**Cultivar**	**Block**	**CO_2×C**	**CO_2×D**	**C×D**	**CO_2×C×D**
LAI	0.609	0.338	0.000	0.120	0.752	0.132	0.001	0.468
Aboveground dry weight at anthesis	0.078	0.000	0.232	0.777	0.803	0.809	0.060	0.214
Aboveground dry weight at maturity	0.010	0.000	0.005	0.231	0.997	0.071	0.015	0.560
Pod number	0.043	0.000	0.036	0.360	0.545	0.047	0.240	0.757
Seed yield	0.024	0.000	0.000	0.175	0.325	0.016	0.031	0.571

Table 3.1. Summary table of the main and interaction effects of elevated CO_2 and drought on different parameters studied on lentils in the first drought experiment (CO_2= CO_2 treatment, C= cultivar, D drought treatment- grey = ns; green = "significant" at between 10 and 5 %; clear = significant at either 5, 1 or 0.1%).

	P value for the main and interaction effects in the second drought exp							
Parameter	**CO_2**	**Drought**	**Cultivar**	**Block**	**CO_2×C**	**CO_2×D**	**C×D**	**CO_2×C×D**
LAI	0.306	0.000	0.244	0.299	0.090	0.221	0.201	0.489
Aboveground dry weight at anthesis	0.064	0.000	0.003	0.450	0.863	0.730	0.344	0.546
Below ground dry weight at anthesis	0.187	0.185	0.276	0.459	0.473	0.756	0.654	0.958
Aboveground dry weight at maturity	0.207	0.000	0.177	0.977	0.894	0.377	0.291	0.891
Below ground dry weight at maturity	0.111	0.000	0.279	0.709	0.489	0.093	0.528	0.153
Root /shoot ratio	0.630	0.000	0.078	0.626	0.473	0.308	0.482	0.254
Pod number	0.099	0.000	0.736	0.487	0.951	0.540	0.211	0.392
Seed yield	0.059	0.000	0.002	0.874	0.795	0.393	0.168	0.653
Harvest Index	0.128	0.000	0.000	0.601	0.231	0.188	0.700	0.299
Nodule number	0.213	0.000	0.005	0.527	0.676	0.498	0.137	0.392
Nodule fresh weight	0.265	0.004	0.198	0.910	0.761	0.437	0.660	0.381
Nitrogen uptake	0.163	0.000	0.814	0.984	0.894	0.489	0.497	0.837
N Concentration (seeds)	0.372	0.019	0.000	0.872	0.729	0.893	0.688	0.267
N concentration (shoots)	0.004	0.000	0.000	0.377	0.456	0.915	0.020	0.797
N concentration (roots)	0.387	0.243	0.000	0.805	0.104	0.118	0.466	0.106
N in residual water	0.126	0.159	0.000	0.085	0.591	0.002	0.788	0.096
Phosphorus uptake	0.973	0.000	0.065	0.297	0.656	0.628	0.218	0.371
P concentration (seeds)	0.257	0.297	0.045	0.933	0.506	0.395	0.685	0.898

P concentration (shoots)	0.009	0.077	0.093	0.416	0.483	0.948	0.585	0.293
P concentration (roots)	0.083	0.107	0.027	0.499	0.002	0.372	0.025	0.314
P in residual water	0.951	0.548	0.000	0.109	0.259	0.847	0.696	0.449

Table 3.2. Summary table of the main and interaction effects of elevated CO_2 and drought on different parameters studied on lentils in the second drought experiment. (CO_2= CO_2 treatment, C= cultivar, D drought treatment-grey = ns; green = "significant" at between 10 and 5 %; clear = significant at either 5, 1 or 0.1%).

Parameter	**First experiment**		**Second experiment**	
	Ambient CO_2	**Elevated CO_2**	**Ambient CO_2**	**Elevated CO_2**
LAI	1.87	2.20	1.49	1.57
Aboveground dry weight at anthesis (kg ha^{-1})	1694	1994	543	753
Below ground dry weight at anthesis(kg ha^{-1})			97	125
Aboveground dry weight at maturity (kg ha^{-1})	1446	1585	2016	2251
Below ground dry weight at maturity(kg ha^{-1})			275	320
Root to shoot ratio			0.16	0.16
Pod number	13.11	15.54	19.40	22.40
Seed yield (kg ha^{-1})	2296	2797	923	1095
Harvest Index	0.34	0.34	0.43	0.46
Nodule number			30.3	41.8
Nodule fresh weight (mg $plant^{-1}$)			26.3	38.5

Nitrogen uptake (mg /pot)			44.4	51
N Concentration (seeds) g. $100g^{-1}$			4.44	4.27
N concentration (shoots) g. $100g^{-1}$			1.23	1.05
N concentration (roots) g. $100g^{-1}$			1.80	1.69
N in residual water (mg /pot)			14.4	14.9
Phosphorus uptake (mg/pot)			4.87	4.97
P concentration (seeds) mg. $100g^{-1}$			531	490
P concentration (shoots) mg. $100g^{-1}$			96.9	69.5
P concentration (roots) mg .$100g^{-1}$			190	149

Table 3.3. Summary table of the CO_2 treatment main effect on the different parameters studied in the two drought experiments. (grey = no data collected/available).

3.5.1. Experiment 1

3.5.1.1. Technical difficulties and problems

During the experiment one of the carbon dioxide regulators malfunctioned and led to problems in meeting the desired CO_2 levels. CO_2 was being pumped out of the chamber and leaking between the chambers. Consequently the CO_2 levels in the control chambers were higher than planned with an overall average of 557 μmol mol^{-1}. The average during the first two months was 670 μmol mol^{-1} and, after fixing the problem, CO_2 levels decreased to 414 μmol mol^{-1} (atmospheric ambient levels are 379 μmol mol^{-1}).

The average CO_2 concentrations in the supplemented chambers were 638, 627, and 890 μmol mol^{-1} in the three replicates respectively with an overall average of 718 μmol mol^{-1}. Thus, although an overall difference in CO_2 concentration between treatments was maintained, the difference was less than originally planned.

3.5.1.2. LAI and total above ground dry weight at anthesis

Leaf Area Index varied between 1.0 and 3.5 between treatments at anthesis. These LAI's compare favourably with field measurements for Lentils of 1.5- 5.5 (Wall, 1996). Both drought and elevated CO_2 affected LAI but overall the differences were not significantly different ($p= 0.305$). There were however, significant differences ($p < 0.001$) between the varieties with Idlib 3 having a higher LAI and a significant interaction ($p = 0.001$) between cultivars and drought (Figure 3.8). The cultivar Idlib 3 showed increased LAI when subjected to drought in both the ambient and elevated CO_2 treatments whereas the cultivar ILL7979 was unaffected by drought in ambient CO_2 but showed a decrease under elevated CO_2. These unusual observations may be attributable to the single sampling date used and an asynchronous development stage in the treatments (An interaction plot for cultivar and drought is presented in Appendix C).

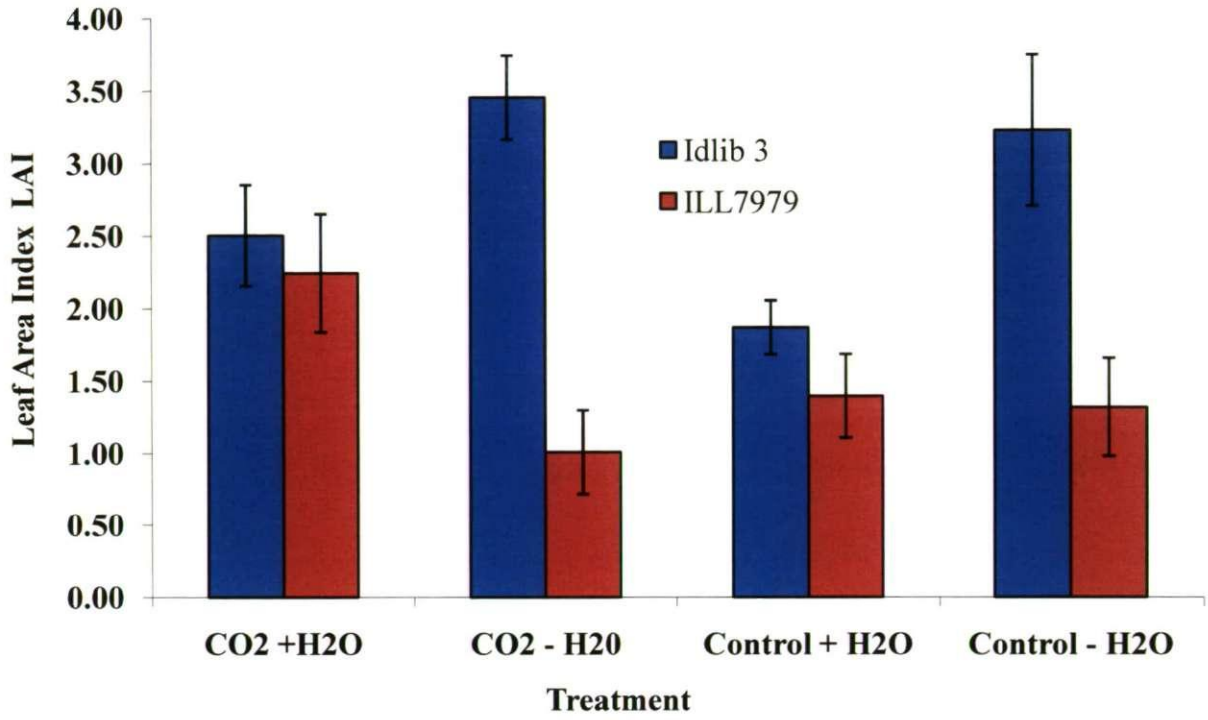


Figure 3.8. Effect of elevated CO_2 and drought on LAI at anthesis of two lentil cultivars (Idlib 3 [ILL6994] and ILL7979) (vertical bars are +/- 1 se) (exp 1). (CO_2= elevated CO_2, +H_2O= full irrigation, - H_2O= water stress, Control= 1 ambient CO_2).

Total above ground dry weight of the plants did not correlate with the LAI measurements but followed a more predictable pattern. Thus, dry weight was significantly ($p < 0.001$) depressed by drought and fully irrigated plants were noticeably thicker stemmed and heavier. Elevated CO_2 marginally increased dry weight ($p=0.078$) in both the fully irrigated and droughted plants (Figure 3.9). There was no significant difference in total dry weight between the two cultivars under all treatments although Idlib 3 was slightly heavier.

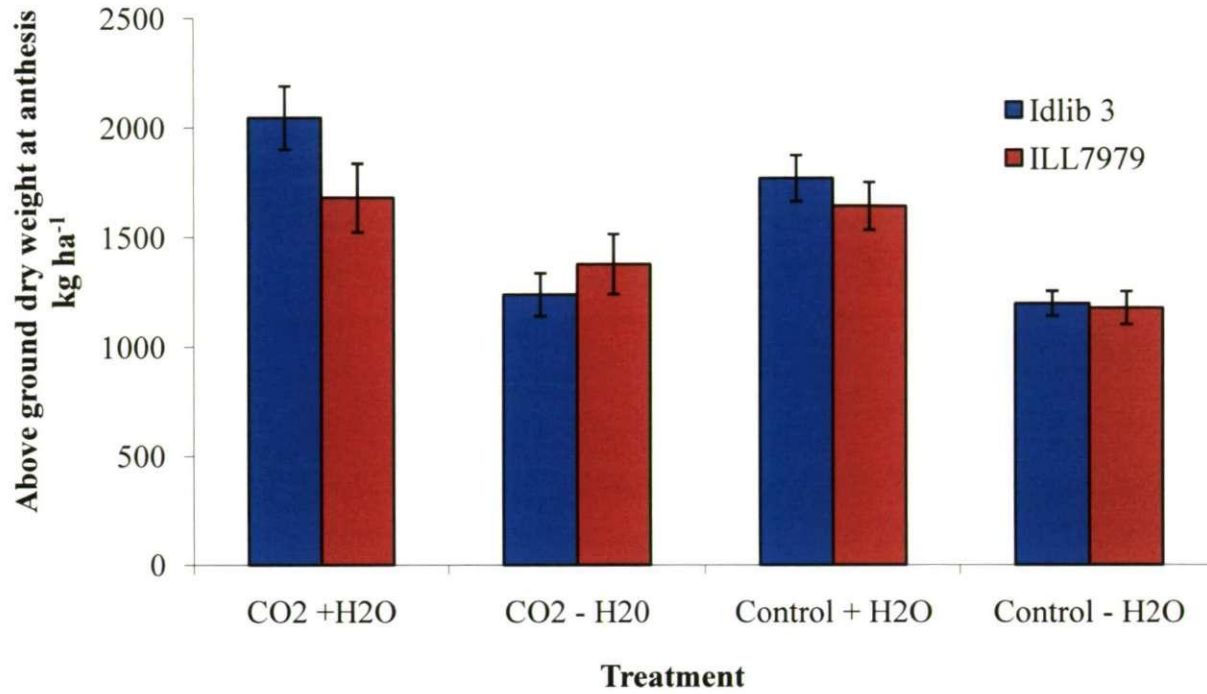


Figure 3.9. Effects of elevated CO_2 and drought on above ground dry weight at anthesis in two lentil cultivars (Idlib 3 [ILL6994] and ILL7979) (vertical bars are +/- 1 se) (exp. 1).
(CO_2= elevated CO_2, +H_2O= full irrigation, - H_2O= water stress, Control= ambient CO_2).

3.5.1.3. Final harvest at maturity

Above ground dry weight, number of pods per plant, and seed yield in the two cultivars were significantly influenced by both elevated carbon dioxide and drought. The three parameters were increased with elevated carbon dioxide, and by contrast reduced by drought, which also accelerated the development in the two varieties leading to an earlier harvest of two weeks than the fully irrigated plants.

The increase in above ground dry weight in fully irrigated plants under elevated CO_2 was more noticeable than that in the drought treatment, as the average increase in the latter was only about 9% compared to about 32% for the former (Figure 3.10).

The number of pods per plant, varied between 8 and 22 for Idlib 3 and between 8 and 17 for ILL7979. It is reported that number of pods per plants varies noticeably with plant density and genotype (Saxena and Hawtin, 1981). Malhotra *et al.*, (1974) found that at spacing of 50 cm × 10 cm, the pods number per plant ranged between 17.2 and 216.2 pods.

The pattern of response to CO_2 and drought of pods per plant followed a similar pattern to that of above ground dry weight, but there was a significant interaction between elevated CO_2 and drought treatment. This means that the drought affected the response to elevated CO_2 and therefore, the slight increase in droughted plants under elevated CO_2 was not significant (the interaction plot is shown in Appendix D)

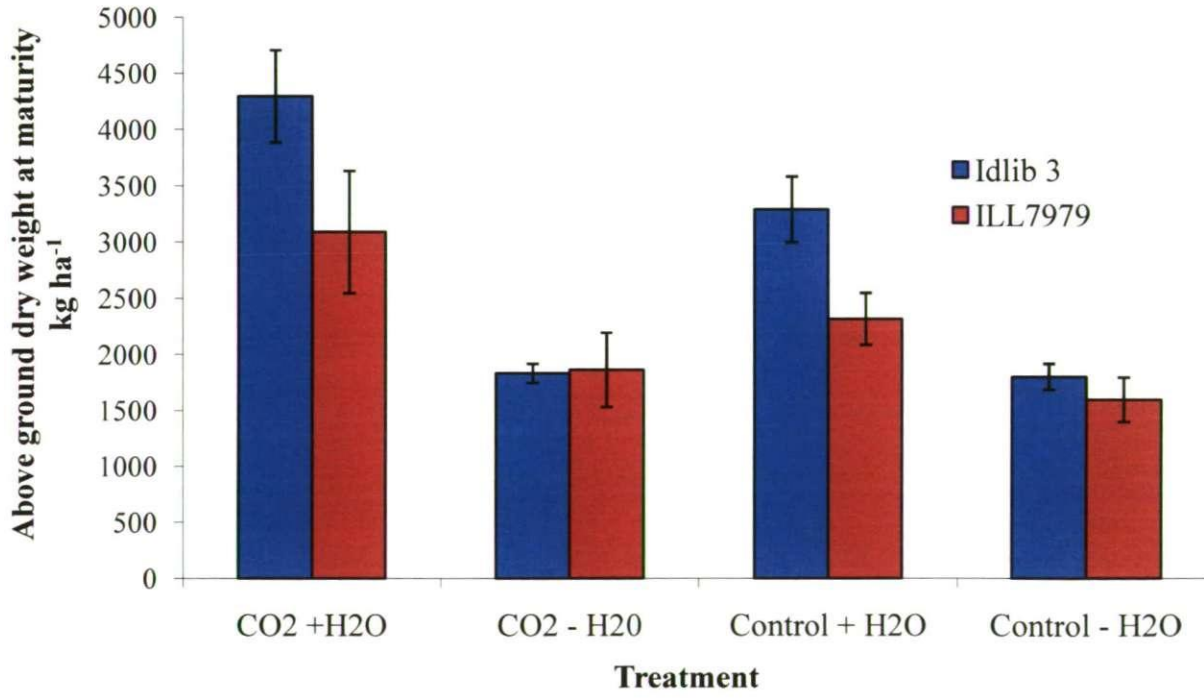


Figure 3.10. Effects of elevated CO_2 and drought on above ground dry weight at maturity in two lentil cultivars (Idlib 3 [ILL6994] and ILL7979) (vertical bars are +/- 1 se). (CO_2= elevated CO_2, +H_2O= full irrigation, - H_2O= water stress, Control= ambient CO_2).

The seed yield varied between 600 and 1800 Kg ha^{-1} equivalent for Idlib 3 (average yield reported by ICARDA is 1010 kg ha^{-1}) (El-Ashkar *et al.*, 2004), and between 500-1100 kg ha^{-1} for ILL7979. Similar to the response in pod number per plant, elevated CO_2 significantly increased the seed yield in the fully irrigated plants and this increase was about 41% for Idlib 3, and 29% for ILL7979, and the significant interaction between CO_2 and drought treatments indicated that CO_2 enrichment effect was not significant when water was restricted (Figure 3.11) (An interaction plot is shown in Appendix E). Seed yield however, was significantly affected by the drought treatment, and was reduced by 53% under ambient CO_2 and by 57% under elevated CO_2 in the cultivar Idlib 3 and by 47% and 40% in the cultivar ILL7979 under ambient and elevated CO_2 respectively.

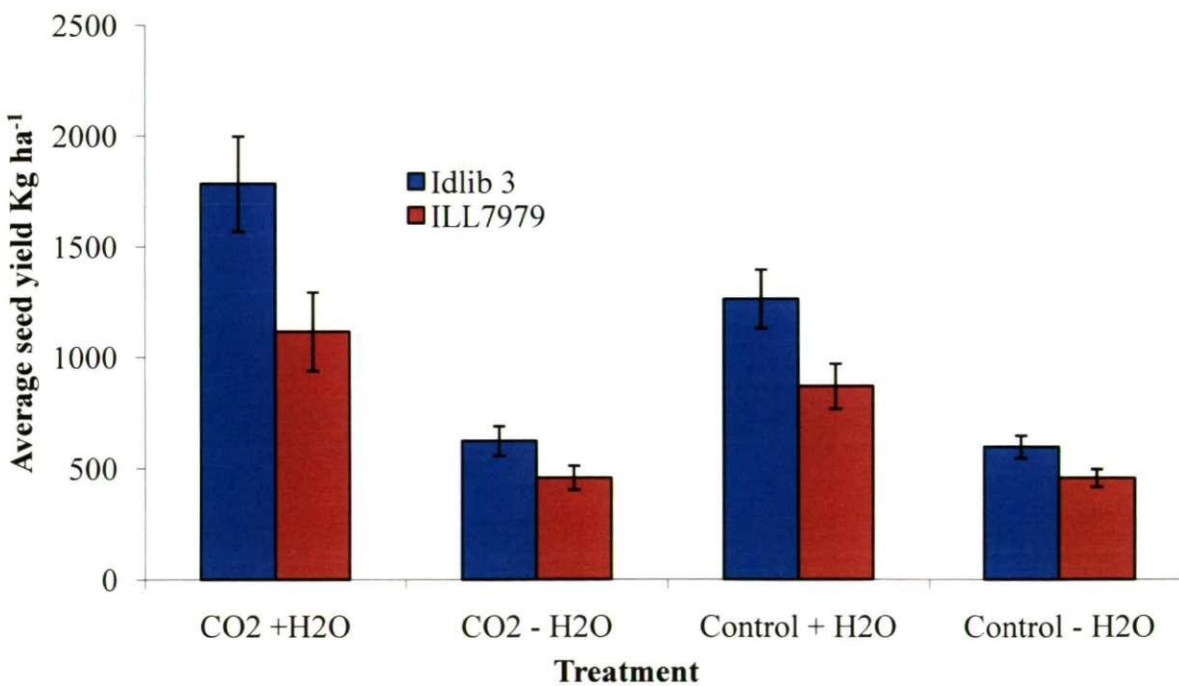


Figure 3.11. Effects of elevated CO_2 and drought on average seed yield in two lentil cultivars (Idlib 3 [ILL6994] and ILL7979) (vertical bars are +/- 1 se). (CO_2= elevated CO_2, +H_2O= full irrigation, - H_2O= water stress, Control= ambient CO_2).

The Harvest Index (HI= seed dry weight/ total aboveground dry weight) ranged between 23% and 41% between treatments (Figure 3.12), which corresponds with values of 19 – 40% for different cultivars of lentils reported by Whitehead *et al.*, (2000). In both varieties HI was not affected by elevated CO_2, but was significantly ($p< 0.001$) reduced by drought under both control and elevated CO_2.

Of the two cultivars, the Harvest Index under all treatments was significantly ($p< 0.001$) higher in Idlib 3. The above ground dry weight,, was significantly higher for Idlib 3 than for ILL7979 under full irrigation conditions, and there was no significant differences between the two cultivars under drought conditions, although it was slightly higher for Idlib 3.

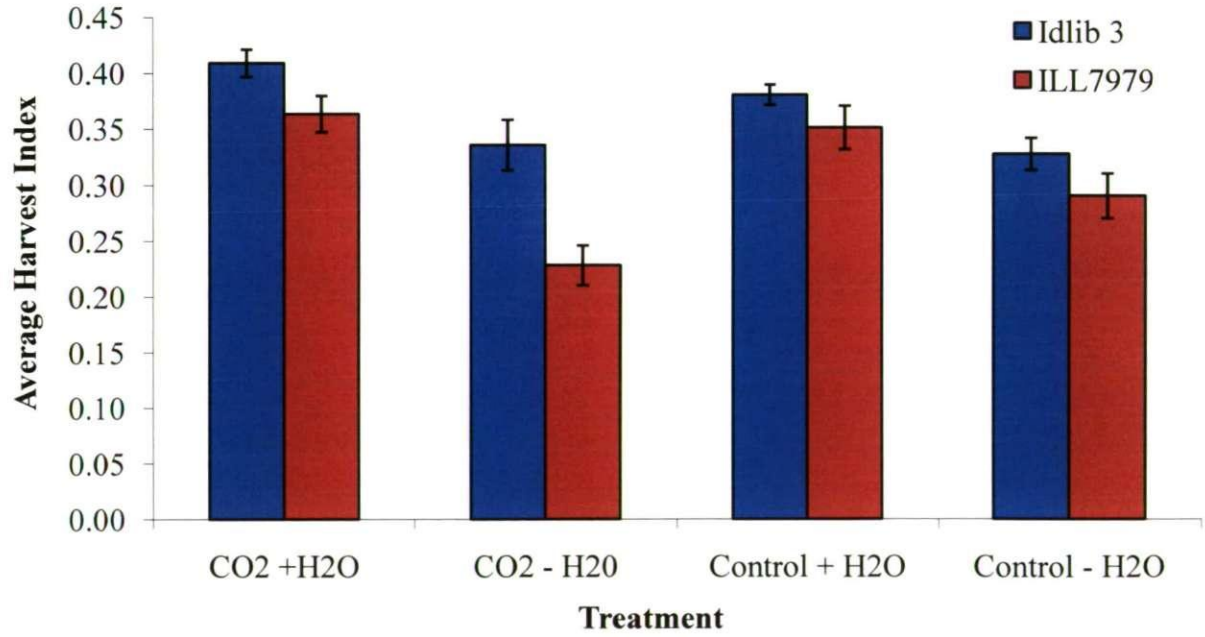


Figure 3.12. Effects of elevated CO_2 and drought on Harvest Index in two lentil cultivars (Idlib 3 [ILL6994] and ILL7979) (vertical bars are +/- 1 se).
(CO_2= elevated CO_2, $+H_2O$= full irrigation, - H_2O= water stress, Control= ambient CO_2).

3.5.2. Experiment 2

3.5.2.1. LAI and total biomass (above and below ground) dry weight at anthesis

The plants of the cultivar ILL7979 flowered 48 days after sowing, and five days earlier than the cultivar Idlib 3. Leaf Area Index at anthesis ranged between 0.92 and 2.44 between treatments, which is less than the recorded values in experiment 1, but still, compares favourably with field measurements for Lentils of 1.5- 5.5 (Wall, 1996). The values of leaf area and LAI were higher under elevated CO_2 but not significantly (p= 0.306). Drought, however, led to a significant decrease of about 33% (P= 0.002) and no significant difference was found between the two cultivars (Figure 3.13).

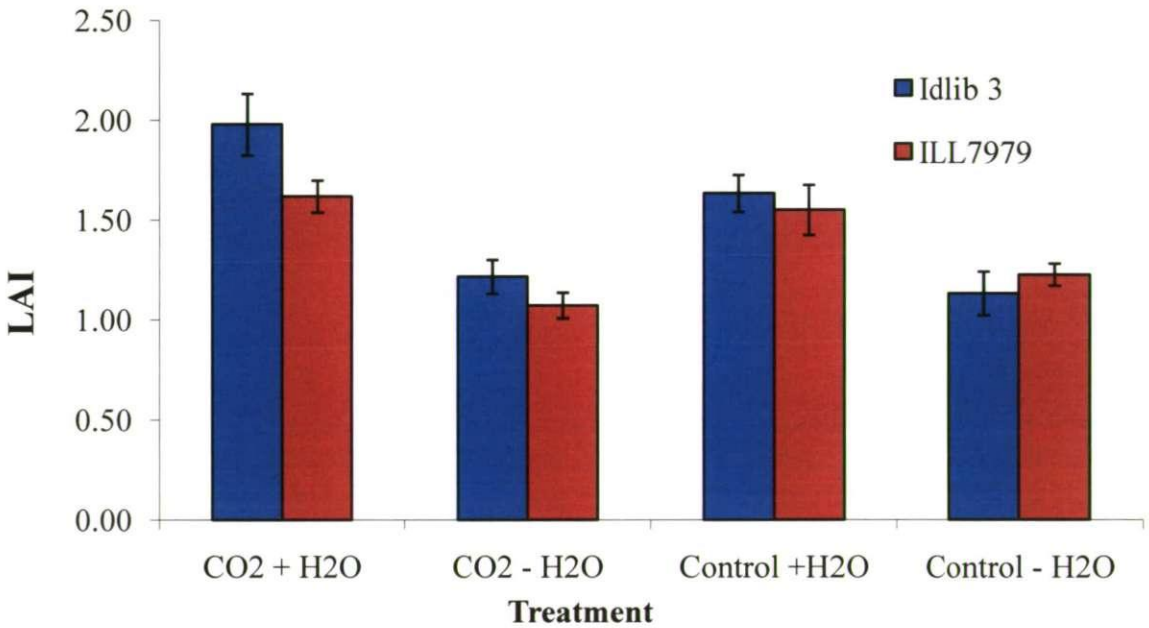


Figure 3.13. Effect of elevated CO_2 and drought on LAI at anthesis of two lentil cultivars (Idlib 3 [ILL6994] and ILL7979) (vertical bars are +/- 1 se) (exp 2). CO_2= elevated CO_2, +H_2O= full irrigation, - H_2O= water stress, Control= ambient CO_2).

Above ground dry weight at anthesis was increased under elevated CO_2, but this increase was only marginally significant ($p = 0.064$). This increase was up to 33 % and 58 % for the cultivar Idlib 3 under full irrigation and water stress conditions respectively. The increase for the cultivar ILL7979 was about 40 % under full irrigation conditions and 22 % under drought (Figure 3.14). On the other hand, drought led to a significant decrease in above ground dry weight ($p < 0.001$) under both ambient and elevated CO_2 for both cultivars. For Idlib 3, the decrease was about 40 % under elevated CO_2 and 48 % under ambient. The decrease was greater in the cultivar ILL7979 and was about 52 % under elevated CO_2 and 47 % under ambient. For all treatments, the cultivar Idlib 3 showed significantly ($p = 0.003$) better results than the cultivar ILL7979 with an average advantage of 20 %.

The root weights for both cultivars under both drought and full irrigation conditions, were higher under the high levels of atmospheric CO_2, however the increase was not statistically significant ($p = 0.187$).

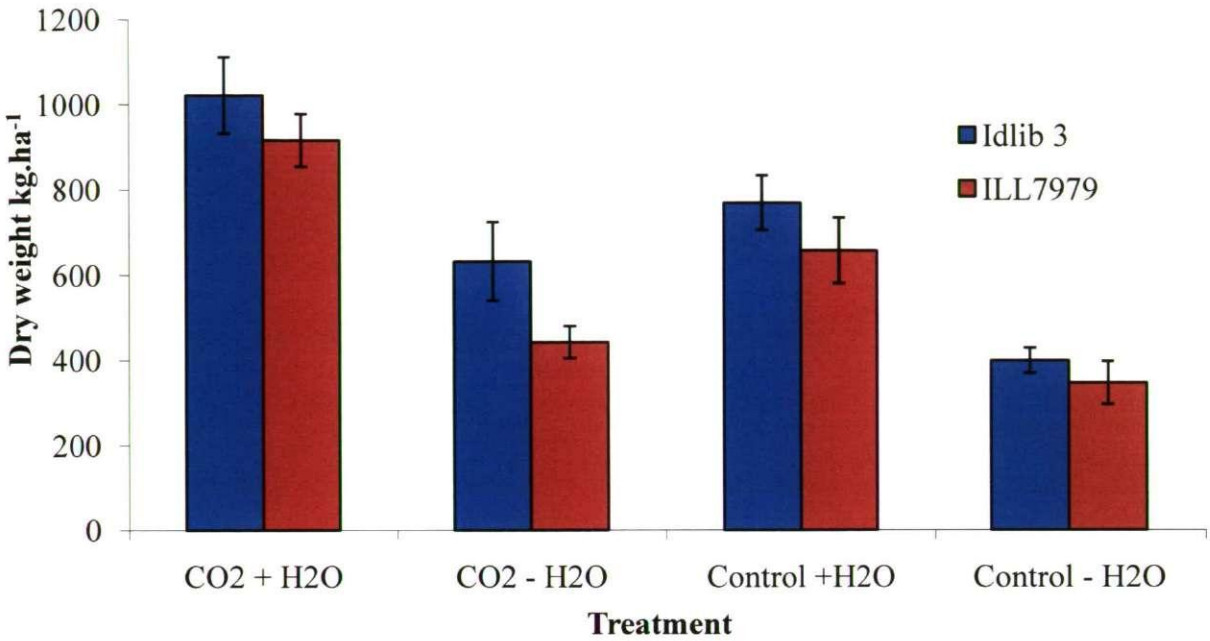


Figure 3.14. Effects of elevated CO_2 and drought on above ground dry weight at anthesis in two lentil cultivars (Idlib 3 [ILL6994] and ILL7979) (vertical bars are +/- 1 se) (exp. 2). (CO_2= elevated CO_2, $+H_2O$= full irrigation, - H_2O= water stress, Control= ambient CO_2).

The roots of both cultivars weighed more under full irrigation conditions but not significantly (p= 0.185), and similarly, there was no significant difference between the two cultivars although it was slightly higher in the cultivar Idlib 3 (Figure 3.15).

Root to shoot ratio, however, did not increase under elevated CO_2 (p= 0.375), and by contrast, when water was limited, the ratio increased for both ambient and elevated CO_2 at a similar pace (p< 0.001). No significant difference was observed between the two cultivars (p= 0.350) (Figure 3.16).

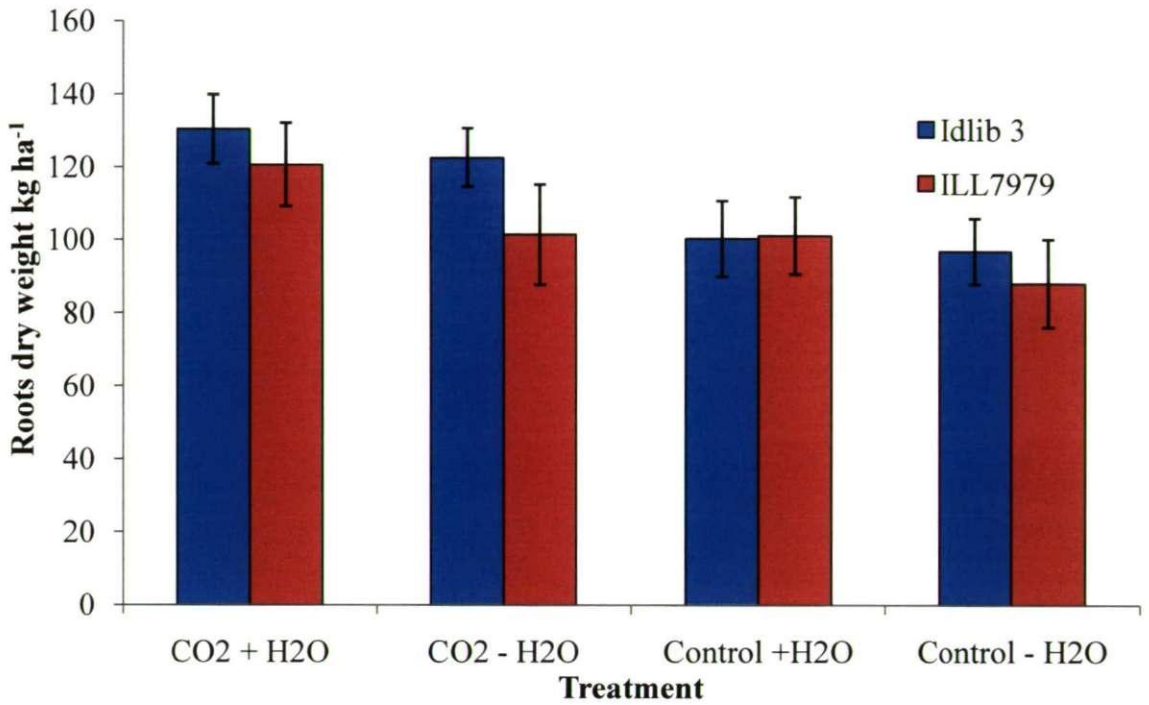


Figure 3.15. Effects of elevated CO_2 and drought on below ground dry weight at anthesis in two lentil cultivars (Idlib 3 [ILL6994] and ILL7979) (vertical bars are +/- 1 se) (exp. 2). (CO_2= elevated CO_2, +H_2O= full irrigation, - H_2O= water stress, Control= ambient CO_2).

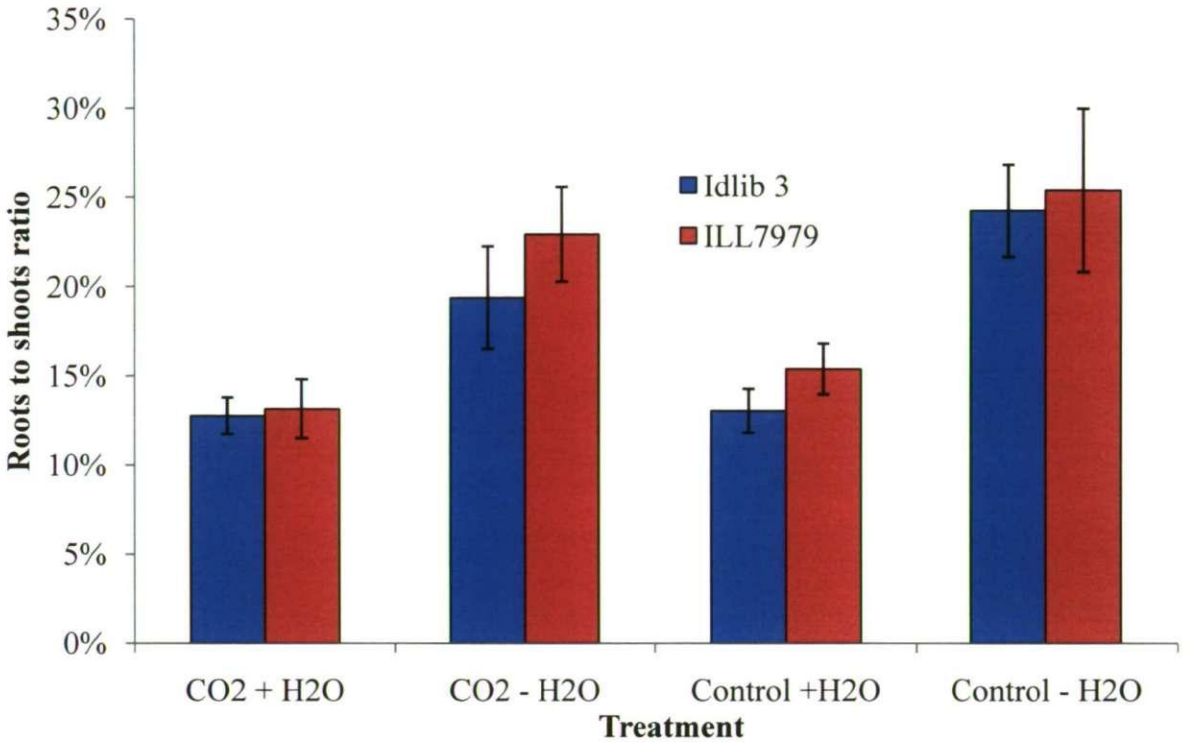


Figure 3.16. Effects of elevated CO_2 and drought on root to shoot ratio at anthesis in two lentil cultivars (Idlib 3 [ILL6994] and ILL7979) (vertical bars are +/- 1 se) (exp. 2). (CO_2= elevated CO_2, +H_2O= full irrigation, - H_2O= water stress, Control= ambient CO_2).

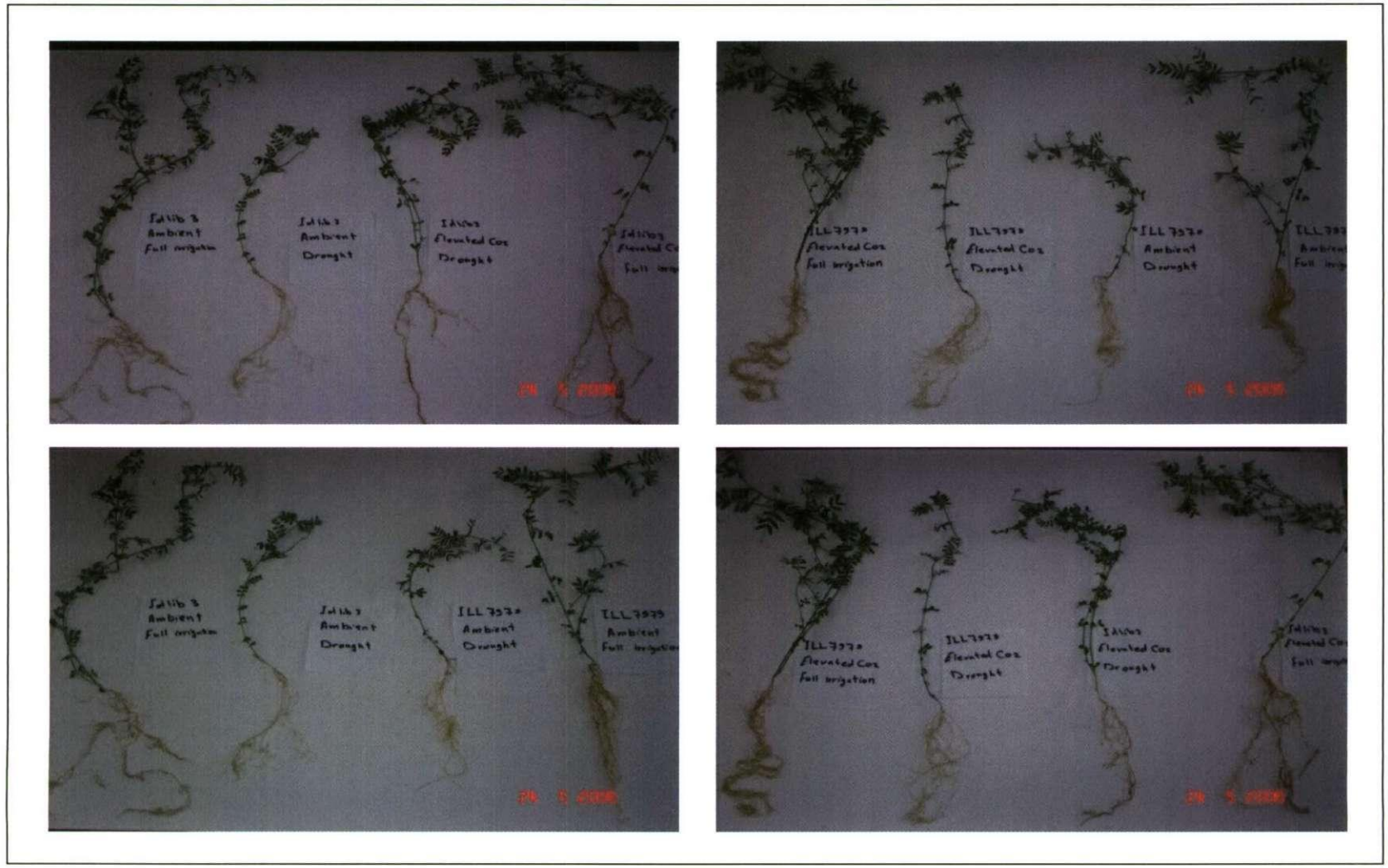


Plate 3.2. To show the plants growth at anthesis under the different treatments.

3.5.2.2. Nodule number and fresh weight at anthesis

Nodule number per plant ranged between 5 and 110 and although it was increased under elevated CO_2 conditions, this increase was not significant (p= 0.213). Nodule number in the cultivar Idlib 3 was increased by an average of 17 % under full water conditions and when water was limited the increase was greater with an average increase of 75 %. However, in spite of this substantial increase, it is still non-significant as the main effect of elevated CO_2 and its interaction with either drought or cultivar were all non-significant. Similarly, nodule number in the cultivar ILL7979 was increased, but non-significantly under elevated CO_2 and this increase was greater under full irrigation conditions with an average of 47 % compared to 29 % when water was limited.. On the other hand, drought significantly reduced nodule number by an average of 42% between the different treatments (Figure 3.17).

.The nodule number in the cultivar Idlib 3 was significantly greater than that for ILL7979 (p= 0.005) under all treatments with an average advantage of about 44%.

Nodule fresh weight was similarly increased under elevated CO_2 by an average of about 45%, but again this increase failed to reach significance (p= 0.265. In contrast drought significantly reduced nodule fresh weight by an average of about 56% (p= 0.004). The nodule fresh weight in the cultivar ILL7979 was slightly higher than that of Idlib 3 but not significantly (p= 0.136). Therefore, although nodule number was greater at all treatments in the cultivar Idlib 3, there was no difference in the fresh weight between the two cultivars as the nodules in the cultivar ILL7979 were heavier and bigger (Figure 3.18).

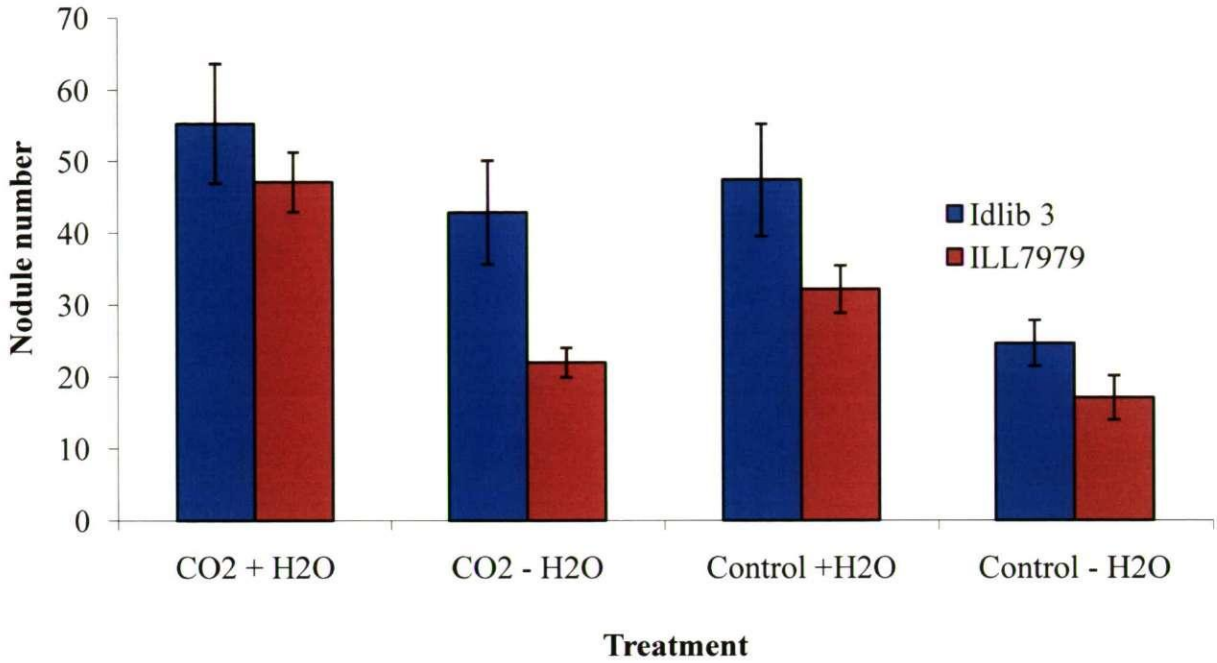


Figure 3.17. Effects of elevated CO_2 and drought on nodule number per plant at anthesis in two lentil cultivars (Idlib 3 [ILL6994] and ILL7979) (vertical bars are +/- 1 se) (exp. 2). (CO_2= elevated CO_2, +H_2O= full irrigation, - H_2O= water stress, Control= ambient CO_2).

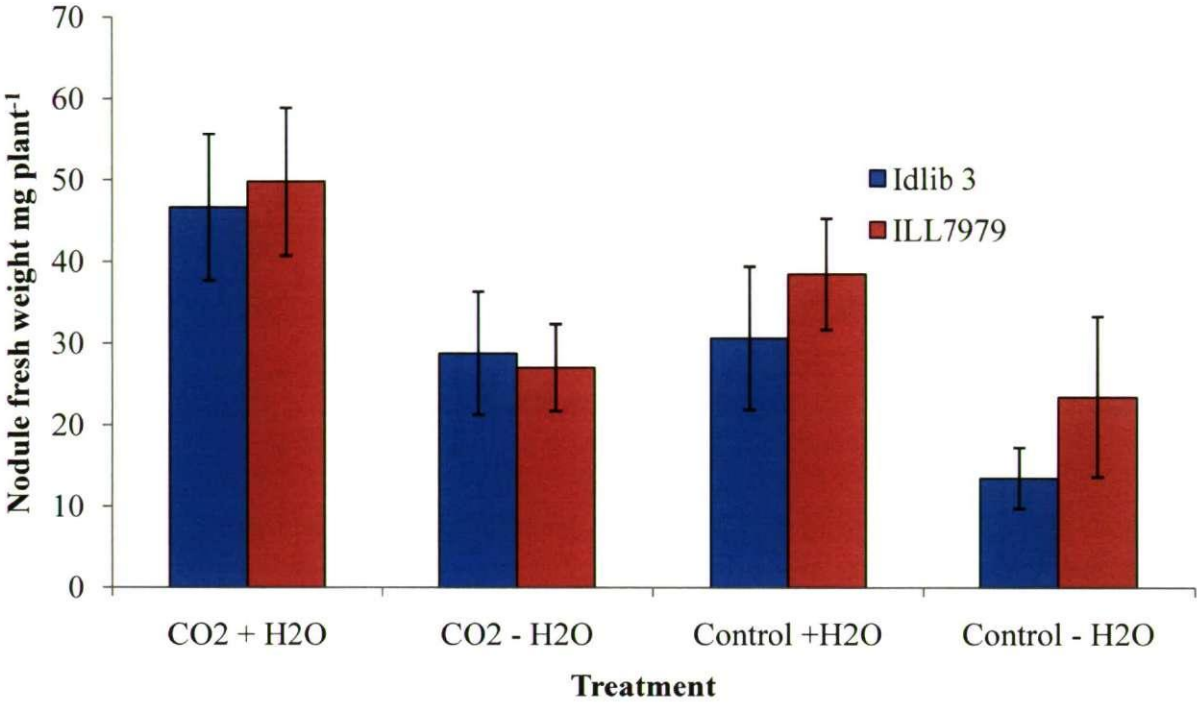


Figure 3.18. Effects of elevated CO_2 and drought on nodule fresh weight per plant at anthesis in two lentil cultivars (Idlib 3 [ILL6994] and ILL7979) (vertical bars are +/- 1 se) (exp. 2). (CO_2= elevated CO_2, $+H_2O$= full irrigation, - H_2O= water stress, Control= ambient CO_2).

3.5.2.3. Final harvest at maturity

The plants were harvested at three dates over a period of three weeks with over 70 % of the water stressed plants being harvested in the first two harvest points. It was also noticed that more than 60% of the plants harvested in the first two harvest points were from elevated CO_2 treatments, which might indicate to faster maturity induced by CO_2 enrichment. In fact, early maturity is a response previously reported in other crops and plant species (Omer and Horvath, 1983; Rogers *et al.*, 1994).

A spider mite infection was diagnosed and a few plants were seriously infected. To manage the problem, the plants were sprayed twice with the recommended chemicals

(Savona' which is a fatty acid "a soap concentrate insecticide and acaricide" (Koppert (UK) Ltd, Homefield Road, Haverhill, Suffolk, CB9 8QP)).

. The infection might have affected the plant growth and the results obtained, but the effect was similar in all treatments and in both cultivars. Compared to ambient CO_2, aboveground dry weight under elevated CO_2 increased by an overall average of about 12%, which was not statistically significant ($p= 0.207$). The effect of drought on the above ground dry weight, however, was more noticeable and led to a significant decrease ($p< 0.001$) of up to 54% with the two cultivars showing no significant difference between them ($p= 0.177$), although the values were slightly higher for the cultivar Idlib 3 (Figure 3.19).

Similarly, below ground dry weight also increased under elevated CO_2 with an overall average of 18.5%, but this increase was not statistically significant ($p= 0.111$). (Figure 3.20). The root weight was significantly decreased by about 30 % when water was limited with a greater decrease under elevated CO_2 conditions. Although, the root weight of the cultivar ILL7979 was slightly higher than that for the cultivar Idlib 3, there was no significant difference between the two cultivars ($p= 0.291$).

As reported in the first harvest at anthesis, the root to shoot ratio was not affected by elevated CO_2 ($p= 0.630$), but it was significantly increased by drought ($p< 0.001$). Although the cultivar ILL7979 showed higher values than the cultivar Idlib 3, these differences were only significant at the 10% level ($p=0.078$) (Figure 3.21).

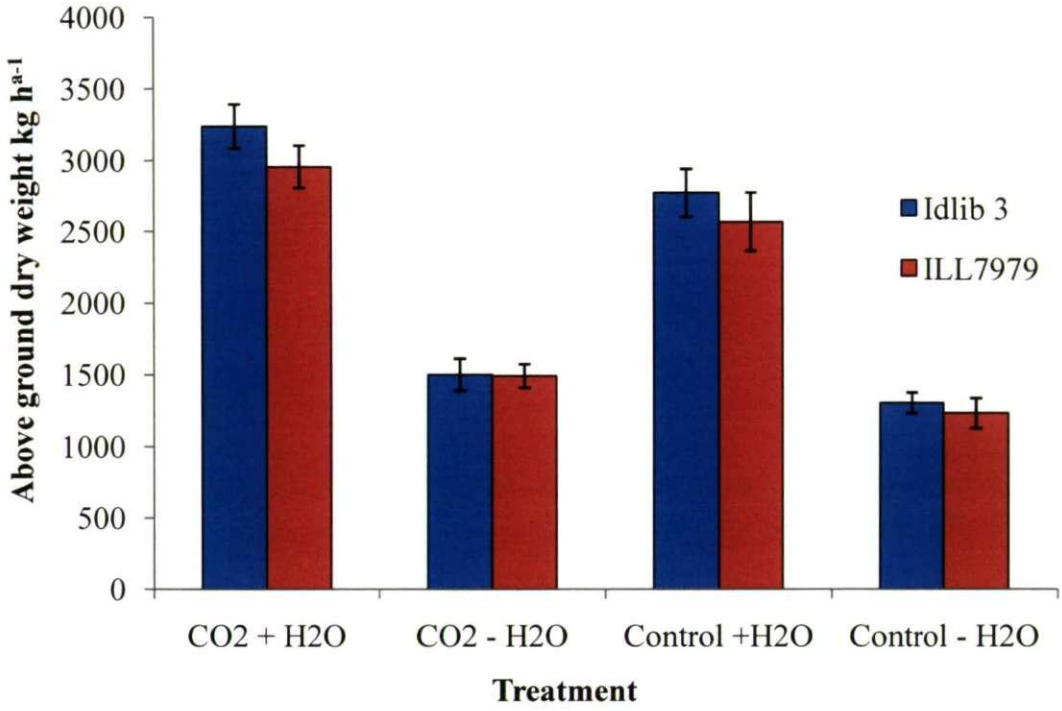


Figure 3.19. Effects of elevated CO_2 and drought on aboveground dry weight at maturity in two lentil cultivars (Idlib 3 [ILL6994] and ILL7979) (vertical bars are +/- 1 se) (exp. 2). (CO_2= elevated CO_2, +H_2O= full irrigation, - H_2O= water stress, Control= ambient CO_2).

Pod number per plant also increased by elevated CO_2 by an average of 16% which was only marginally significant at 10% level ($p= 0.099$), and on the other hand, pod number was significantly reduced by drought ($p<0.001$) with no significant difference between the two cultivars. The number of pods for the cultivar Idlib 3 under elevated CO_2 ranged between 9 and 47 with an average 32 pod per plant under full irrigation conditions and 13.13 under drought conditions compared to 26.9 and 12 pods per plant under full and limited irrigation conditions under ambient CO_2 respectively. Average pod number per plant for the cultivar ILL7979 under high levels of atmospheric CO_2, was 28.6 under full irrigation conditions and 15.25 when water was limited, and under ambient conditions the numbers were 26 and 12.1 with the latter under drought conditions. Such

variation in pod number has been reported before in lentils depending on the genotype and other factors (Saxena and Hawtin, 1981; Malhotra *et al.*, 1974).

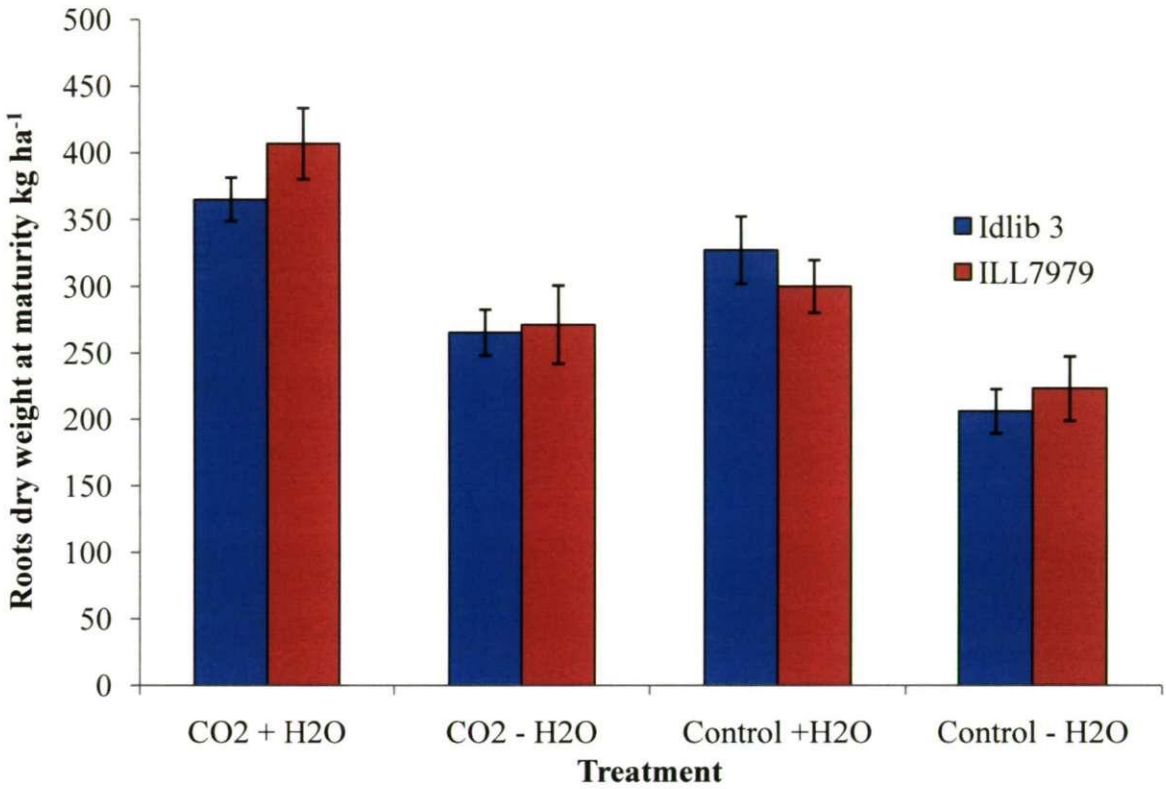


Figure 3.20. Effects of elevated CO_2 and drought on below ground dry weight at maturity in two lentil cultivars (Idlib 3 [ILL6994] and ILL7979) (vertical bars are +/- 1 se) (exp. 2). (CO_2= elevated CO_2, +H_2O= full irrigation, - H_2O= water stress, Control= ambient CO_2).

The seed yield followed a similar pattern of response towards elevated CO_2 and drought as that of pod number (Figure 3.22). However, the two cultivars showed a significant difference with greater yields being achieved by Idlib 3 with an overall average of 1102 kg ha^{-1} (reported average of 1010 kg ha^{-1} by El-Ashkar *et al.*, (2004)) compared to 904 kg ha^{-1} for the cultivar ILL7979. Elevated CO_2 led to an average increase in seed yield of 16 % and 11 % for the cultivar Idlib 3 under full and limited irrigation conditions respectively, and for the cultivar ILL7979, the average increase was 17 % under full irrigation conditions and 33 % under water stress conditions. Therefore, although the

seed yield was higher in the cultivar Idlib 3, the relative increase was higher in the cultivar ILL7979 especially when water was limited.

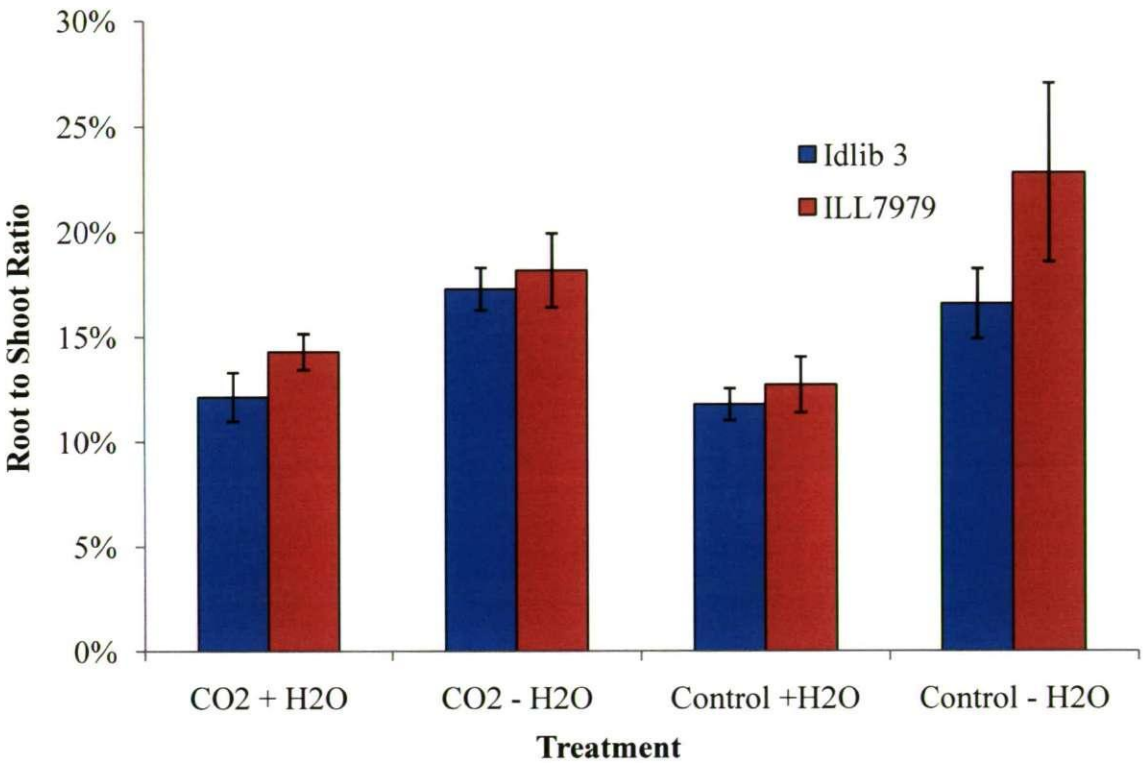


Figure 3.21. Effects of elevated CO_2 and drought on root to shoot ratio at maturity in two lentil cultivars (Idlib 3 [ILL6994] and ILL7979) (vertical bars are +/- 1 se) (exp. 2). (CO_2= elevated CO_2, +H_2O= full irrigation, - H_2O= water stress, Control= ambient CO_2)

The drought effect was very noticeable on seed yield for both cultivars under both ambient and elevated CO_2, and led to a significant decline. The seed yield in the cultivar Idlib 3 decreased by an average of 57 % under ambient CO_2 and even at a higher rate of 61 % under elevated CO_2 as a result of drought. The seed yield in the cultivar ILL7979 reduced by an average of 52 % and 54 % under ambient and elevated CO_2 respectively.

Harvest Index (HI) (ranged between 0.2 and 0.56) was slightly higher under elevated CO_2 but not significantly ($p= 0.128$) and this increase was up to 23 % in the cultivar

ILL7979 when water was limited (Figure 3.23). HI was also significantly reduced by drought ($p< 0.001$), and the cultivar Idlib 3 showed significantly ($p< 0.001$) higher indexes than the cultivar ILL7979 under all treatments.

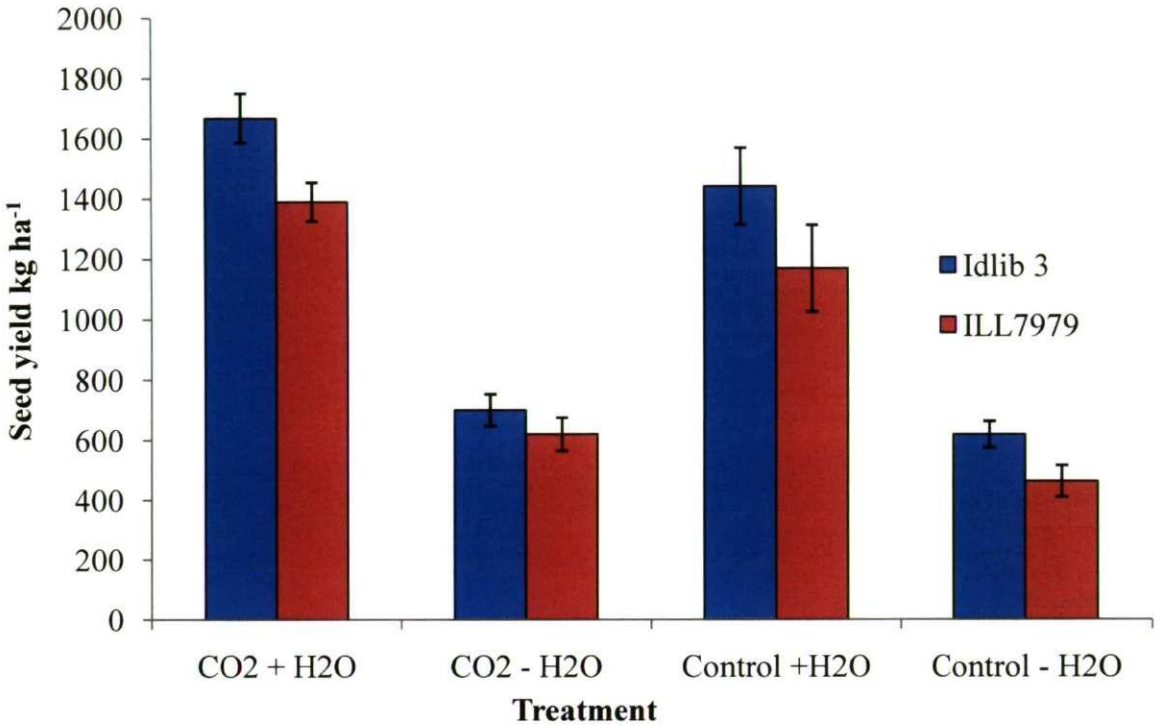


Figure 3.22. Effects of elevated CO_2 and drought on seed yield in two lentil cultivars (Idlib 3 [ILL6994] and ILL7979) (vertical bars are +/- 1 se) (exp. 2). (CO_2= elevated CO_2, +H_2O= full irrigation, - H_2O= water stress, Control= ambient CO_2).

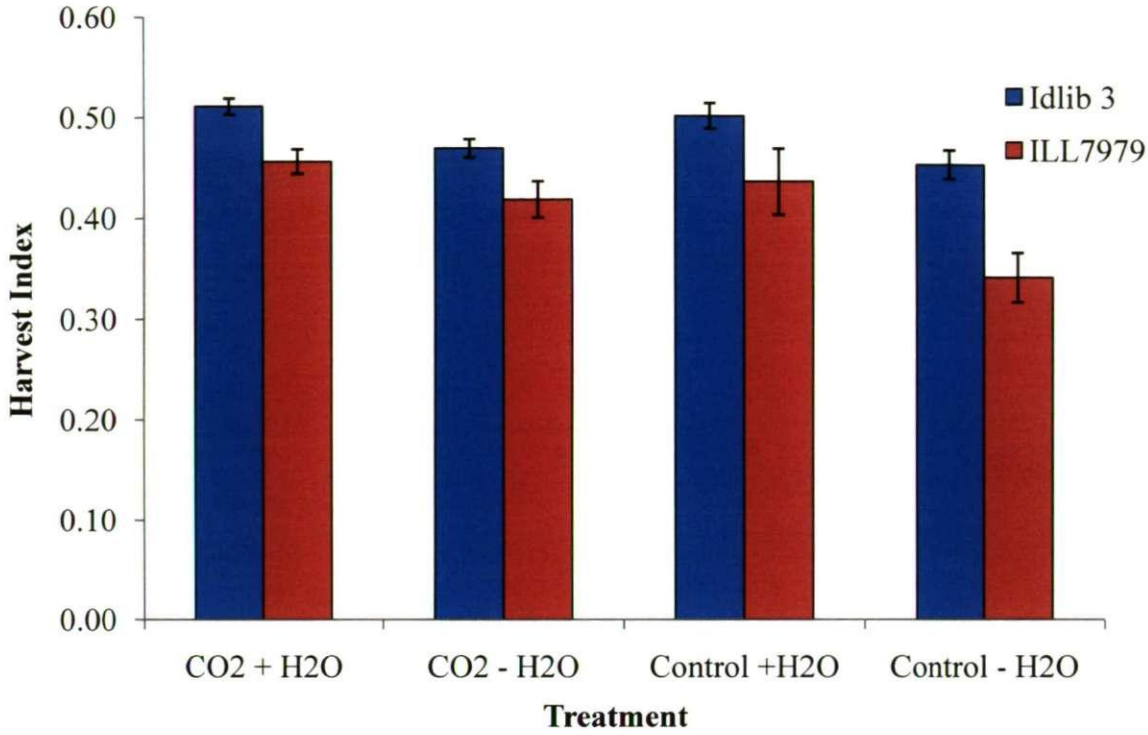


Figure 3.23. Effects of elevated CO_2 and drought on Harvest Index (HI) in two lentil cultivars (Idlib 3 [ILL6994] and ILL7979) (vertical bars are +/- 1 se) (exp. 2). (CO_2= elevated CO_2, +H_2O= full irrigation, - H_2O= water stress, Control= ambient CO_2).

3.5.2.4. Nutrient content

Total nitrogen and protein levels in the seeds which ranged from 21 to 35 % were lower but not significantly affected by elevated CO_2 (p= 0.372) (Figure 3.24) and therefore, the dilution effect usually expected under elevated CO_2 is not significant in this experiment. On the other hand, protein levels were significantly increased under drought conditions (p= 0.019) by an average of about 10 %. Additionally, the cultivar ILL7979 showed significantly higher protein levels (average of 28.35 %) than that of the cultivar Idlib 3 (average of 26.06 %). The nitrogen concentration in the roots followed the same pattern as that in the seeds, but in contrast to that, the shoots N concentration was significantly reduced at high levels of CO_2 (p= 0.004) (Table 3.4).

This suggests that the plants grown under high atmospheric CO_2 concentration were more efficient in diverting nitrogen towards the seeds which are the main final product.

	CO_2 + H_2O		CO_2 - H_2O		Control + H_2O		Control - H_2O	
	Roots	Shoots	Roots	Shoots	Roots	Shoots	Roots	Shoots
Idlib 3	1.51	**0.80**	1.68	**0.96**	1.45	**0.94**	1.65	**1.09**
ILL7979	1.61	**0.98**	1.95	**1.45**	2.17	**1.19**	1.92	**1.72**

Table 3.4. Total nitrogen concentration (g. $100g^{-1}$) in the roots (roots + nodules) and shoots of two lentil cultivars (Idlib 3 [ILL6994] and ILL7979) grown at elevated and ambient CO_2 under drought and full irrigation conditions. (CO_2= elevated CO_2, +H_2O= full irrigation, - H_2O= water stress, Control= ambient CO_2).

The amount of nitrogen uptake was greater under high levels of CO_2, but not significantly so ($p = 0.163$). Although not significant, this slightly higher uptake could be a consequence of the increased nitrogen demand required for the increased growth. The uptake was also significantly increasing when water was not limited with almost double the amounts absorbed compared to that when water was restricted ($p=0.000$). No significant difference in the total nitrogen uptake observed between the two cultivars ($p= 0.814$) (Figure 3.25).

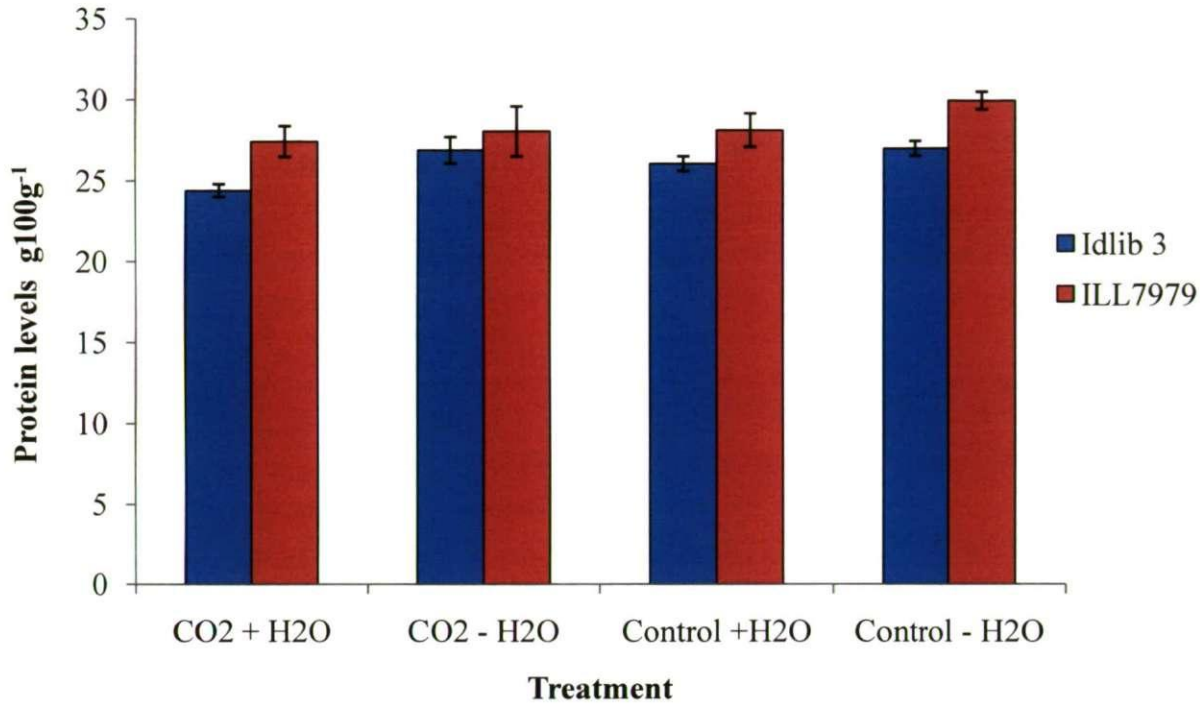


Figure 3.24. Effects of elevated CO_2 and drought on protein levels in the seeds of two lentil cultivars (Idlib 3 [ILL6994] and ILL7979) (vertical bars are +/- 1 se) (exp. 2). (CO_2= elevated CO_2, +H_2O= full irrigation, - H_2O= water stress, Control= ambient CO_2).

Phosphorus levels in the seeds,, followed a similar pattern to that of nitrogen and were reduced by elevated carbon dioxide where biomass production was increased but not significantly (p= 0.257).This reduction was more obvious in the cultivar Idlib 3 as P concentrations reduced by about 8 and 11 % under ample and limited water conditions compared to 3 and 8% for the cultivar ILL7979 (p= 0.045) (Figure 3.26). Furthermore, the phosphorus concentration in water limited plants was not significantly different from that in fully irrigated plants (p= 0.298). Similarly, phosphorus levels in the shoots were significantly reduced at high CO_2 concentration (p= 0.009 for shoots,), and in the roots, the reduction was not significant at 5% level (p=0.083).The concentration in water stressed plants were not significantly different from those of fully irrigated plants (p= 0.077 for shoots, and p= 0.107 for roots). Additionally, phosphorus concentration in

the roots of the cultivar ILL7979 was significantly higher than that of the cultivar Idlib 3 (Table 3.5), but however, these differences in the shoots were only significant at the 10% level (p= 0.093).

	$CO_2 + H_2O$		$CO_2 - H_2O$		Control + H_2O		Control - H_2O	
	Roots	Shoots	Roots	Shoots	Roots	Shoots	Roots	Shoots
Idlib 3	151.65	59.89	158.99	70.97	147.32	75.86	158.97	100.74
ILL7979	156.12	63.90	129.73	83.35	215.76	110.48	187.72	120.07

Table 3.5. Average phosphorus concentration (mg. $100g^{-1}$) in the roots (roots + nodules) and shoots of two lentil cultivars (Idlib 3 [ILL6994] and ILL7979) grown at elevated and ambient CO_2 under drought and full irrigation conditions. (CO_2= elevated CO_2, +H_2O= full irrigation, - H_2O= water stress, Control= ambient CO_2).

The phosphorus uptake, on the other hand, was not significantly affected by elevated CO_2 (p= 0.973), and the cultivar Idlib 3 showed slightly higher uptake, which was only significant at 10% (p= 0.065), than the cultivar ILL7979. By contrast, a considerable reduction of more than 50% in phosphorus uptake was reported when water was limited (p< 0.001) (Figure 3.27).

Examining nitrogen content in the water residual at the final harvest, showed that on average the amount of nitrogen left unabsorbed, although not significantly (p= 0.189), was greater to some extant under elevated CO_2 than that under ambient. This probably can be attributed to the greater number of nodules under elevated CO_2 (although not significantly) which could lead to more nitrogen being released to the roots zone. In addition, the residual nitrogen amounts were significantly higher under drought

conditions ($p< 0.001$) where the nitrogen uptake was significantly reduced. In general, the amounts of unabsorbed nitrogen by the plants of the cultivar Idlib 3 were slightly lower than the cultivar ILL7979 but not significantly ($p= 0.081$) (Figure 3.28). However, Idlib 3 showed higher amounts than that of ILL7979 under elevated CO_2 when water was limited, and in fact a significant interaction was reported (CO_2 × drought: $p= 0.002$ Appendix D) as the two cultivars acted contradictory when water was limited under elevated and ambient CO_2.

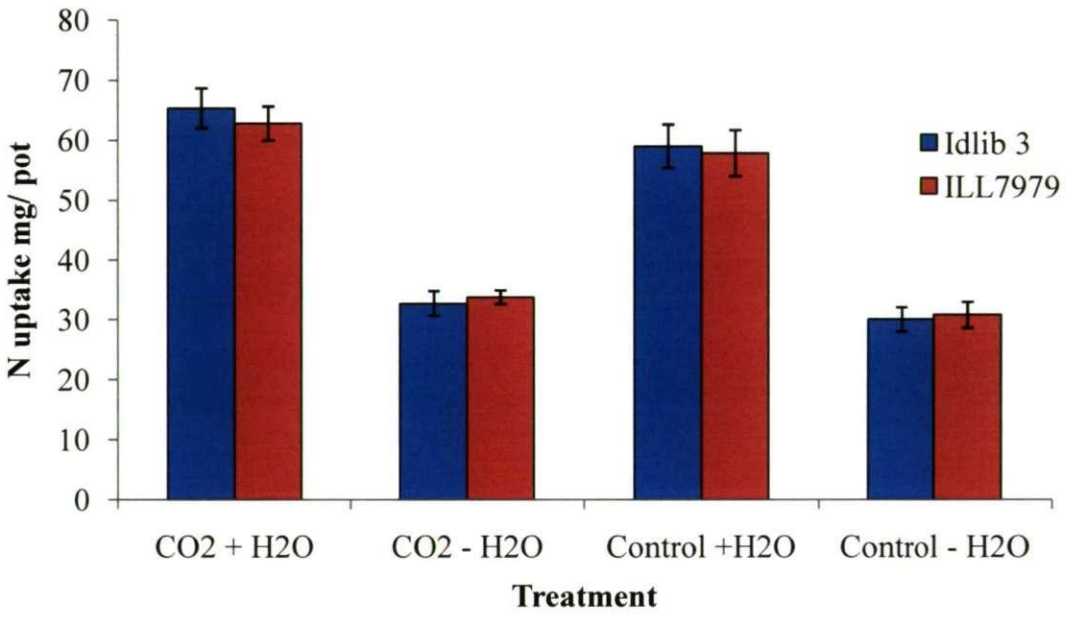


Figure 3.25. Effects of elevated CO_2 and drought on Nitrogen uptake in two lentil cultivars (Idlib 3 [ILL6994] and ILL7979) (vertical bars are +/- 1 se) (exp. 2). (CO_2= elevated CO_2, $+H_2O$= full irrigation, - H_2O= water stress, Control= ambient CO_2).

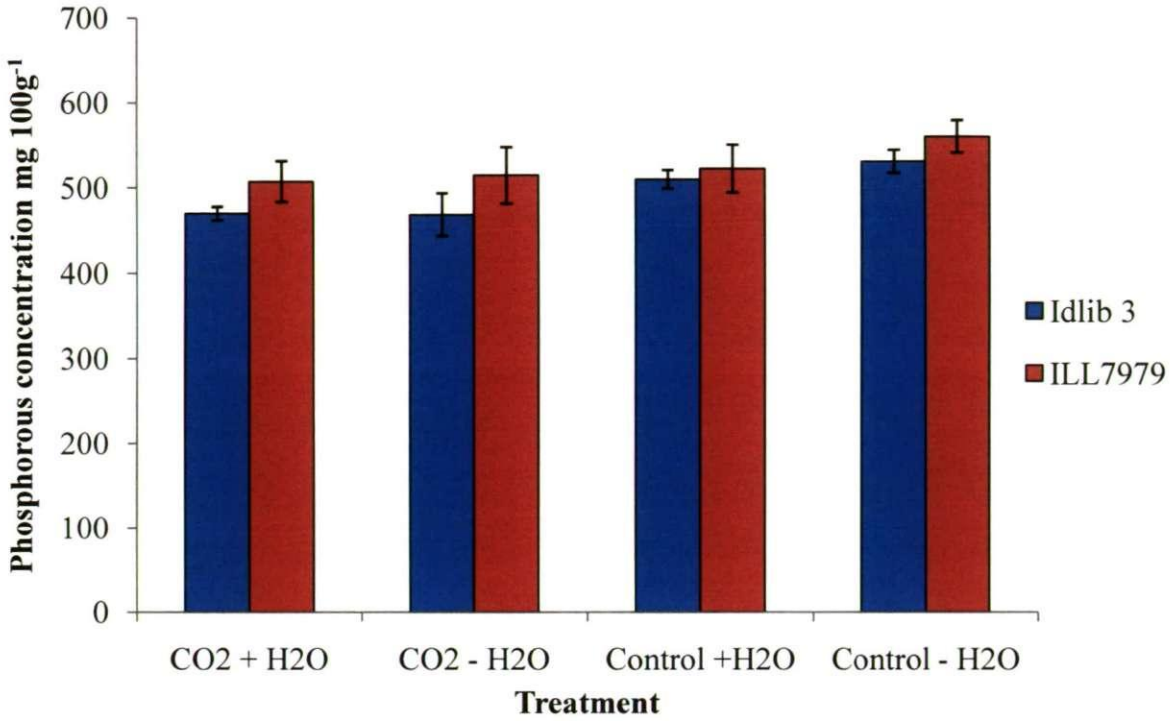


Figure 3.26. Effects of elevated CO_2 and drought on Phosphorous concentration in the seeds of two lentil cultivars (Idlib 3 [ILL6994] and ILL7979) (vertical bars are +/- 1 se) (exp. 2). (CO_2= elevated CO_2, +H_2O= full irrigation, - H_2O= water stress, Control= ambient CO_2).

Similarly, the amounts of unabsorbed phosphorus was not significantly affected by elevated CO_2 (p= 548), although the values were slightly lower under high levels of CO_2 indicating to higher uptake by the increased biomass (Figure 3.29). When water was limited, significantly greater amounts of phosphorus were found in the residual water since the uptake was largely reduced by the restricted roots and shoots growth (p< 0.001). Additionally, the two cultivars showed no significant difference in the amount of phosphorus left in the residuals (p= 0.109), but however these amounts were somewhat lower in the cultivar Idlib 3, which showed slightly higher uptake.

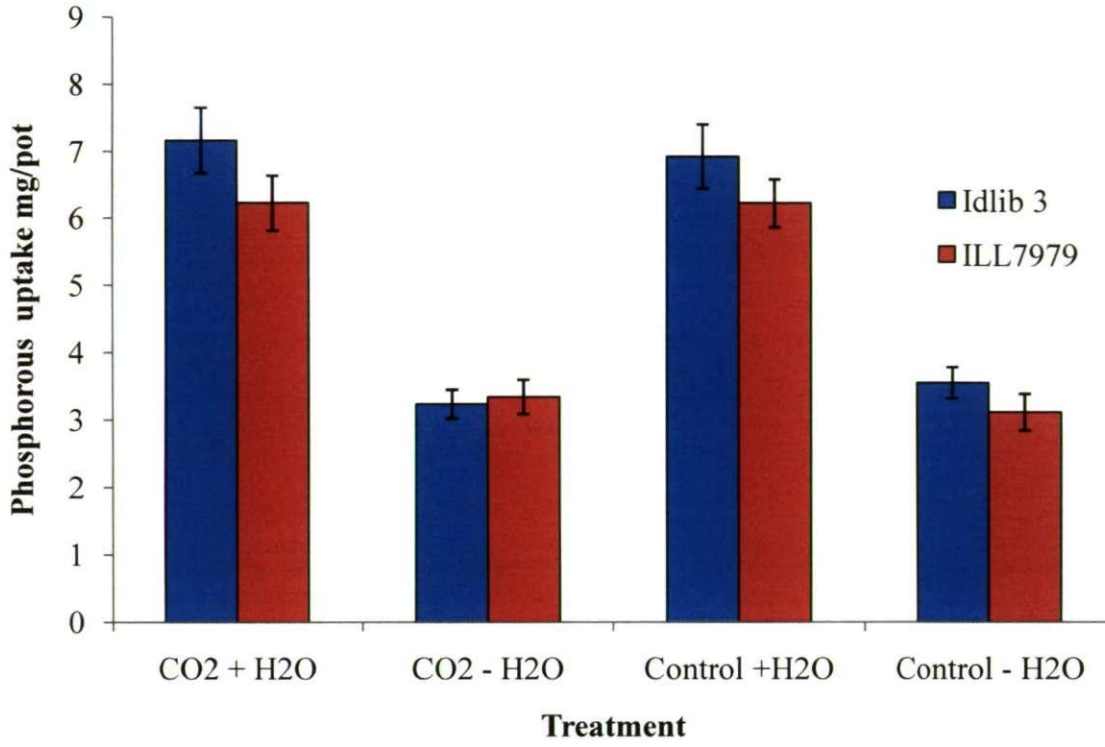


Figure 3.27. Effects of elevated CO_2 and drought on Phosphorous uptake in two lentil cultivars (Idlib 3 [ILL6994] and ILL7979) (vertical bars are +/- 1 se) (exp. 2).
(CO_2= elevated CO_2, $+H_2O$= full irrigation, - H_2O= water stress, Control= ambient CO_2).

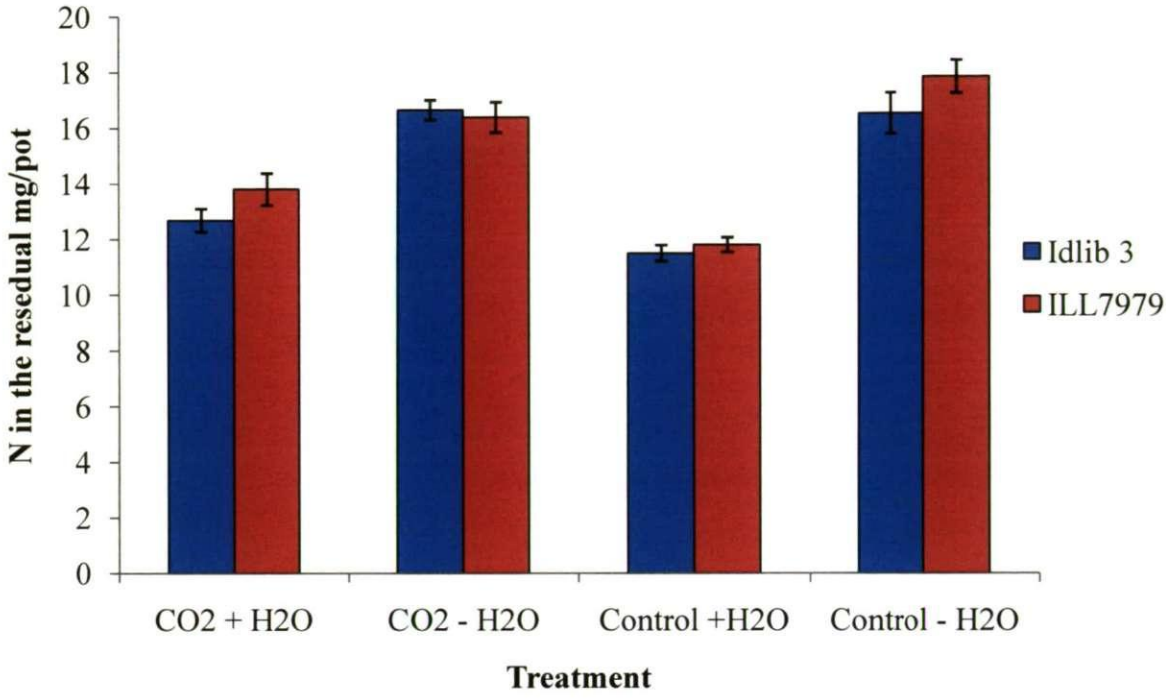


Figure 3.28. Effects of elevated CO_2 and drought on the amount of unabsorbed N found in the residuals of two lentil cultivars (Idlib 3 [ILL6994] and ILL7979) (vertical bars are +/- 1 se) (exp. 2). (CO_2= elevated CO_2, +H_2O= full irrigation, - H_2O= water stress, Control= ambient CO_2).

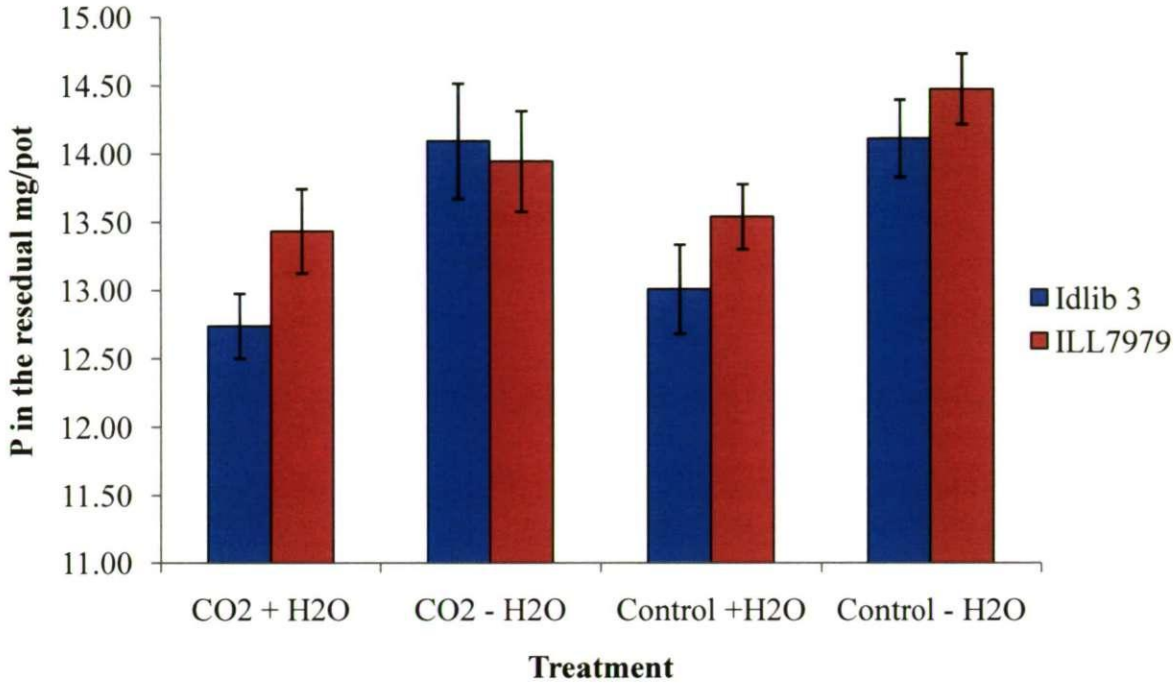


Figure 3.29. Effects of elevated CO_2 and drought on the amount of unabsorbed P found in the residuals of two lentil cultivars (Idlib 3 [ILL6994] and ILL7979) (vertical bars are +/- 1 se) (exp. 2). (CO_2= elevated CO_2, +H_2O= full irrigation, - H_2O= water stress, Control= ambient CO_2).

3.6. Discussion

When harvesting the plants at the first harvest in the first experiment, the average CO_2 in the control chambers was recorded to be about 670 μmol mol $^{-1}$, which was very close to the levels in the elevated chambers (average of 792 μmol mol $^{-1}$) at the same stage. This similarity in treatments meant that, at this stage, although LAI and dry weight were slightly higher at the higher levels of carbon dioxide in the elevated chambers, differences were small and not statistically significant for LAI and marginally significant for dry weight (p=0.078). The experiment however, also showed that LAI in the two varieties was not influenced by drought, but despite this, the

biomass dry weight was significantly reduced by drought. It can be interpreted that the leaves and the stems in the fully irrigated plants must have been heavier and thicker since leaf area was unchanged (i.e. changes in SLA (Specific Leaf Area)). Similarly, in the second experiment the LAI at anthesis was not significantly increased by elevated CO_2 although it was higher, but unlike the results from the first experiment, LAI followed more predictable response and was considerably reduced by drought which is a response reported before (Clifford *et al.*, 1993; Kaddour and Fuller, 2004). Furthermore, although not significantly at 5% level, aboveground dry weight at anthesis for both cultivars in the second experiment was increased considerably under elevated CO_2 (significant at 10%), and this increase in the cultivar Idlib 3 was greater when water was limited (58%) compared to full irrigation conditions (33%). The increase in the cultivar ILL7979 due to elevated CO_2 was higher under full irrigation conditions (40%) than under water stress (27 %).

In the same way, drought in the second experiment affected the growth of both cultivars under ambient and elevated CO_2 conditions leading to a significant decrease in the aboveground dry weight at anthesis. Although there was not any interaction between elevated CO_2, drought and cultivars, it seems that the severity of drought on the cultivar Idlib 3 was less pronounced under elevated CO_2 (40% reduction compared to 48 % under ambient), but this was not the same for the cultivar ILL7979 which in general was more affected by drought and the dry weight was always significantly less than the cultivar Idlib 3. In previous work on durum wheat, Kaddour and Fuller (2004) LAI was not affected by elevated CO_2 (approx 1000 μmol mol $^{-1}$) compared to ambient CO_2, while, the biomass dry weight was increased by elevated CO_2 and this was interpreted as improved photosynthetic capacity leading to improved net assimilation rate. Without

either sequential destructive harvest or direct measurement of photosynthetic rate this can only be inferred. In agreement with the current experiments, Kaddour and Fuller (2004) showed that biomass dry weight was reduced by drought, but that was associated with a drop in LAI, an observation that was not found in the first experiment. The unusual observation of LAI in the first experiment can be attributed to the single sampling date used and an asynchronous development stage in the treatments. Furthermore, it is also reported that in winter sown lentils, the leaf area can continue to increase after flowering (Saxena and Hawtin, 1981), which means that maximum leaf area may not be at flowering.

After the initiation of the flowering stage in the first experiment, CO_2 levels in the control chambers were returned to the target level of approx 400 μmol mol $^{-1}$ creating the difference originally planned between the two treatments of carbon dioxide and thus had significant effects at final harvest. The high levels of CO_2 under well irrigated conditions increased the biomass production and the number of pods per plant, which resulted in an increase in the seed yield of the plants. Since the majority of dry matter in the seeds of lentils comes from assimilation post-flowering (Kumar *et al.,* 1977), it is concluded that the high levels of CO_2 in elevated chambers enhanced photosynthetic capacity and net assimilation rate, and eventually resulted in higher seed yield. This increase in seed yield was about 29% in Idlib 3, and 22% in the cultivar ILL7979. This experiment also demonstrated that when water was restricted in the drought treatment, number of pods and seed yield) were not significantly increased under elevated carbon dioxide. However, it is difficult in this experiment (exp 1) to tell whether the crop is not responsive to CO_2 enrichment under drought conditions, or whether it was a result of the early maturation caused by drought, that did not give the crop enough time to

benefit from the high levels of CO_2 experienced after flowering in the elevated chambers compared with the control.

In the second experiment, the difference in the levels of CO_2 between the chambers maintained over the entire growth period, and biomass production after flowering continued to increase, although not significantly, under elevated CO_2 at full and limited irrigation conditions leading to improved pod number per plant and consequently greater seed yield, which were marginally significant at 10% level ($p=0.099$ for pod number and $p= 0.059$ for seed yield), for both cultivars with the cultivar Idlib 3 showing better results under all treatments (Table 3.6).

	$CO_2 + H_2O$		$CO_2 - H_2O$		Control + H_2O		Control - H_2O	
	Exp 1	Exp 2	Exp 1	Exp 2	Exp 1	Exp 2	Exp 1	Exp 2
Idlib 3	1784.09	1667.71	623.11	683.24	1202.95	1441.83	594.55	618.92
ILL7979	1115.91	1372,02	458.37	614.86	867.51	1169.56	455.91	462.76

Table 3.6. Average seed yield (kg ha^{-1}) in the two experiments of the two the lentils cultivars (Idib 3 [ILL6994] and ILL779) under elevated and ambient CO_2 at full and limited irrigation conditions. (CO_2= elevated CO_2, +H_2O= full irrigation, - H_2O= water stress, Control= ambient CO_2).

Harvest Index is a crude measure of assimilate partitioning and the harvest index (HI) in both experiments was not significantly affected by elevated CO_2although it was slightly higher particularly in the second experiment where it was improved by an average of about 7%. . Results from previous experiments of the effect of elevated CO_2 on HI are mixed. For example, Baker *et al.* (1989) stated a decline in HI in soybean under high levels of atmospheric CO_2, whereas, a significant increase was reported in Wheat

(*Triticum aestivum* L.) (Wu *et al.*, 2004). Also, in a study on two cultivars of rice (*Oryza sativa*), the HI was significantly increased for one of the cultivars (IR-36) but not for the other one (Fujiyama-5) (Ziska and Teramura, 1992). A slight improvement in harvest index was also reported in woody perennial cotton (Mauney *et al.*, 1994), and in woody perennial grape (Pinter *et al.*, 1996).

Generally, other legume crops have shown a positive response to high levels of atmospheric CO_2. For example, seed yield in soybean increased by 32% when grown under carbon dioxide concentration of 630 μmol mol^{-1} and similarly, the increase in cowpeas has been reported to be about 78% (Allen, 1998). In fact, the yield of many crops has been reported to increase under high concentration of CO_2, and levels of about 1000 μmol mol^{-1} are practically exploited in greenhouse production of tomato and cucumber, leading to noticeable rises in the yield (Wittwer, 1986).

In the current second drought experiment, although not statistically significant at 5% level (significant at 10% level), biomass production and seed yield were increased under elevated CO_2 by an overall average of about 12 and 19% respectively with no significant interaction between elevated CO_2 with either drought or cultivar. Likewise, some research showed an enhancement for elevated CO_2 which was not always statically significant at 5%. For example, Navas *et al.*, (1997) in a study on 19 Mediterranean species reported an average enhancement of 20% for final dry mass under CO_2 enriched conditions, but this increase was not statistically significant, Miyagi *et al.*, (2007) in their work on 11 annuals in open top chambers also reported an increase in seed yield in response to elevated CO_2 (700 μmol mol^{-1}) with high interspecific variation (0.84-2.12 elevated/ambient) and the increase was only significant in six plant species. Also, in a study on potato plants (*Solanum tuberosum*)

grown in open-top chambers under different levels of CO_2 and O_3, Lawson *et al.*, (2000) concluded that the effect of elevated CO_2 on above-ground dry weight and tuber yield at the final harvest was no longer significant.

Failing to reach significance at 5% level in the current second experiment in spite of the consistent positive response to elevated CO_2 in all production parameters, is probably due to the experimental design which was restricted by a limited number of chambers or blocks. Furthermore, encountering a spider mite infection during growth could have also probably contributed in restricting the full benefits of elevated CO_2. Also, at one stage after flowering, the pH of the hydroponics dropped to 4.5, and although it was quickly repaired this might have restricted the absorbance of nutrients which may also attributed in reducing the full potential benefits of elevated CO_2. Prasad *et al.*, (2005) indicated that the absence of abiotic and biotic stresses boosts the response of grain legumes to elevated CO_2. A combination of the experimental conditions mentioned above may have influenced the magnitude of the plants response to elevated CO_2.

In the second experiment, the increase in seed yield (significant only at 10% level) in water stressed plants under elevated CO_2 was about 20% on average. In the cultivar ILL7979 the increase in seed yield was proportionately even greater when water was restricted (33 %) than that under full irrigation conditions (17 %), which is in fact an observation supported in previous works on other crops (Gifford, 1979; Kimball *et al.*, 1995) but, this can not be conclusive as no such observation was reported in the first experiment and further investigation is needed. Gifford (1979) and Kimball *et al.*, (1995) both reported a greater response to elevated CO_2 in wheat when water was

limited, and in general, the enhancement of elevated CO_2 to the economic yield of different crops was not reduced by water limitations (Gifford, 1979; Sionit *et al.,* 1980). On the whole, previous experiments on different plants under drought have shown positive effects of elevated CO_2 on production. In work on durum wheat, Kaddour and Fuller (2004) found that production and yield under water restriction conditions was improved by CO_2 enrichment and with no increase in LAI, the yield increase was attributed to an increase in net assimilation rate and water use efficiency. Similar results were also found in earlier work on *Triticum sativum* (Sionit *et al.,* 1980). Additionally, in work conducted on 1 year-old peach seedling, CO_2 enrichment under water stress conditions increased total dry mass by 31%,and improved water use efficiency (WUE) by 63% (Centritto, 2002). Therefore, it is believed that the increased production under elevated CO_2 can mainly be attributed to the increase in photosynthesis and net assimilation rate (Allen, 1998), and partly to the reduced transpiration rate due to reduced stomatal opening which delays the onset of drought (Rogers *et al.,* 1984; Samarakoon and Gifford, 1996).

The demand of increased assimilation rate under high levels of atmospheric CO_2 places a demand for other resources to increase, which put a pressure on the capture of these resources from the environment (Gifford, 1979). Therefore, under atmospheric CO_2 enrichment, root density, root length and root spread could be enhanced and lead to greater water capture and consequently improving the growth rate (Rogers *et al.,* 1994). In the current investigation, results showed that although there was not a significant difference, root biomass increased under elevated CO_2 at both irrigated and water restricted conditions by an overall average of about 17 %. It is well known that roots biomass respond positively to elevated CO_2 sand in crops such as *Lolium perenne* and

Trifolium repens (Jongen *et al.*, 1995), and cotton (*Gossypium hirsutum* L), the length and roots dry weight and density were increased (Prior *et al.*, 2005). In wheat, root growth increased during all development stages (Wechsung *et al.*, 1999) and maximum length was faster achieved under CO_2 enrichment conditions (Chaudhuri *et al.*, 1990). In sour orange tree (*Citrus aurantium* L.) the fine roots biomass was also improved (Idso and Kimball, 1992). Rogers *et al.*, (1994) in their review, indicated that in all the examined studies of the effect of elevated CO_2, the dry weight of the roots always increased by CO_2 enrichment regardless of the species or the experimental conditions.

As mentioned earlier, in the current investigation (exp 2), the increase in root biomass was not statistically significant, and this could be a result of the use of small pots to grow the plants, and in fact, previous reports indicated that root growth under elevated CO_2 can be restricted in small pot experiments (Arp, 1991; Nobel *et al.*, 1993; Rogers *et al.*, 1994). Additionally, it is possible that the spider mite infection and the drop in pH levels have affected the overall growth of the plants including the roots.$_2$, The root to shoot ratio did not change under elevated CO_2 indicating that the assimilated carbon allocation between roots and shoots was not affected by elevated CO_2. In fact, little change or even reduced root to shoot ratio is recorded by other workers (Norby, 1994; Gavito, 2000; Ainsworth *et al.*, 2002). Chu *et al.*, (1992) in a study on wild radish, stated that elevated CO_2 showed no effect on root/ shoot partitioning. Eamus and Jarvis (1989) also found no effect of CO_2 enrichment on root/shoot ratio of temperate tree saplings. Some other studies, however, have shown an increase of root/shoot ratio under elevated CO_2. For example, Rogers *et al.*, (1994) when examined results from previous studies conducted on the effects of elevated CO_2 on different plant species, he reported increased root/shoot ratio in 41 % of the cases.

The nodule number and nodule fresh weight for both cultivars at both water conditions were increased by CO_2 enrichment, by an average of 38% and 45% respectively, but this increase was not significant. Although not significant, this considerable increase can be explained as a response to the greater source of the extra amounts of carbohydrate assimilated in the plants due to the increase in photosynthetic rates (Arnone and Gordon, 1990). It is not unusual that nodule biomass and nitrogen fixation increase under high levels of atmospheric CO_2 (Philips *et al.,* 1976; Masterson and Sherwood, 1978; Finn and Brun, 1982; Norby, 1987; Temperton *et al.,* 2003; Cen and Layzell, 2004). Serraj *et al.,* (1998) reported that when soybean plants were grown under water limited conditions, CO_2 enrichment delayed the decrease in nitrogen fixation associated with the drying soils and increased nodule number and weight, which is an observation in agreement to some extent with the outcomes of the current experiment (exp 2) as nodule biomass in water stressed plants was doubled under CO_2 enrichment conditions, although this was not statistically significant It was also noticed that relatively, the average improvement in nodule number and fresh weight under elevated CO_2 was higher in water stressed plants as nodule number and fresh weight were increased by an average of 55% and 50% respectively compared to 29% and 40% under full irrigation conditions. In general, higher nodule number and biomass was previously reported in response to atmospheric CO_2 enrichment (Philips *et al.,* 1976; Masterson and Sherwood, 1978; Finn and Brun, 1982; Temperton *et al.,* 2003). On the other hand, a non significant response to elevated CO_2 was also previously reported. For example Feng *et al.,* (2004) indicated that although nodule number in plants of 1-year-old Robinia pseudoacacia was doubled in response to high levels of CO_2 (700 μmol mol^{-1}), this was not statistically significant, and this was attributed to the great variability of root nodule formation. Aranjuelo *et al.,* (2008) also reported that nodule

production was not significantly increased in alfafa plants grown at elevated CO_2 levels of 700 μmol mol^{-1}.

When nitrogen content is concerned, results from the second experiment showed that total nitrogen uptake by the plants was higher under high levels of atmospheric CO_2 by an overall average of 15%, but this was not statistically significant. Analysis of the unabsorbed nitrogen in the residual water showed that there was not any significant difference between the ambient and elevated CO_2 treatments, and even the unabsorbed nitrogen in the elvated CO_2 treatment was higher by an average of 3% than that under ambient CO_2. This suggest that the 15% greater uptake observed under elevated CO_2 was mainly due to the increase in the process of nitrogen fixation, and the extra amounts of nitrogen found in the residual water of the elevated CO_2 treatment can be another indication of improved nitrogen fixation where some nitrogen could be released from the nodules to the surrounding area of the roots.

As photosynthesis and symbiotic nitrogen fixation are closely linked with the latter depending on the former (Wheeler, 1971; Lawrie and Wheeler, 1974; Hardy and Havelka, 1976), hence, it is not unusual that nitrogen fixation would be improved under elevated CO_2, which in actual fact was previously reported as a result of increased nodule weight and/or activity (Zanetti *et al.,* 1995; Díaz, 1996, Zanetti *et al.,* 1996). Reich *et al.,* (2001), in a study on 16 grassland species, found that CO_2 enrichment led to an increase in biomass and in total plant nitrogen in two of the four legume species studied. Significant enhancement to nitrogen fixation under elevated CO_2 was also reported in white clover (Soussana and Hartwig (1996), pea (Philips *et al.,* 1976), soybean (Hardy and Havelka, 1976) and other legumes.

In the current study, nitrogen and protein concentration in the seeds, which was increased under elevated CO_2, but at the 10% level ($p=0.059$) tented to be lower but not statistically significant, and this was most likely due to a clear enhancement of symbiotic nitrogen fixation. Although these findings contradict with previous findings on other crops which in general indicate to lowered nitrogen and other minerals concentration under elevated CO_2 (Wong, 1979; Baxter *et al.*, 1994; Reich *et al.*, 2001; Fangmeier *et al.*, 2002; Taub *et al.*, 2008), it comes in agreement with other works. For example, Rogers *et al.*, (1983) reported no change in protein percentage in the soybean seeds of plants grown under high levels of CO_2, and similarly, Havelka *et al.*, (1984) reported a similar response in wheat.

Results from the current experiment also showed a significant reduction in nitrogen concentration of the shoots of the plants grown under elevated CO_2, unlike that of the seeds and the roots, and a suggestion that elevated CO_2 altered N allocation between the plant parts could be made. In fact, in a review of the effects of rising levels of CO_2 on pasture species and communities, Newton (1991) reported a general decrease in nitrogen concentration in the shoots while that in the seeds was unchanged. A reduction in shoot N to root N in plants of *Danthonia richardsonii* (Wallaby Grass) under elevated CO_2 was also reported by Lutze and Gifford (1998).

Phosphorus concentration, however, followed the general trend and was significantly lower at elevated CO_2, and despite the non-significant slightly higher total uptake, the effect of elevated CO_2 was not significant.

Generally, total nutrient uptake decreases when water is limited (Garg, 2003; Hu and Schmidhalter, 2005), and the outcomes of the current study stress the same results as the uptake of nitrogen and phosphorus was halved in most of the cases.

Furthermore, usually the decreased nutrients uptake when drought is imposed frequently results in reduced concentration for these nutrients (Garg, 2003; Brown *et al.,* 2006), which in fact an observation was not reported in the current experiment.

Although the uptake of both nitrogen and phosphorus was greatly reduced by drought, by contrast, the concentration of nitrogen was significantly increased (apart from that in the shoots), and that for phosphorus was unchanged. Symbiotic nitrogen fixation must have helped in providing additional amounts of nitrogen and with reduced growth and biomass under water stress the concentration increased. Jin *et al.*, (2006) reported similar outcomes where nitrogen concentration in soybean increased when water was limited, and when phosphorus was sufficiently adequate, the effect of drought on plants growth, phosphorus accumulation and grain quality was improved. Also, in a study on corn (*Zea mays* L.), Rafiee *et al.,* (2004) found that, nutrient concentration in the flag leaves decreased but that in the seeds were increased as the seed yield decreased.

Conclusions

LAI in the first experiment was not affected by elevated CO_2 , and dry weight at anthesis was marginally significantly increased (p=0.078) at both drought and full irrigation conditions. At maturity, elevated CO_2 increased above ground dry weight, number of pods per plant and seed yield. However there was a significant interaction between CO_2 and drought treatments, which indicated that pod number and seed yield were not affected by elevated CO_2 in water stressed plants and only significantly increased in fully irrigated plants. Technical difficulties encountered and uncertainty about the statistical analysis cast some doubts on the conclusions made from this first experiment, and therefore, less confidence can be attached to them.

Results from the second experiment showed that LAI at anthesis was not significantly affected by elevated CO_2 although it was slightly higher. Above ground dry weight at anthesis was only marginally significantly higher at elevated CO_2 treatment (p= 0.064), and root weight also was increased but this increase was not statistically significant. At maturity, elevated CO_2 increased above and below ground dry weight by an average of 12 % and 17% respectively, but this effect was not statically statistically significant. Pod number and seed yield were also increased by elevated CO_2 by an average of 15% and 19% respectively and this increase however, was only significant at 10% level (p= 0.099 for pod number and p= 0.059 for seed yield). There was no significant interaction between drought and CO_2 treatments in any of the production parameters, and the response to elevated CO_2 in the water stressed plants was in the same pattern to that in the fully irrigated plants, but sometimes, the percentage or relative increase was even greater than that in the fully irrigated plants.

Although not statistically significant, both nodule number and fresh weight per plant responded positively to elevated CO_2 in both water stressed and fully irrigated plants, and nodule number increased by an average of 38% and nodule fresh weight by an average of 45%. This improvement in nodule biomass must have helped improve the process of nitrogen fixation, as nitrogen uptake was increased by an average of 15% which is considered to be mainly due the enhancement of nitrogen fixation due to increased nodule biomass or/and nitrogenase activity. In response, although seed yield marginally significantly increased under elevated CO_2, nitrogen concentration in the seeds was not significantly altered. Similarly, phosphorus concentration in the seeds was not significantly affected.

Drought depressed the LAI, the above and below ground dry weight at anthesis and maturity, the nodule number, the number of pods and the seed yield, but on the other hand nitrogen concentration was improved and phosphorus concentration was not affected by the water stress treatment. Although, elevated CO_2 did not fully compensate for the loss due to drought, the effect of drought was at least marginally mitigated and seed yield in the droughted plants was increased by an average of 20% in the second experiment. Therefore, overall, it can be concluded that lentils positively responded to elevated CO_2 and the effects of drought can be partially mitigated by a rise atmospheric carbon dioxide, this response however is only marginally significant (10% level).

The cultivar Idlib 3 showed higher yields results under all treatments, despite the fact that proportionate increases were sometimes relatively higher under elevated CO_2 in the cultivar ILL7979. The cultivar Idlib 3 still emerges as the most favourable cultivar for future cultivation in the Mediterranean region.

3.7. Critique of experimental conditions in the first experiment (open-top chambers)

Some critiques of the experimental conditions can be levelled at the first experimental set up. In standard conditions in enclosed environments, irradiance levels of about 400 μmol m^{-2} s^{-1} are normally required for cool season crops (Tibbitts and Langhans, 1993), such as lentils. In our experiment, the irradiance varied between the chambers in a range of 180-280 μmol m^{-2} s^{-1}, and this may have affected photosynthetic capacity and maximum production in the crop, and it is possible that these levels of lighting may be preventing the plants from making the maximum use of elevated levels of carbon dioxide.

Despite this criticism, seed yields and harvest indices were comparable to reported field levels which lend confidence to the growing conditions. Achieving high irradiance levels in growth chambers are technically demanding with high energy usage and overbearing problems. Consideration of moving the whole experimental set-up into natural light conditions in a glasshouse was given and with relocation to the Plymouth campus in the midst of this study (closure of the Seal-Hayne campus), the second chamber design was implemented.

Another major problem was the control of carbon dioxide levels in the chambers. It is known that CO_2 is the most difficult environmental parameter to control as its concentration can easily be affected by people around, heating systems and other sources producing CO_2 (Anon,2004). Due to technical malfunction and leakage between chambers, CO_2 levels were very difficult to control in the first chambers and this affected the results during the early stages of the experiment. As a result, the

experimental set up was considered unreliable, and move to tightly sealed and ventilated chambers was proposed for future experiments and the newly designed chambers were used in the second experiment in this chapter and in all subsequent experiments.

4. Chapter 4

The Effect of Elevated CO_2 and Nitrogen Levels on the Growth and Nodulation of Lentils

4.1. Introduction

The photosynthetic rate in C_3 species under the current levels of ambient CO_2 is below physiological saturation levels and it is anticipated that photosynthesis should be stimulated by the higher levels of atmospheric carbon dioxide (Allen, 1998; Drake *et al.*, 1996). As a consequence, it is expected that the primary productivity for most crops will increase under increased atmospheric levels of CO_2 (White, 2001; Houghton, 1997), and a large number of experiments have shown that doubling CO_2 levels leads to an average increase in yield of about 30 % to 40% (Kimball, 1983; Cure and Acock, 1986). However, plant responses to elevated CO_2 are species dependent and are limited by other factors such as light, temperature, water and nutrients (Acock, 1990; Patterson and Flint, 1990). Under elevated CO_2, the demand for nutrients is increased as the growth increases (Rogers *et al.*, 1997; Stitt and Krapp, 1999), and over time nutrient limitation, particularly nitrogen, will increasingly restrict production under high levels of CO_2 (Wong, 1979; Bazzaz, 1990; Newton, 1991). It is therefore believed that nitrogen fixing species can have a stronger response to elevated CO_2 than non- fixing species since root nodules provide a large source of nitrogen (Soussana & Hartwing, 1996) and as N is reduced as a limiting factor (Soussana & Hartwing, 1996; Clark *et al.*, 1997; Reich *et al.*, 2001). It is also believed that for plant growth to be enhanced by elevated CO_2 for extended periods, sustained strong carbon sinks are essential (Arp, 1991; Stitt, 1991). Root nodules represent extra sinks and put more demand for carbohydrate to be transported to the root system which could lead to enhanced carbon fixation overall (Lewis *et al.*, 1994; Díaz, 1996). Schenk *et al.*, (1996) reported that in a perennial ryegrass/white clover sward mixture, the response in yield to high levels of CO_2 was an increase of 16 to 42% in white clover, while the response of ryegrass yield

ranged between -33 and +9 % depending on N supply, mixture and year. It is also believed that the increased growth of legumes will lead to an increased nitrogen fixation rate as the demand for nitrogen from the plant increases (Reddy *et al.*, 1989; Reardon *et al.*, 1990; Soussana & Hartwing, 1996). Generally, the addition of combined nitrogen, which can be readily absorbed by the plants, leads to a reduction in symbiotic nitrogen fixation (Herdina and Salisbury, 1989; Buttery *et al.*, 1990; Walsh, 1995), which is considered a very costly process (Pate *et al.*, 1981; Dixon and Wheeler, 1983). The degree of reduction, however, depends on different factors such as the form of nitrogen compound, the application time, the type of the host plant, the host plant growing conditions, and the bacterial strain used (Lie, 1974). Adding small starter amounts of fertilizer, however, is believed to enhance plant and nodule growth which means greater amounts of nitrogen can be fixed (Herdina and Salisbury, 1989; Buttery *et al.*, 1990; Walsh, 1995).

The interaction between elevated carbon dioxide and nitrogen supply have been studied in different crop species such as winter wheat (Wolf, 1993), cotton (Coviella *et al.*, 2000), spring wheat (Schutz and Fangmeier, 2001), grasses (Hunt *et al.*, 1995), sunflower (Zerihun *et al.*, 2000), rice (Bannayan *et al.*, 2005) soybean (Sims *et al.*, 1998), and others. Generally, nitrogen uptake increases under elevated CO_2 when nitrogen resources are adequate outside the plants, and at the same time a stronger response to elevated CO_2 is frequently reported in well fertilized plants (Stitt and Krapp, 1999). Under limited nitrogen supply however, results were mixed depending on the plant species, the degree of nitrogen deficiency and the way the fertilizer was added (Pettersson and MacDonald, 1994). Some studies showed no effect of elevated CO_2 on biomass production when nitrogen was limited such in soybean (Sionit, 1983), tobacco

(Geiger *et al.*, 1998) and rice (Ziska *et al.*, 1996). Other crops however, showed a significant increase such as wheat (Hocking and Meyer, 1991a; McKee and Woodward 1994), cotton (Wong, 1979) and others. Nitrogen concentration in the plant tissue is usually reduced under elevated CO_2 (Wong 1979; Coleman and Bazzaz, 1992) especially when nitrogen is limited, but it is not necessary the case in well fertilized plants (Stitt and Krapp, 1999). Until recently, no work has been reported on the legume crop lentils (*Lens culinaris* Medic), which is a major pulse crop grown in over 40 countries on around 4 million hectares (Andrews and McKenzie, 2007). The seeds of lentils are a valuable human food product containing a high amount of protein (22-34.5%) and carbohydrate (65%) (Muehlbauer *et al.*, 1985), and in many countries, lentils are used as a meat substitute (Duke, 1981). Because of the process of symbiotic nitrogen fixation, nitrogen is fixed and will be available to the lentil crop and subsequent crops, improving soil nitrogen status (Sarker *et al.*, 2002).

The work reported here is the initial investigation of the effect of elevated CO_2 and different levels of nitrogen fertilizer on the growth and nodulation of this crop.

4.2. Aim

To investigate the effects of elevated atmospheric CO_2 and different levels of nitrogen fertilizer on the growth and nodulation of lentils.

4.3. Objectives

4.3.1. Objective 1.

Test the effects of elevated CO_2 and nitrogen levels and any interaction between them on leaf area index (LAI), biomass production, nitrogen and phosphorus concentration in the dry mass materials, and nodule number per plant at different growth stages of the crop development.

4.3.2. Objective 2.

Investigate the effects of elevated CO_2 and nitrogen levels on the seed yield, the main economic production of the crop.

4.3.3. Objective 3.

Test if nodule number will increase under high levels of CO_2, and by calculating the nitrogen budget, test whether nitrogen fixation could be enhanced by elevated CO_2.

4.3.4. Objective 4.

Examine the level of nitrogen fertilizer at which nodultion is suppressed at both ambient and elevated CO_2 conditions and study if there is any difference in the response of the plants under the two growth conditions.

4.3.5. Objective 5.

Examine the level of nitrogen fertilizer at which maximum production is achieved without significantly reducing nitrogen fixation at both ambient and elevated CO_2 conditions.

4.4. Materials and Methods

Syrian lentil (cultivar Idlib 3) was grown under ambient (400 μmol mol^{-1}) and elevated (700 μmol mol^{-1}) CO_2 at five nitrogen levels equivalent to 5, 25, 50, 75 and 100 kg ha^{-1}. The plants were grown in tightly sealed ventilated chambers (60×60×80 cm) placed in a glasshouse located at the University of Plymouth. Two chambers were supplied with elevated CO_2 and two with ambient air, and there were 25 pots (5 replicates of the five nitrogen levels) placed at random in each chamber. Carbon dioxide supplementation was achieved using cylinders of compressed CO_2 (BOC gases) coupled to an IRGA Eurotherm™ controller which constantly coupled the air in the chamber and pulsed CO_2 from the bottled gas to a set point of 750 μmol mol^{-1} (twice ambient). Telaire ™ monitors were used to measure CO_2, temperature and relative humidity at 15 minutes intervals, and data logged to Hobo™ dataloggers. From these data, daily average CO_2 (Figure 4.1), temperature (Figure 4.2) and relative humidity (Figure 4.3) were calculated. Mean daily temperature did not vary between chambers, but there was a significant difference in relative humidity (RH) between chambers and in CO_2 concentration within the two elevated chambers. However there were no detectable chamber effects on most of the production parameters (apart from seed yield), and it was concluded that the chamber variations did not significantly affect the results. The RH differences may be worthy of further investigation in the future. Empirical adjustment showed that a setpoint of 750μmol mol^{-1} in the pre-mix chamber led to an actual concentration in the growing chambers of 700 μmol mol^{-1}.

The seeds were inoculated with *Rhizobium leguminosarum* bacteria (Soya UK Ltd) immediately before sowing (24/03/2005), and the growth medium used was medium-

grade horticultural perlite which facilitated easy root system recovery. Three seeds were sown in each pot (constructed from cylindrical polypropylene pipe 30cm high × 10cm in diameter), and thinned to two plants after establishment. A complete Hoagland's solution minus nitrogen was irrigated every 10-14 days (100-150 ml), and similar amounts of tap water supplied in between according to demand. Nitrogen (NH_4NO_3) was added in equal amounts in the first week and in three subsequent doses 18 – 20 days apart. Five destructive harvests were conducted after 30, 52, 72, 86, and 103 days from sowing (23/04/2005, 15/05/2005, 04/06/2005, 18/06/2005, and 05/07/2005). Measurements of leaf area using a Delta-T Image Analysis System- type (DIAS^{TM}), LAI, above and below ground dry weight (after 48 h at 80 °C in a Gallenkamp 250 °C drying ovens), nodule number, and seed yield (final harvest only) were recorded. At the end of the experiment, the pots were soaked in water overnight, drained, washed with fresh water and samples of the solutions collected and later analysed for N, P (using a flow injection analyser), and K content.

Crop growth rate (CGR), which is the dry matter accumulation rate per unit area, was calculated using the following equation:

$$CGR = (W_2 - W_1)/SA\ (t_2 - t_1)$$

Where, CGR is crop growth rate expressed in g. m^{-2} per day or kg. $ha^{-1}day^{-1}$, W_1 and W_2 are the crop dry weight at beginning and end of intervals, t_1 and t_2 are the corresponding days, and SA is the soil area occupied by the plant at each sampling (Fageria, 1992).

Dried plant material was subsequently analysed for total nitrogen by Kjeldhal analysis according to Cerdá *et al.*, (1997) with a digestion block used instead of a microwave, and phosphorus was analysed according to Murphy and Riley (1962) using a Bran and Luebbe Autoanalyser 3 (flow injection analyser).

Nodule number per plant was calculated by counting the active nodules identified by their pink, red and sometimes light brown colour showing leghmoglobin activity (Somasegaran and Hoben, 1994). Data were analysed using Minitab 13.1, using a split plot analysis design (see Chapter 2). All data were tested for normality using the Anderson-Darling normality test. When data was not normal (nodule number, nitrogen uptake, nitrogen concentration, phosphorus uptake and concentration and above and below ground dry weight), the log data was used in the analysis. All the figures are drawn from the actual data.

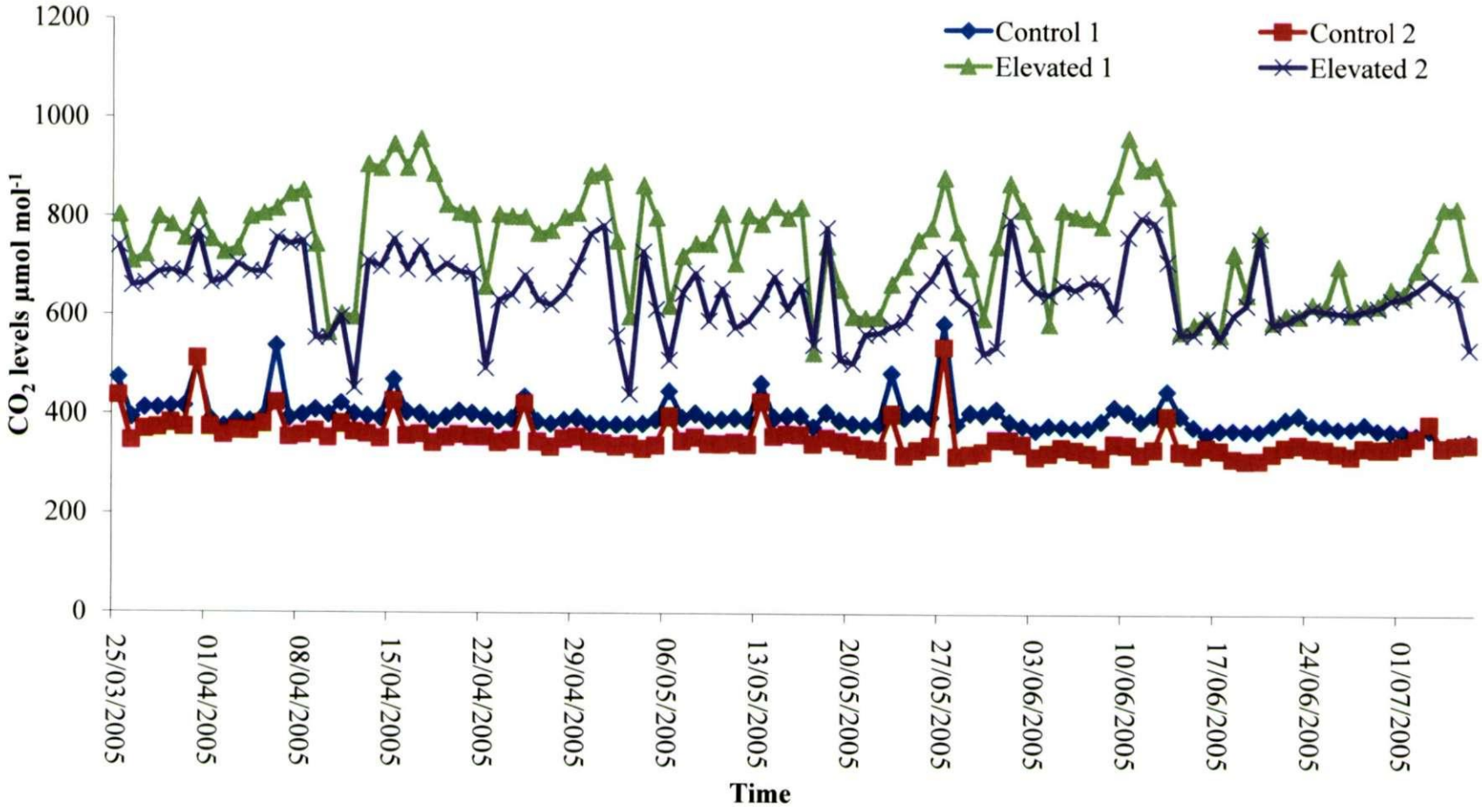


Figure 4.1. Daily average CO_2 concentration in the growth chambers over the growth period.

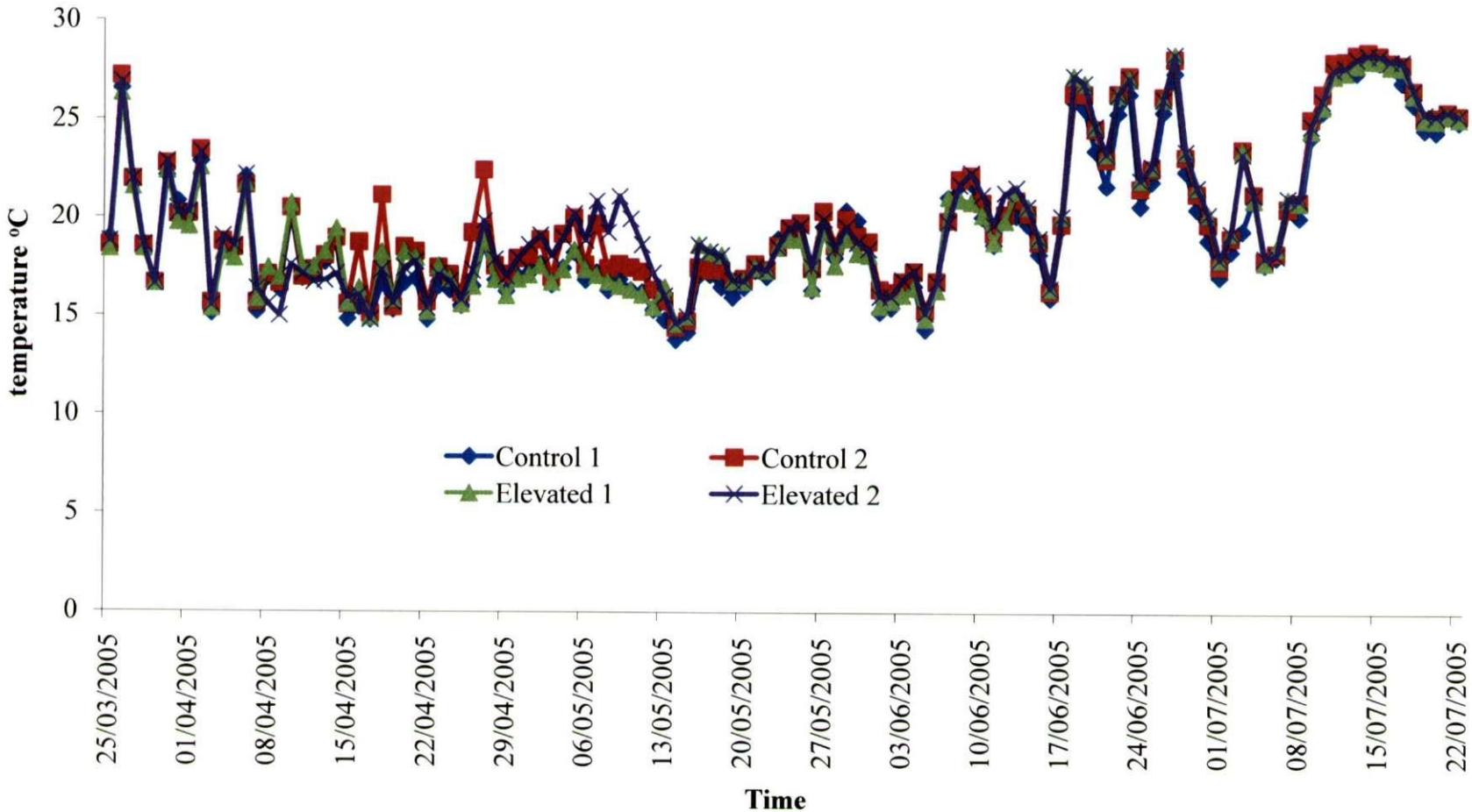


Figure 4.2. Daily average temperature in the growth chambers over the growth period.

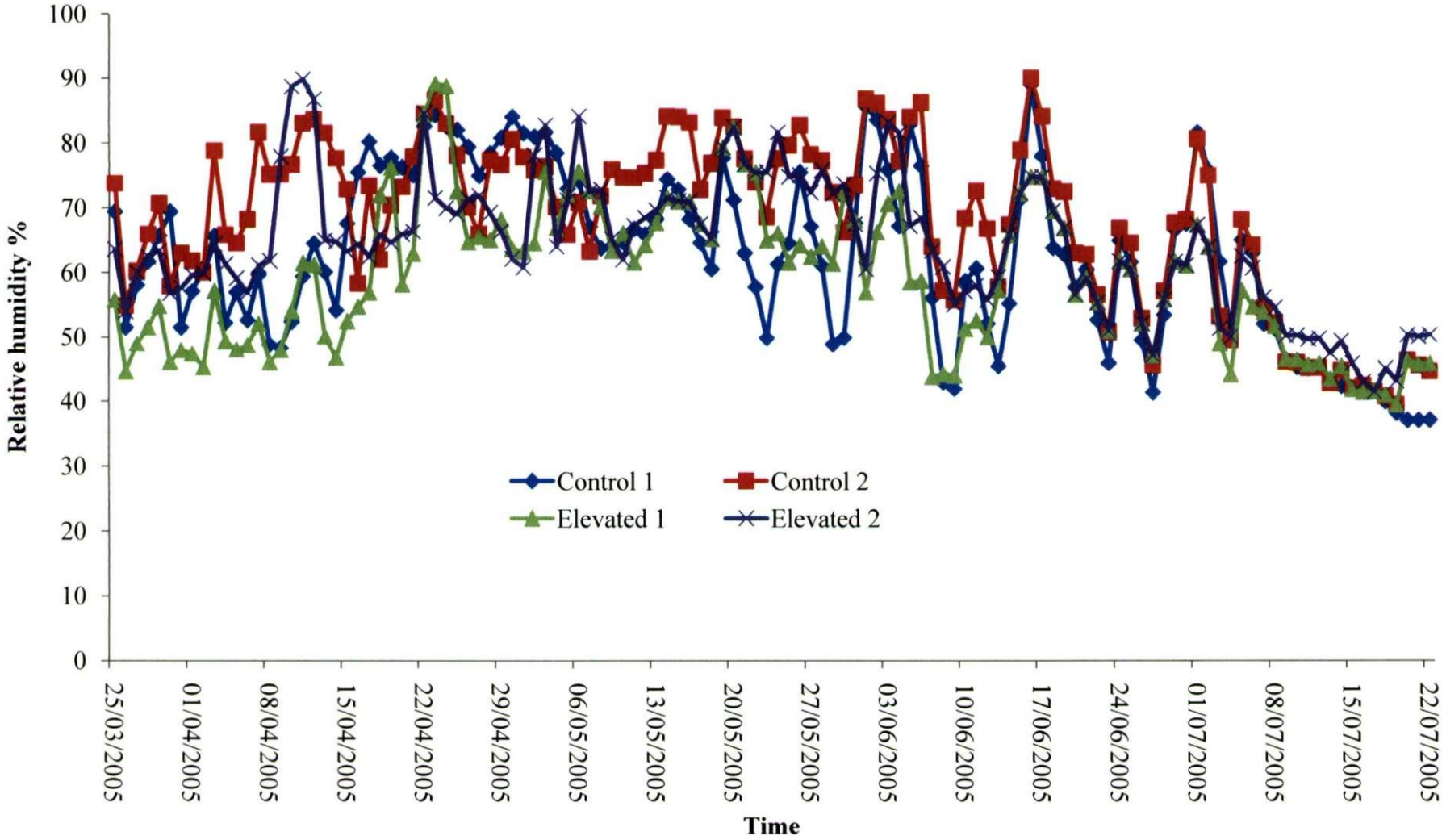


Figure 4.3. Daily average relative humidity in the growth chambers over the growth period.

4.5. Results

A summary of the results is presented in the table below (Table 4.1), and each of the results is discussed later in the text.

	Block	CO_2	N level	Harvest	$CO_2 \times N$
LAI	0.053	0.023	0.000		0.027
Shoot dry weight	0.126	0.032	0.000	0.000	0.070
Root dry weight	0.343	0.078	0.000	0.000	0.386
Root to shoot ratio	0.184	0.847	0.517	0.000	0.452
Nodule number	0.270	0.294	0.154	0.000	0.589
Pod number per plant	0.170	0.020	0.000		0.847
Seed yield	0.029	0.003	0.000		0.187
Harvest Index	0.467	0.269	0.741		0.615
Nitrogen concentration	0.669	0.294	0.000	0.000	0.117
Phosphorus concentration	0.361	0.259	0.569	0.000	0.261
Nitrogen uptake	0.535	0.221	0.000	0.000	0.990
Phosphorus uptake	0.881	0.455	0.000	0.000	0.525

Table 4.1. A summary of p values of main and interactions effects of elevated CO_2 and nitrogen levels on different parameters studied on the lentil cultivar Idlib 3. (grey= insignificant, green=<10%; clear = significant, 5, 1 & 0.1% , dotted= not included).).

	First harvest		Second harvest		Third harvest		Fourth harvest		Final harvest	
	Ambient CO_2	**Elevated CO_2**	**Ambient CO_2**	**Elevated CO_2**	**Ambient CO_2**	**Elevated CO_2**	**Ambient CO_2**	**Elevated CO_2**	**Ambient CO_2**	**Elevated CO_2**
LAI			0.35	0.42	1.67	2.33				
Shoot dry weight (kg ha^{-1})	78.	105	557	597	1385	2041	2230	3062	2080	2896
Root dry weight (kg ha^{-1})	75	101			209	257	241	350	254	326
Root to shoot ratio	1.01	1.08			0.17	0.15	0.09	0.1	0.13	0.12
Nodule number	15.5	31.9	71.0	94.1	83.4	105	21.7	26.8	2.3	1.7
Pod number per plant									12.5	17.2
Seed yield (kg ha^{-1})									1293	1968
Harvest Index									0.48	0.52
Nitrogen concentration (g $100g^{-1}$)	2.10	2.32	1.87	1.94	1.37	1.55	1.95	1.97	1.95	1.97
Phosphorus concentration (mg $100g^{-1}$)	434	444	386	409	334	310	318	254	677	602
Nitrogen uptake (mg/pot)	1.67	2.33	12.4	13.1	21.5	36.7	46.4	65.6	47.3	67.0
Phosphorus uptake (mg/pot)	0.34	0.50	2.19	2.42	4.43	5.65	6.73	6.83	8.22	10.3

Table 4.2. A summary table of the main effect of CO_2 treatment on the different production parameters studied on lentils, (dotted cells= not included).

4.5.1. LAI, Dry weight, and seed yield

Leaf area and LAI were calculated over the first three harvests and the results showed that the leaf area continued to increase up to and after anthesis, which was first recorded at 50 days after sowing. Maximum values of leaf area were observed at the third harvest (72 days after sowing). Elevated CO_2significantly increased LAI by the third harvest ($p = 0.023$), LAI increased with increasing nitrogen inputs ($p< 0.001$) with the higher values always recorded under elevated CO_2 (Figure 4.4). LAI at the third harvest ranged between 0.65 and 4.9 under the different treatments, and these LAI's compare favourably with field measurements for lentils of 1.5 to 5.5 (Wall, 1996).

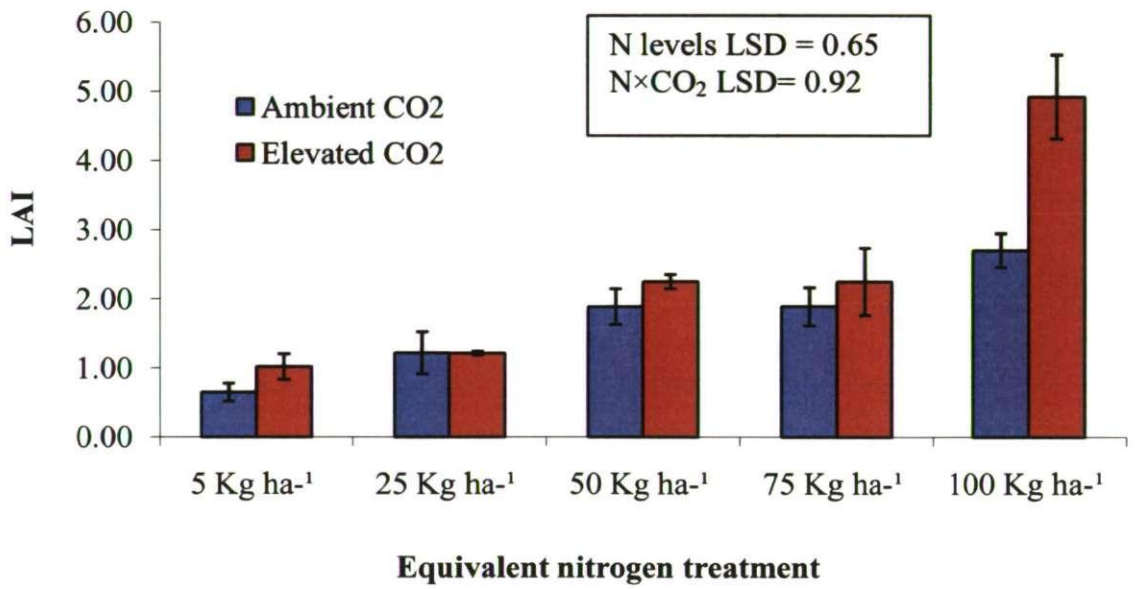


Figure 4.4. The effect of elevated CO_2 and different levels of nitrogen fertilizer on LAI in the lentil cultivar Idlib 3 at 72 days after sowing. (Vertical bars are +/- 1 se).

Above ground dry weight showed a significant increase under elevated CO_2 ($p = 0.032$) at each nitrogen level and was up to 76% higher than at ambient CO_2 (Figure 4.5, Table 4.3), and the higher the nitrogen input the higher the value (Table 4.4). Root dry weight was also increased under elevated CO_2 with an overall average of 32% compared to ambient, but this increase was not statistically significant at the 5% level ($p = 0.078$), although it was significant at 10% level. Whenever the nitrogen increased, the roots weight increased (Figure 4.6, Table 4.5). The root/shoot ratios, however, were slightly higher but not significant ($p = 0.847$). The highest root/shoot ratio was observed in the first harvest (80%) when shoot growth was not very strong, and thereafter the values decreased to about 10%, and there was no significant difference observed between the different nitrogen levels. Similarly, elevated CO_2 significantly increased the average pod number per plant ($p = 0.020$), which ranged between 7.00 and 23.25, with the highest value recorded under the highest level of nitrogen (Figure 4.7).

Harvest (days from sowing)	5 kg N ha^{-1}	25 kg N ha^{-1}	50 kg N ha^{-1}	75 kg N ha^{-1}	100 kg N ha^{-1}
30	60	7	18	47	47
52	66	-3	14	14	-9
72	67	16	43	48	63
86	-28	42	31	76	41
103	66	21	25	39	53

Table 4.3. Percent increases of above ground dry weight under elevated CO_2 compared to ambient CO_2 over the growth period of the lentil cultivar Idlib 3 at different nitrogen levels.

Harvest (days from sowing)	Mean value for the aboveground dry weight at different nitrogen levels (kg ha^{-1})					LSD
	5 kg ha^{-1}	25 kg ha^{-1}	50 kg ha^{-1}	75 kg ha^{-1}	100 kg ha^{-1}	
30	65.0	97.5	92.5	95.0	107.5	19.5
52	224.5	413.0	631.5	776	839.0	229.3
72	1297.0	1113.0	2001.0	1750.0	2406.0	598.7
86	1534.0	2314.0	2360.0	3391.0	3631.0	614.5
103	1604.0	1718.3	3077.0	2759.0	3283.0	466.9

Table 4.4. A summary table of mean values of above ground dry weight at the different nitrogen levels and the least significant difference (LSD) at each harvest point.

Harvest (days from sowing)	Mean value for the belowground dry weight at different nitrogen levels (kg ha^{-1})					LSD
	5 kg ha^{-1}	25 kg ha^{-1}	50 kg ha^{-1}	75 kg ha^{-1}	100 kg ha^{-1}	
30	73.25	85.99	86.0	105.1	89.172	ns
72	118.15	237.5	210.1	301.6	298.3	96.77
86	168.92	231.6	312.5	396.3	369.5	90.28
103	190.8	249.08	269.0	349.1	392.8	ns

Table 4.5. A summary of mean values of below ground dry weight at the different nitrogen levels and the least significant difference (LSD) at each harvest point. Note: unfortunately values of second harvest were lost due to human error.

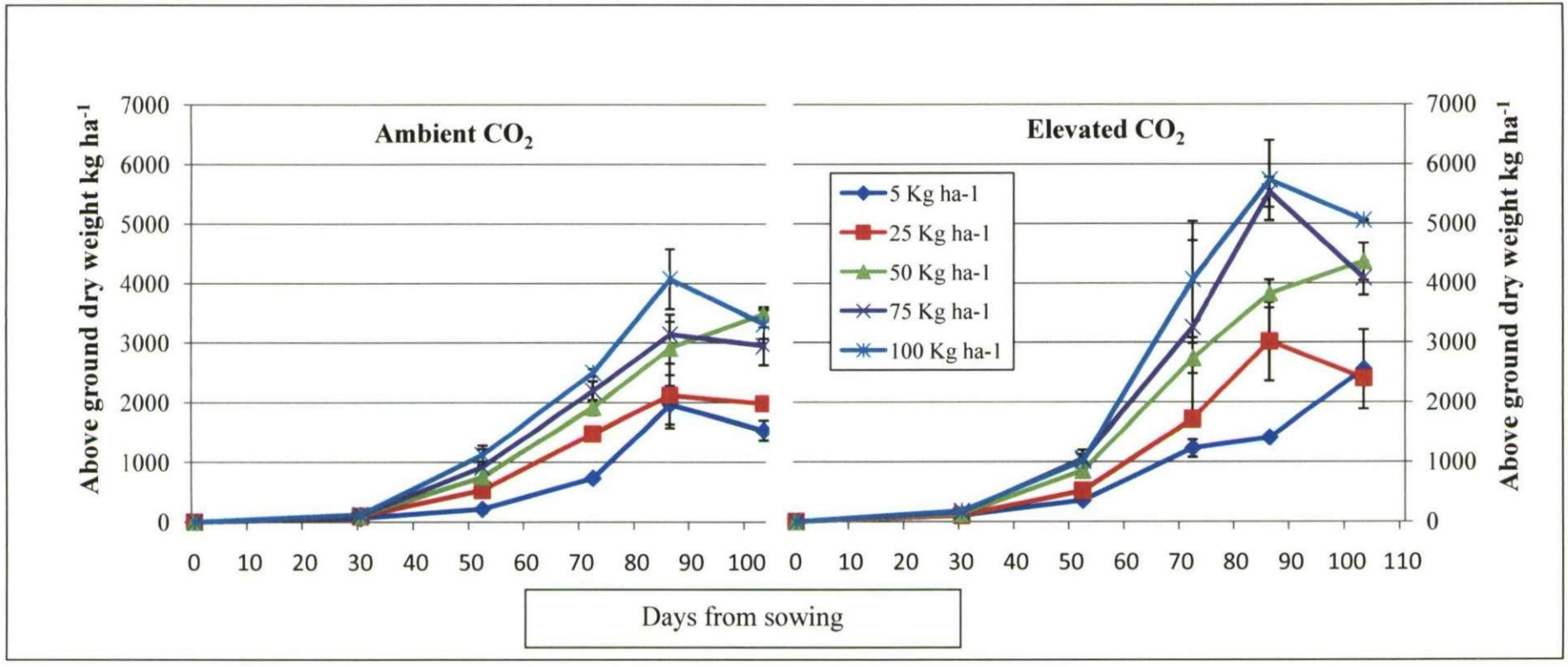


Figure 4.5. The effect of elevated CO_2 and different levels of nitrogen fertilizer on the above ground dry weight of the lentil cultivar Idlib 3 over the growth period.

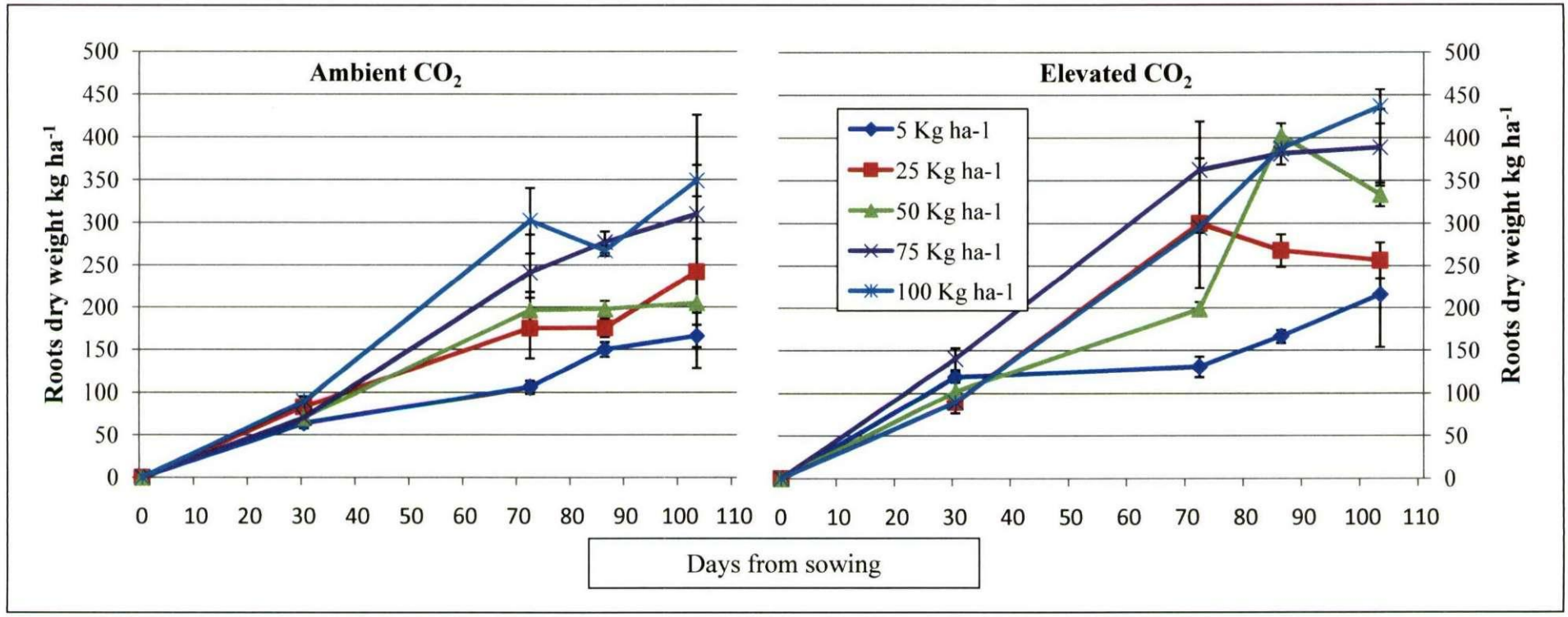


Figure 4.6. The effect of elevated CO_2 and different levels of nitrogen fertilizer on the roots dry weight of the lentil cultivar Idlib 3 over the growth period.

Seed yield under all nitrogen treatments was higher under elevated carbon dioxide than under ambient (p= 0.003) and this increase was more obvious at the high levels of nitrogen (equivalent to 75 and 100kg ha^{-1}). However, a relatively greater response of more than 100% was also reported under the lowest nitrogen treatment (Figure 4.8). In fact, the seed yield under both ambient and elevated CO_2 increased steadily with the increasing inputs of nitrogen up to the level of 50kg ha^{-1}. There was then a slight decrease under ambient with the higher levels of nitrogen (75, 100 kg ha^{-1}), whereas under elevated carbon dioxide, the seed yield continued to increase albeit at a lower rate.

Over the growth period, the crop growth rate increased significantly under elevated CO_2 up until the fourth harvest (p=0.020), and thereafter the increase was not statistically significant (p= 0.766). Similarly, these rates significantly increased with the increasing levels of nitrogen up until the fourth harvest (p= 0.004), and afterwards, the effect of nitrogen levels was non significant (p= 0.175) (Figure 4.9). Under ambient CO_2, the maximum growth rate ranged between 4.6 and 11.3 g. m^{-2} d^{-1}, and these rates were achieved at the reproductive stage (from 72 to 86 days after sowing), after which, the rates decreased sharply. Under elevated CO_2 however, maximum rates, which ranged between 4.4 and 16.3 g m^{-2} d^{-1}, were achieved at different stages, and while that for 5, 50 and 100 kg N ha^{-1} were reached in the period between 52 and 72 days after sowing, that for 25 and 75 kg N ha^{-1} were achieved in the period between 72 and 86 days after sowing. The values obtained from the current investigation compare favourably with values reported in lentils which range from 9 to 24 g m^{-2} d^{-1}(Ishag and Dennett, 1998).

Although in most treatments, HI values were increased by elevated CO_2, the overall effect was not significant (p= 0.146), and these values were also not affected by the different nitrogen levels applied (p= 0.704) (Figure 4.10).

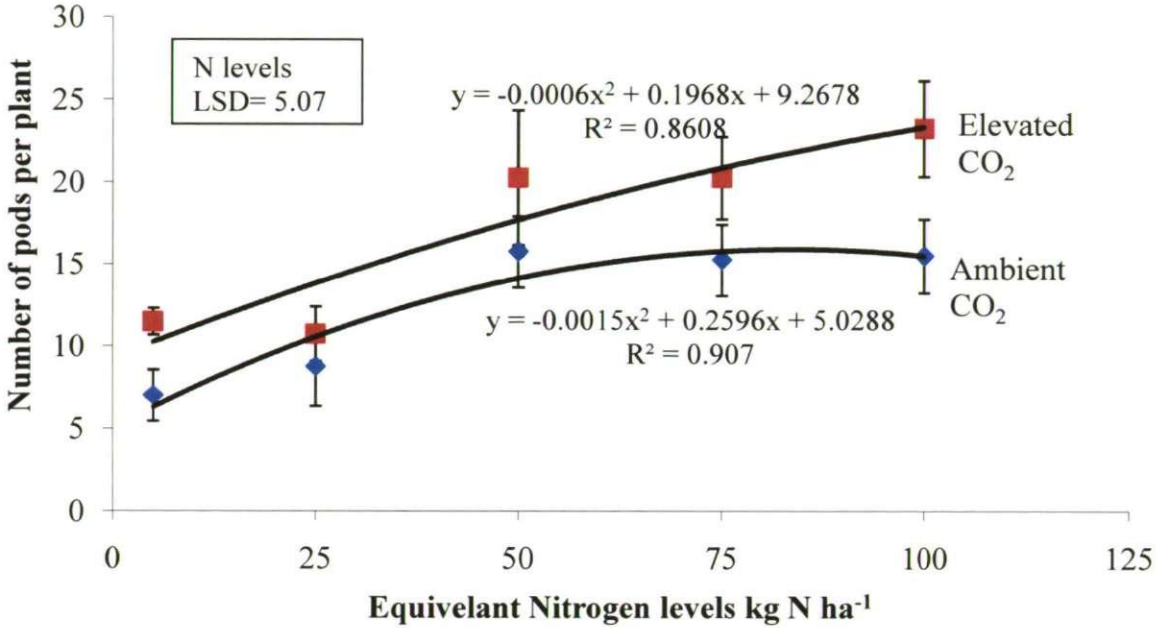


Figure 4.7. The effect of elevated CO_2 and different levels of nitrogen fertilizer on pod number per plant of the lentil cultivar Idlib 3 (Vertical bars are +/- 1 se).

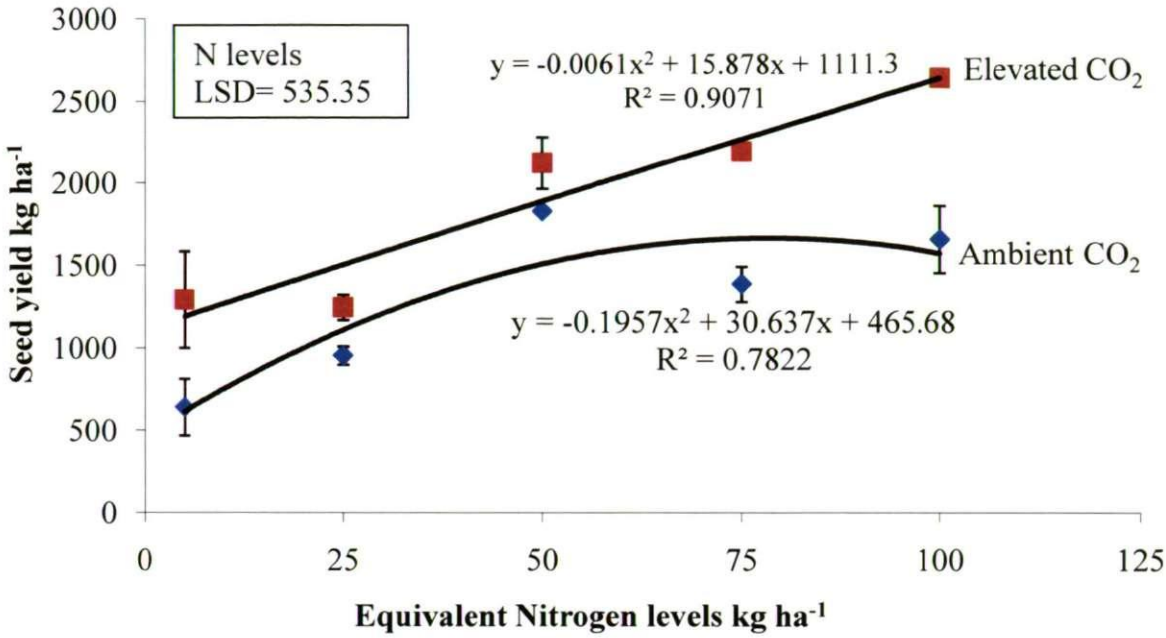


Figure 4.8. The effect of elevated CO_2 and different levels of nitrogen fertilizer on the seed yield of the lentil cultivar Idlib 3 (Vertical bars are +/- 1 se).

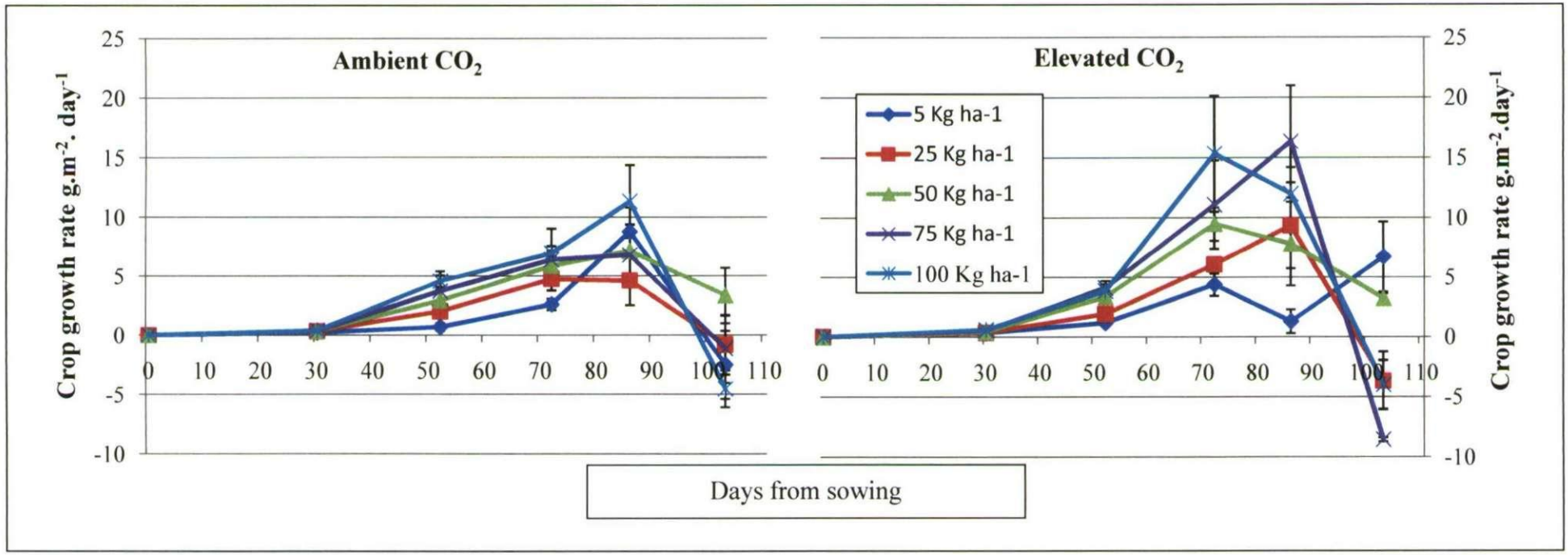


Figure 4.9. The effect of elevated CO_2 and different levels of nitrogen fertilizer on the crop growth rate of the lentil cultivar Idlib 3.

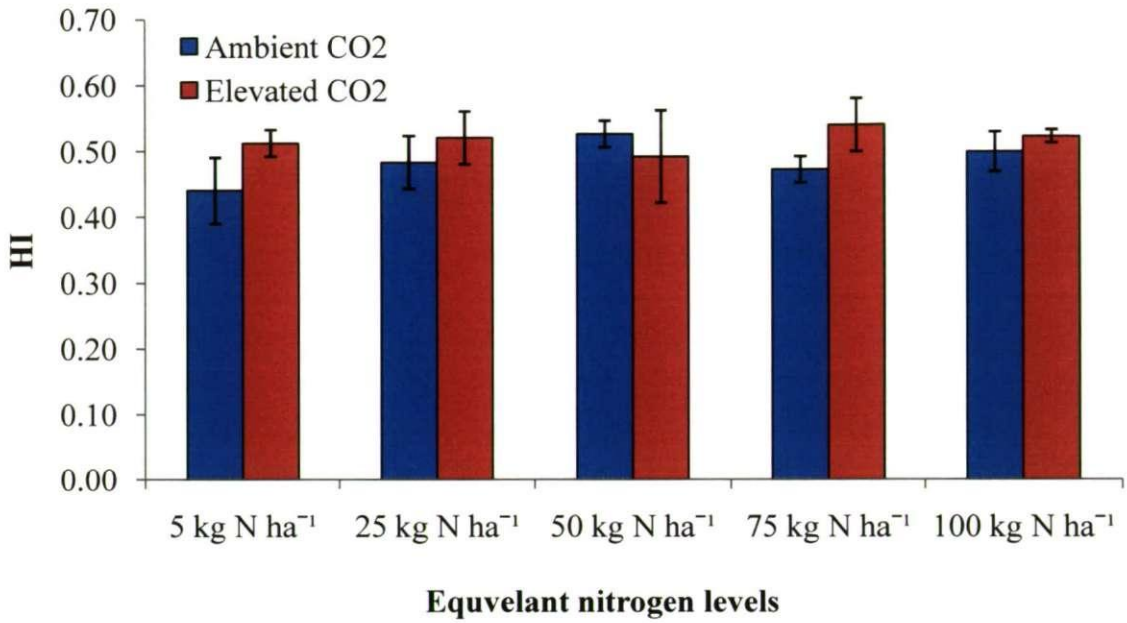


Figure 4.10. The effect of elevated CO_2 and different levels of nitrogen fertilizer on the HI of the lentil cultivar Idlib 3. (Vertical bars are +/- 1 se).

4.5.2. Nodule number

Over the growth period, and under both ambient and elevated carbon dioxide, active nodule number per plant increased steadily up until the third harvest (72 days after sowing) (Plate 4.1), but decreased rapidly thereafter (Figure 4.11).

Although average nodule numbers under all nitrogen treatments were always higher under elevated CO_2, the effect of elevated CO_2 was not statistically significant ($p = 0.294$). There was also no overall significant difference between the different levels of nitrogen input ($p= 0.154$).The lowest nodule numbers were found under the highest (100 kg. ha^{-1}) nitrogen treatment which also showed a slower increase in numbers during early plant growth.

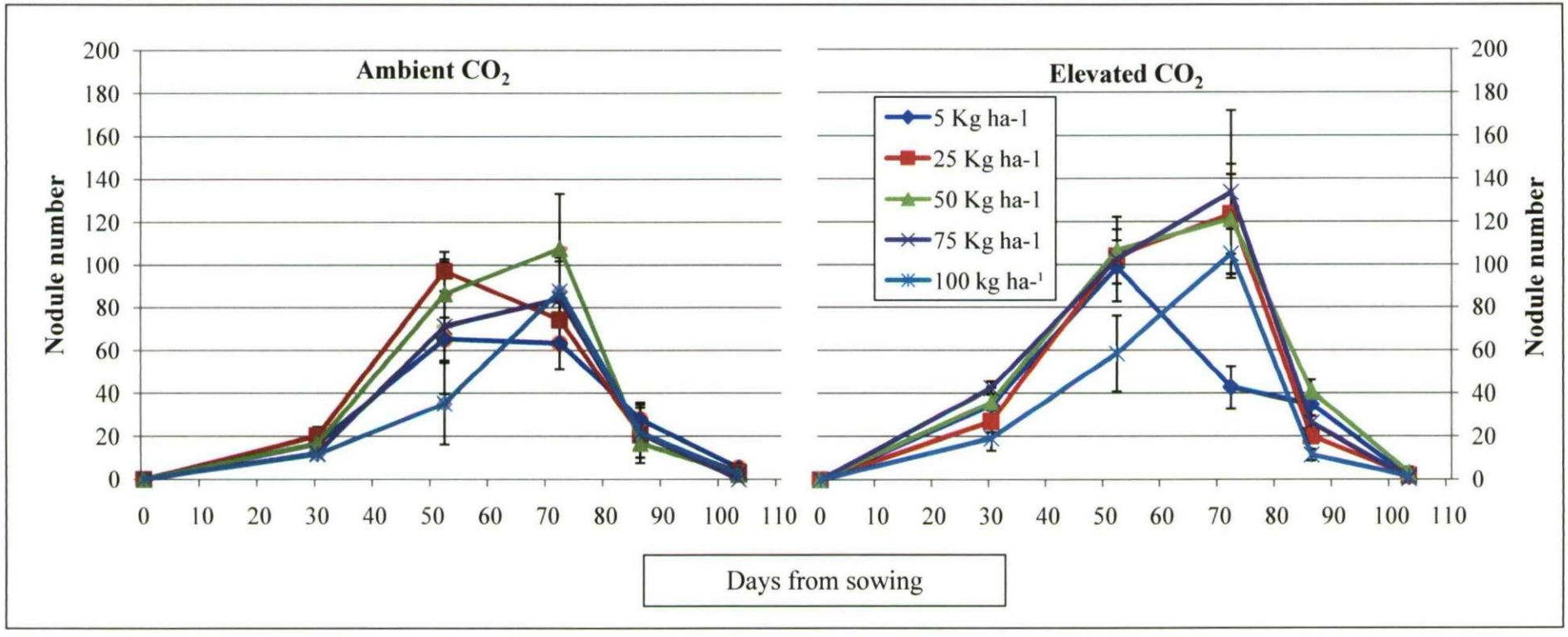


Figure 4.11. The effect of elevated CO_2 and different levels of nitrogen fertilizer on the nodule number per plant of the lentil cultivar Idlib 3.

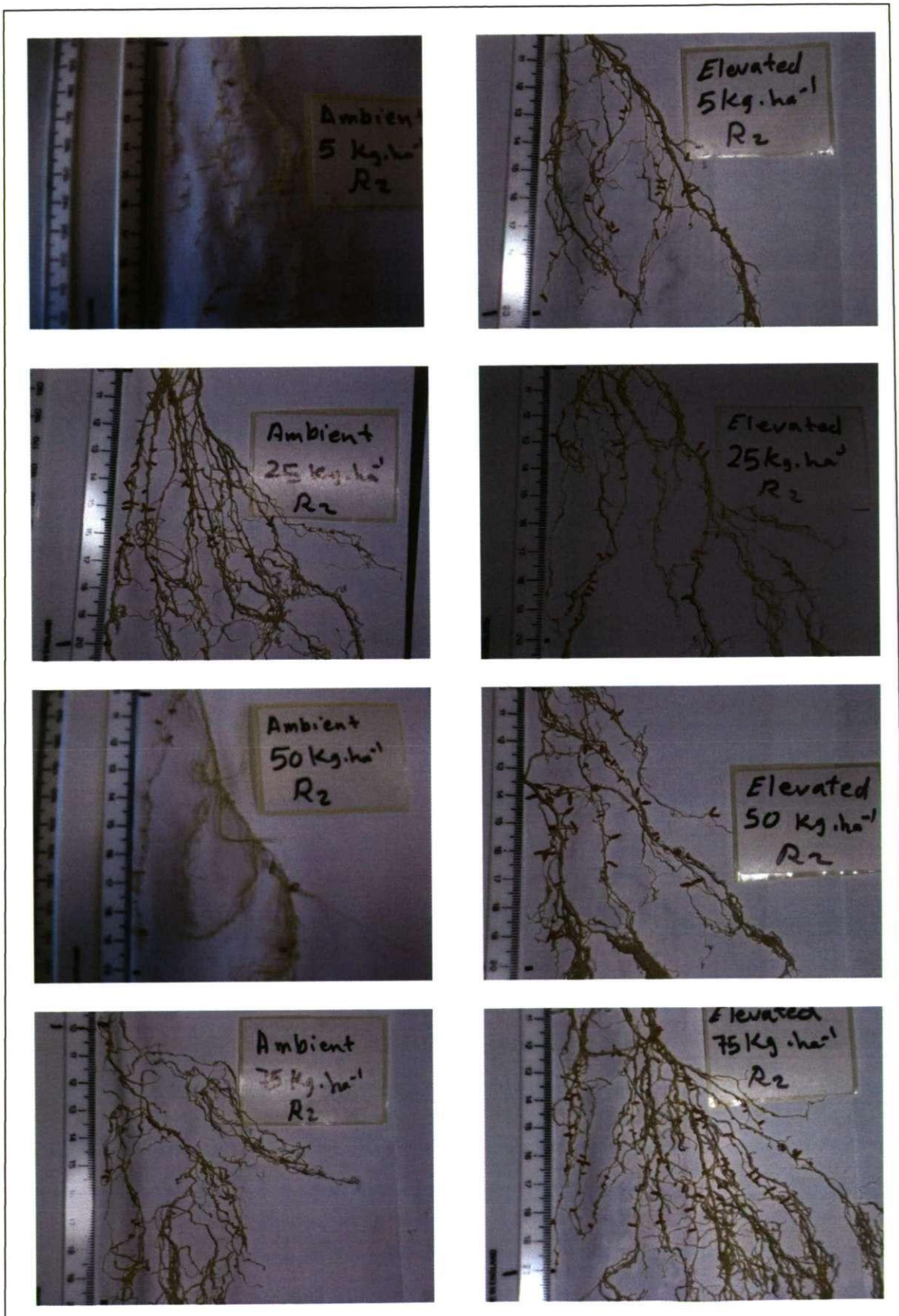
Interestingly the lowest (5 kg ha^{-1}) nitrogen level also showed relatively low nodule numbers. The highest nodule numbers occurred when moderate amounts of nitrogen fertilizer (25-50 kg N ha^{-1} at ambient CO_2, and 25- 75 kg N ha^{-1} at elevated CO_2) were added.

4.5.3. Nutrient content

The total amount of nitrogen absorbed by the above ground dry matter increased incrementally throughout the growth cycle of the plants, and also increased significantly ($p< 0.001$) with increasing inputs of nitrogen (Table 4.6). In spite of the greater nitrogen uptake under elevated CO_2, the effect was not statistically significant ($p=0.200$). (Figure 4.12).

Harvest (days from sowing)	**Mean value for nitrogen uptake at different nitrogen levels (mg/pot)**					**LSD**
	5 kg ha^{-1}	**25 kg ha^{-1}**	**50 kg ha^{-1}**	**75 kg ha^{-1}**	**100 kg ha^{-1}**	
30	1.37	2.06	1.77	2.42	2.36	ns
52	2.284	5.33	13.77	18.33	24.02	8.06
72	6.72	10.60	19.71	47.9	60.40	21.01
86	12.85	42.80	62.33	80.20	81.86	25.15
103	29.20	35.42	73.91	64.99	82.11	12.06

Table 4.6. Summary table of mean values of nitrogen uptake at the different nitrogen levels and the least significant difference (LSD) at each harvest point.


Ambient
5 Kg.ha-1
R2
Elevated
5kg.ha-1
R2
Ambient
25 Kg.ha-1
R2
Elevated
25kg.ha-1
R2
Ambient
50 Kg.ha-1
R2
Elevated
50 Kg.ha-1
R2
Ambient
75 Kg.ha-1
R2
Elevated
75 Kg.ha-1
R2

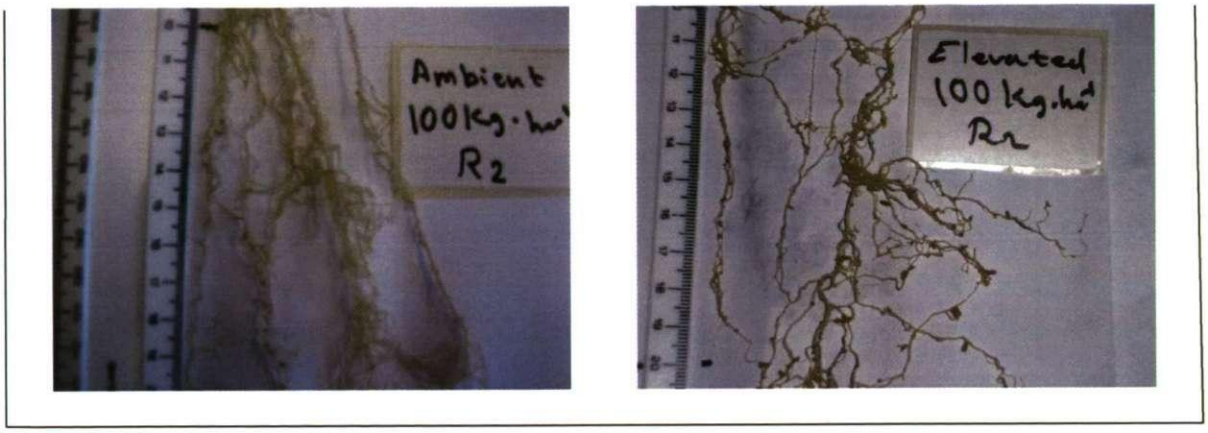


Plate 4.1. To show the difference in nodule number between ambient and elevated CO_2 and that between different levels of nitrogen fertilizer at the third harvest (72 days after sowing.

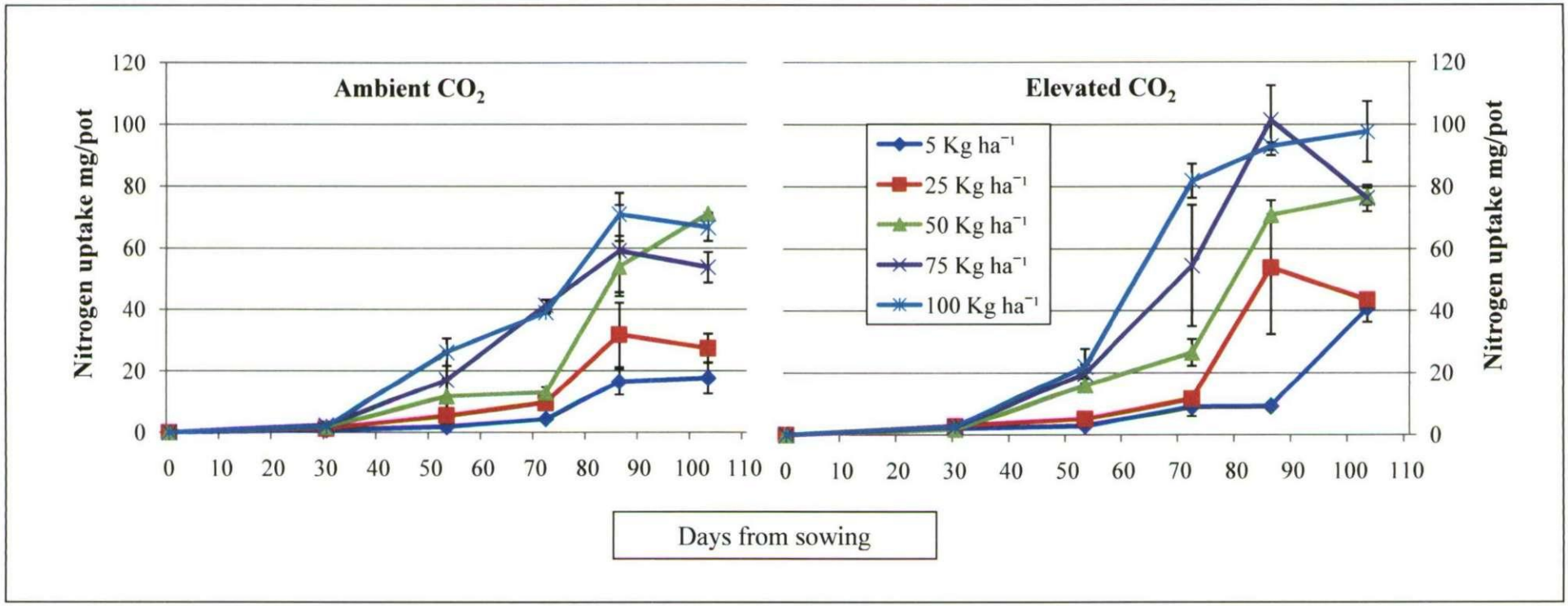


Figure 4.12. The effect of elevated CO_2 and different levels of nitrogen fertilizer on the total amount of nitrogen absorbed over the growth period of the lentil cultivar Idlib 3.

Nitrogen content however, was lower but not always significantly so and elevated CO_2 had no effect on nitrogen content of the plants (Table 4.7) so the slightly higher uptake observed (Figure 4.12) was a result of increased biomass. Protein content in the seeds, which is calculated by multiplying nitrogen content by a conversion factor of 6.25 (Jin *et al.*, 2006), varied between 13.30-24.30 % and similarly elevated CO_2 had no effect on the concentrations which increased with the higher levels of nitrogen inputs.

Harvests (days from sowing)	5 kg N ha^{-1}		25 kg N ha^{-1}		50 kg N ha^{-1}		75 kg N ha^{-1}		100 kg N ha^{-1}	
	Ambient	Elevated	Ambient	Elevated	Ambient	Elevated	Ambient	Elevated	Ambient	Elevated
30	1.62	2.46	1.75	3.53	2.16	1.73	2.89	1.93	2.10	1.94
52	1.07	1.00	1.31	1.28	1.84	2.35	2.18	2.36	2.94	2.71
72	0.76	0.92	0.83	0.86	0.85	1.27	2.41	2.02	1.99	2.20
86	1.05	0.83	1.74	2.23	2.36	2.37	2.71	2.33	2.22	2.09
103	2.69	2.69	3.10	3.97	4.45	4.08	4.36	4.02	4.51	4.23

Table 4.7. The effect of elevated CO_2 and different levels of nitrogen fertilizer on nitrogen concentration in the dry matter (g $100g^{-1}$) over the growth period of the lentil cultivar Idlib 3.

The pattern of phosphorus uptake was similar to nitrogen being significantly greater ($p< 0.001$) under higher levels of nitrogen input (Table 4.8) and higher but not significantly so under elevated CO_2 (Figure 4.13). The phosphorus concentration tended to be lower with increased biomass although these differences were not always significant (Table 4.9).

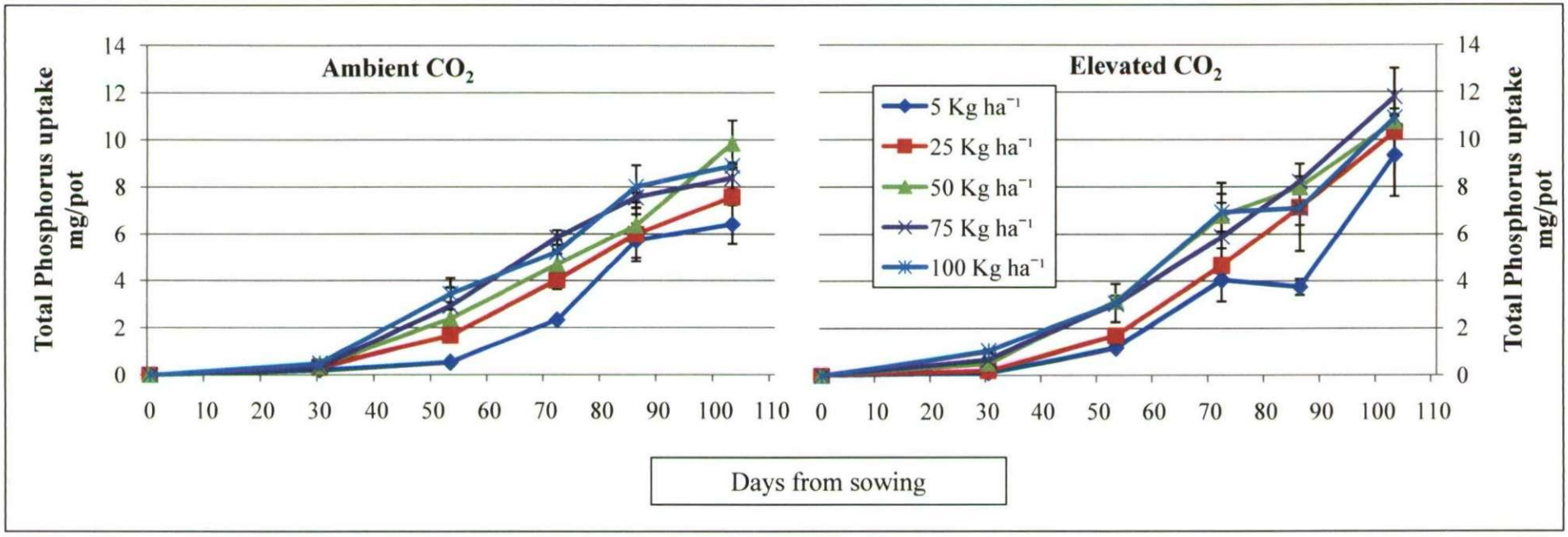


Figure 4.13. The effect of elevated CO_2 and different levels of nitrogen fertilizer on total phosphorus absorbed over the growth period of the lentil cultivar Idlib 3.

Harvest (days from sowing)	Mean value for phosphorus uptake at different nitrogen levels (mg/pot)					LSD
	5 kg ha^{-1}	25 kg ha^{-1}	50 kg ha^{-1}	75 kg ha^{-1}	100 kg ha^{-1}	
30	0.1425	0.2625	0.4475	0.502	0.753	0.25
52	0.853	1.670	2.765	2.982	3.255	0.87
72	3.183	4.335	5.737	5.857	6.075	2.07
86	4.735	6.555	7.178	7.892	7.553	2.04
103	7.87	8.940	10.30	9.65	9.475	ns

Table 4.8. A summary table of mean values of phosphorus uptake at the different nitrogen levels and the least significant difference (LSD) at each harvest point.

Examination of the residual nutrient concentrations in the perlite at the end of the experiment showed that the unabsorbed nitrogen and phosphorus amounts were slightly, but not significantly, higher in the ambient CO_2 treatment reflecting the slightly greater uptake by the improved biomass production under elevated CO_2.

As the amount of nitrogen added to the plants and that absorbed are known, and by assuming that the amount of nitrogen lost by denitrification or volatilization is negligible, and that all the amount added is absorbed, a minimum amount of nitrogen fixed by the plants can be calculated (Table 4.10). Accurate values, however, are very difficult to estimate in this experiment since it is impossible to know the amounts of nitrogen lost to the air or whether the amounts found in the residues are from added nitrogen or from fixed nitrogen, therefore these amounts are crude values that clearly indicate a minimum amount.

Harvests (days from sowing)	5 kg N ha^{-1}		25 kg N ha^{-1}		50 kg N ha^{-1}		75 kg N ha^{-1}		100 kg N ha^{-1}	
	Ambient	Elevated	Ambient	Elevated	Ambient	Elevated	Ambient	Elevated	Ambient	Elevated
30	414.0	163.5	422.9	255.2	427.9	525.4	407.2	539.9	496.4	735.7
52	317.8	420.0	398.1	414.0	400.5	467.0	428.0	367.1	387.8	377.9
72	405.3	412.9	347.7	345.0	308.5	312.5	340.0	295.7	266.8	194.8
86	373.2	338.6	365.6	310.7	282.0	267.2	269.8	189.5	258.4	161.5
103	866.9	683.8	751.7	800.0	569.1	537.8	655.3	531.0	567.9	524.8

Table 4.9. The effect of elevated CO_2 and different levels of nitrogen fertilizer on phosphorus concentration in the dry matter (mg $100g^{-1}$) over the growth period of the lentil cultivar Idlib 3.

Harvests (days from sowing)	5 kg N ha^{-1}		25 kg N ha^{-1}		50 kg N ha^{-1}		75 kg N ha^{-1}		100 kg N ha^{-1}	
	Ambient	Elevated	Ambient	Elevated	Ambient	Elevated	Ambient	Elevated	Ambient	Elevated
N found inthe shoots	17.7	40.7	27.3	43.5	70.9	76.9	53.7	67.2	66.6	97.6
N absorbed by roots	1.2	1.6	1.7	1.7	1.5	2.5	2.2	2.6	2.6	3.0
N found in the water	4.2	3.9	4.6	3.1	4.5	4.2	6.7	5.0	6.8	6.0
N added to the plants	3.9	3.9	19.6	19.6	39.2	39.2	58.8	58.8	78.4	78.4
Minimum amounts of N fixed	19.2	42.3	14.0	28.7	37.7	44.4	3.8	25.0	-2.4	28.2

Table 4.10. Average of minimum amounts of nitrogen fixed by the plants in each pot (mg/pot) of the lentil cultivar Idlib 3.

4.6. Discussion

This investigation confirmed that lentils respond positively to elevated CO_2 in a manner similar to other crops and plants. The leaf area at all nitrogen levels increased under elevated CO2 by 20-30% after flowering and maximum LAI achieved after flowering as previously reported in winter sown lentil in the field (Saxena and Hawtin, 1981). . Increased leaf area under elevated CO_2 is commonly reported (Newton, 1991; Mulholland *et al.*, 1998; Suter *et al.*, 2001; Ainsworth *et al.*, 2006) and is an essential aspect of the crops physiological response to the improvement of a limiting factor. Nitrogen availability is essential to increase both leaf area and to maintain leaf longevity which combines to improve LAI (Grindly, 1997) and in this experiment LAI's increased proportionally with increased nitrogen inputs of nitrogen under both ambient and elevated CO_2 treatment.

Aboveground dry weight was increased under elevated carbon dioxide by up to 76%, and increased with the higher levels of nitrogen. Dry weights were up to two to three times higher under the highest nitrogen level compared to the lowest (Figure 4.5). Root dry weight was higher under elevated CO_2 and significant at a 10% level, but not at 5% level. Failing to achieve significant increase at 5% level could be a result of the limited volume of the pots used in the experiment as it has been previously reported that the roots growth under elevated CO_2 could be restricted in small pot experiments (Arp, 1991; Rogers *et al.*, 1994). In general, increased roots biomass under elevated CO_2 is commonly resported (Rogers *et al.*, 1994: Jones et al., 1984), and frequently roots show the largest relative increase among the plant parts (Rogers *et al.*, 1983; Imai *et al.*, 1985; Norby et al., 1992). The root/shoot ratios in the CO_2 enriched plants were slightly

higher but not significantly so. A little change or even reduced root to shoot ratio under high levels of CO_2 is also recorded (Morison and Gifford, 1984; Norby, 1994; Gavito, 2000), but on the other hand, other studies reported a significant increase in root to shoot ratio (Rogers *et al.*, 1992; Rogers *et al.*, 1994).

The increases in biomass were always associated with increases in pod number and thereby seed yield (Figure 4.8). Productivity for different crops, especially C_3 plants, increases under elevated CO_2 as a result of increased net assimilation rate due to the increased carboxylation to oxygenation rate of ribulose bis phosphate carboxylase/ oxygenase (Rubisco) (Danks *et al.*,1983; Bowes, 1996; Stitt, 1991; Larcher, 2003) and the subsequent increase in carbohydrate production increases biomass and seed yield (Fuller and Jellings, 2004). The response shown here for lentils is similar to that for other legumes, for example, Torbert *et al.*, (2004) reported a 40 % increase in biomass production of soybean when grown under high levels of carbon dioxide. Similarly, Prasad *et al,* (2002) reported a 25% increase in the seed yield of kidney bean (*Phaseolus vulgaris* L.) under high levels of CO_2. In general, in the absence of biotic and abiotic stresses, the yield of grain legume crops (soybean, dry bean, peanut and cowpea) are increased by elevated atmospheric CO_2 (Prasad *et al.*, 2005).

Values of crop growth rates obtained from the current investigation showed an average increase of about 39% under CO_2 enriched conditions, which can be interpreted as a result of the increase in the rate of photosynthesis which directly affect the rate of dry matter accumulation (Streck, 2005). Increased growth rates in response to elevated CO_2 have been previously reported in other crops and plant species (Allen *et al.*, 1991; Hamelton *et al.*, 2002). It was noted that growth rates under elevated CO_2 at some nitrogen levels (5, 50 and 100 kg N ha^{-1}) continued to increase up until 72 days after

sowing and thereafter decreased to levels closer to those under ambient, but at 25 and 75 kg N ha^{-1} levels maximum rates were achieved at a later stage (72 to 86 days after sowing). Maximum growth rates under ambient CO_2 were also reached at the same stage (between 72 and 86 days after sowing). The decreased growth rates under elevated CO_2 for some nitrogen treatments could be an indication of photosynthesis acclimation to the prolonged exposure to the high levels of CO_2, which is a response previously reported (Baker *et al.*, 1990; Chaves and Pereira, 1992; Baker and Allen, 1993). The difference observed at the different nitrogen treatments in the stage of growth at which maximum growth rates were archived under elevated CO_2 is probably due to differences in the strength of the sinks represented by both nodule biomass and pod number.

In the current investigation, the response of seed yield to the higher levels of nitrogen input was more obvious under elevated CO_2 compared to that at ambient condition. This can be interpreted as due to increased atmospheric carbon dioxide concentration, the assimilation rate and hence growth rate increases, which means the demand for nitrogen increases, and the more the nutrient available the more the growth can be sustained. At ambient conditions the higher inputs of nitrogen increase the productivity, but since photosynthetic rate is below saturation level, the benefit is not as big. However, calculations of LSD for seed yield (Figure 4.8) at the different nitrogen treatments, showed that there was no significant difference between the 50, 75, and 100 kg ha^{-1} treatments, but these treatments were significantly higher than the treatments 5 and 25 kg ha^{-1}. This was true for both ambient and elevated CO_2 as results showed that there was not any significant interaction between CO_2 and nitrogen levels treatments. Data collected from different experiments conducted on different crops such as rice, cotton, and tobacco, showed that when grown at varying levels of nitrogen fertiliser, elevated

CO_2 led to a greater increase in biomass at the higher levels of nitrogen, whereas the increase was often small at the moderate levels, with little or no effect at the lowest N level (Stitt and Krapp, 1999). However, the yield response to elevated CO_2 in this experiment was relatively largest under the lowest nitrogen level, which can be explained due to improved nitrogen fixation as it can be seen from table 5 that a minimum amount of 42.3 mg N/pot (second largest) was fixed in this treatment. A similar response was previously reported in red clover where a stronger response to elevated CO_2 was observed at the lower nitrogen availability (Meier and Fuhre, 1997). Kimball *et al.*, (2002) also reported that the growth of white clover was strongly enhanced by elevated CO_2 at low and ample nitrogen levels.

It is not unusual that legume species respond positively to elevated availability of mineral nitrogen since nitrogen fixation has a metabolic cost to the plant (Pate *et al.*, 1981; Dixon and Wheeler, 1983). It was clear in this investigation that lentils are no different in this respect and maximum yields were always obtained under moderate to high levels of N inputs but nodulation was not greatly depressed by the availability of N fertilizer, only really being restricted by the highest N level (100 kg ha^{-1}). Although not significantly, nodule number was always higher under elevated CO_2, which may be a response to the larger source of the extra carbohydrate in the plants caused by the increased photosynthetic rates (Arnone and Gordon, 1990). Aranjuelo *et al.*, (2008) reported that nodule production was not significantly increased in alfalfa plants grown at elevated CO_2 levels of 700 µmol mol^{-1}. Feng *et al.*, (2004) also stated that in plants of 1-year-old *Robinia pseudoacacia* grown under CO_2 enriched conditions (700 µmol mol^{-1}), nodule number was doubled compared to ambient conditions but this effect was not significant because of the great variability of root nodule formation.

In the current experiment, calculations of the minimum amounts of nitrogen fixed by the plants showed that the highest amounts were fixed under the moderate levels of nitrogen (50 kg ha^{-1}) followed by the lowest level (5 kg ha^{-1}) at both ambient and elevated CO_2, and there was a decrease under the higher nitrogen inputs (Figure 9). It can be seen here that in spite of the higher nodule number at the nitrogen level 75 kg ha^{-1} under elevated CO_2, the amounts fixed were not the highest, although they could be higher. This suggests that nodule number alone does not necessarily indicate the efficiency of nitrogen fixation, and should be considered in association with nodule weight and nitrogenase activity. Wilson (1940) indicated that the inhibitory effect of NO_3^- can be decreased by adding sugars to the growth media or by increasing photosynthesis by increasing light or carbon dioxide levels. In general if there is an adequate amount of nitrogen available, the nitrogen uptake can be increased under elevated CO_2 (Stitt and Krapp, 1999) and this fact can be partly supported by the results of this investigation as the nitrogen uptake at all nitrogen levels was greater under elevated CO_2 but not significantly so. By analysis of the nitrogen budget, it was concluded that there was a significant increase in symbiotic N-fixation under elevated CO_2 and this supports previous reports that elevated CO_2 usually increases total N_2 fixation due to increased nodule weight and/or activity and/or number (Wilson *et al.*, 1933; Shivashankar and Vlassak, 1978; Zanetti *et al.*, 1995; MacDowall, 1982; Díaz, 1996; Zanetti *et al.*, 1996). Soussana and Hartwig (1996) also showed that in pure and mixed clover swards using a ^{15}N isotope dilution technique, the nitrogen derived from nitrogen fixation was significantly higher under elevated carbon dioxide.

Despite the significant increase in biomass under elevated CO_2 in this experiment, the dilution effect on nitrogen concentration was not significant, which is more likely a

result of the increased nitrogen fixation under the high levels of CO_2. Generally, nitrogen (and other minerals) concentration is reduced in plants grown under elevated CO_2 (Wong, 1979). However, some studies and in support of our findings, showed no significant effect of elevated carbon dioxide on nitrogen and protein concentration. Havelka *et al.*, (1984) reported no significant reduction in protein concentration in wheat when grown under CO_2 enrichment conditions. Additionally, the percentage of protein in soybean and corn seeds of plants grown over a range of CO_2 levels from 340 to 910 ppm did not change (Rogers *et al.*, 1983). Similarly to nitrogen, phosphorus concentration in the dry matter was also reduced under elevated CO_2 but not significantly and this indicates that the amounts of phosphorus in the feeding solution were adequate for plant need even with the additional growth observed under increased CO_2.

The results from this study are from a chamber based pot experiment, and there is a question as to whether the results can be considered robust enough to reflect actual field conditions. In this experiment, the use of perlite in small pots allowed the supply of sufficient amounts of nutrients which can be much more limited under field conditions. The sterile perlite provided good conditions for root growth with a medium free of soil borne diseases and pests to remove limitations and potentially enhance the response to CO_2 enrichment but at the same time, the small size of the pots can restrict the root growth and present some limitations to the benefits of CO_2 fertilization. Furthermore, under field conditions many different strains of rhizobium bacteria exist in the soil, from which some strains may be more responsive to elevated CO_2 than the strain used in this experiment. In fact, it is very difficult to compare conditions of that of enclosed chambers with that in the field and plants have the potential to respond differently under

each set of conditions. Nevertheless, Kimball *et al.*, (2002) compared results from free air CO_2 enrichment (FACE) experiments under field conditions with previous chamber based experiments and concluded that for CO_2 enrichment there is a high degree of consistency of responses and this gives confidence to the conclusions obtained from each approach. However, some argue that the results obtained from enclosed CO_2 enrichment experiments are over estimated by about 50% compared to that from FACE experiments (Long *et al.*, 2006). It was also reported by Idso and Idso (1997) that under the conditions of resource limitation and environmental stress of the natural ecosystem, it is expected the percentage growth response to elevated CO_2 could be greater than that of managed agricultural and horticultural systems. It can be predicted therefore that similar results to those presented here for lentils under controlled conditions can be expected under field conditions although the degree of response may be somewhat less.

4.7. Conclusions

It is concluded that lentils are responsive to elevated CO_2 and biomass and seed yields can increase by an average of 35% and 53% respectively under CO_2 levels of 700 µmol mol^{-1}. Yield response to elevated CO_2 is maximised by raising the availability of exogenous nitrogen and high yields were obtained at the moderate to high levels of nitrogen (50-100 kg ha^{-1}), which were not significantly different from each other.. Levels of 75 kg N ha^{-1} did not have an undue detrimental effect on nodule number which continued to increase under elevated ,, but this was not necessarily associated with the highest levels of nitrogen fixation efficiency. The implications of these findings are that higher yield potentials can be expected from field grown lentils as atmospheric levels of CO_2 rise but higher fertilizer inputs are not necessarily needed, and levels of 50 kg N ha^{-1} could be ideal as both yield and nitrogen fixation are improved under these conditions.

5. Chapter 5

The Effects of Elevated CO_2 on Nitrogenase Activity in Lentils and White Clover

5.1. Introduction

The effect of rising atmospheric carbon dioxide on nitrogen fixing plant species is expected to be greater than those of non-fixing species since the extra demand for nitrogen required by the increased plant growth will be met by the process of increased symbiotic nitrogen fixation and the expected nitrogen limitation in the long term can be avoided (Poorter 1993; Stulen and den Hertog, 1993; Rogers *et al.*, 1997). In fact, total plant nitrogen fixation is predicted to increase under elevated CO_2 conditions as a result of increased nodule weight or/and specific nodule activity (Díaz, 1996). Zanetti *et al.*, (1996) indeed reported an increase in total nitrogen content in white clover plants grown under high levels of CO_2 and indicated that all of the additional nitrogen was derived solely from nitrogen fixation.

An increase in whole plant nitrogenase activity in response to CO_2 enrichment has been reported for different legume crops including soybean (Finn and Brun, 1982), pea (Philips *et al.*, 1976) and white clover (Masterson and Sherwood, 1978), and also in nodulated nitrogen fixing woody trees (Norby, 1987). However, mixed conclusions have been derived from experiments studying the effect of CO_2 enrichment on specific nitrogenase activity. A significant increase in specific nitrogenase activity was reported in some studies (Hardy and Havelka, 1976; Shivashankar and Vlassak, 1978; Tissue *et al.*, 1997; Arnone and Gordon, 1990). But in contrast, other studies have found that specific nitrogenase activity did not change under elevated atmospheric CO_2, and the increase in total nitrogen fixation was attributed to an increase in nodule biomass

(Philips *et al.*, 1976; Masterson and Sherwood, 1978; Finn and Brun, 1982; Norby, 1987; Temperton *et al.*, 2003).

Different methods have been used for measurements of nitrogen fixation in legumes, and each has advantages and disadvantages, and choosing one method from another depends largely on the facilities available and the objectives of the study (Azam and Farooq, 2003). These methods briefly are: 1- Determination of dry matter: this is the simplest and easiest method that gives a rough estimate about nitrogen fixation as up to 90% of a plant's demand of nitrogen are derived from nitrogen fixation. This method can be used to compare the efficiency of nitrogen fixation between different cultivars, but it cannot be used to obtain reliable quantitative estimations (Azam and Farooq, 2003).

2-Nodule number and mass: this method can be unreliable if used for comparison, since rhizobia are species and sometimes cultivar specific. Additionally, the number and mass of nodules may not reflect the amount of nitrogen fixed because of changes in the amount of carbonaceous compounds available at the time of the sampling, and furthermore there is the problem of ineffective nodulation. This method however, would be particularly suitable in the case where the effect of agro climatic conditions on nodulation and nitrogen fixation of a specific plant species is the aim of the study (Azam and Farooq, 2003).

3- Content of ureides and other metabolites: generally, ureides or amides are compounds that originate from nitrogen fixation, and therefore, the xylem sap in well nodulated plants will be rich in these compounds. This contrasts with plants that depend mainly on soil available nitrogen, which will be rich in NO_3 as NO_3 reduction at the root level is negligible. Hence measurements of ureides or amides in the xylem sap can give rough estimate concerning the efficiency of nitrogen fixation in different types of

legumes (Pate *et al.*, 1980; Herridge, 1982; Dakora *et al.*, 1992). This technique can also be very useful in studies aimed at determining NO_3 tolerance in legumes (Azam and Farooq, 2003). However, genotypes can be different in the level of NO_3 reduction at the root level, which can be used as an argument against the principle of this method which suggests minimum rates of nitrate reductase (Azam and Farooq, 2003).

4- Methods involving the use of ^{15}N: in these methods, nitrogen fixation is measured using the stable isotope of nitrogen (^{15}N), and is called the isotopic dilution approach (McAulife *et al.*, 1958). These methods give true values of nitrogen fixation, and more details about these methods can be found in Azam and Farooq, (2003). Limitations in the use of these methods revolve technical capabilities in the laboratory and the ability to handle radionucletides.

5- Acetylene reduction assay: this method is widely used in measuring nitrogen fixation in both glass house and field experiments. It is considered simple, rapid, inexpensive, sensitive and accurate (Hardy *et al.*, 1968; Hardy *et al.*, 1973; Bergersen, 1980; Hudd, 1980; Turner and Gibson, 1980; Vessy, 1994). The basis of this method is that nitrogenase, the enzyme responsible for reducing nitrogen to ammonia in the nitrogen-fixing bacteria, can also reduce other substrates other than nitrogen including acetylene (C_2H_2). The ability of the enzyme to reduce acetylene (C_2H_2) to ethylene (C_2H_4) has been used to indirectly measure specific nitrogen fixation and to give an estimate of the activity of nitrogenase in a wide range of legumes (Hardy 1973; Bergersen, 1980; Azam and Farooq, 2003). In the standard assay, detached nodules, nodulated root portions or soil cores are incubated in enclosed known volumes with 10% acetylene for a period of time, and then gas samples are analysed for ethylene concentration using gas chromatography (Hardy 1973; Bergersen, 1980; Azam and Farooq, 2003). The assay has also been conducted on intact plants in pot experiments (Singh and Wright, 2003;

Arnone and Gordon, 1990; Minchin *et al.*, 1983). Nitrogenase activity is usually expressed in nanomols or micromols C_2H_4 per unit of sample (grams of nodule fresh or dry weight, milligrams dry weight of cells etc.) per unit time (Bergersen, 1980). One of the main limitations for the assay is the optimisations of the gas chromatography protocol (Bergersen, 1980).

Previous experiments on lentils reported in the current investigation showed an increase in nodule number and weight under elevated CO_2 which was associated with an increase in plant nitrogen fixation (based on a nitrogen budgeting model). Increased nodule biomass clearly contributed to the increase in nitrogen fixation, but whether specific nitrogenase activity was also a contributory factor to this increase is unknown. Therefore the aim of this experiment was to attempt to investigate the effects of elevated atmospheric carbon dioxide on specific nitrogenase activity in lentils (*Lens culinaris* Medic). Additionally a preliminary investigation on white clover (*Trifolium repens*) was also included as a potential reference point.

5.2. Lentil experiment

5.2.1. Aim: To determine the effect of elevated CO_2 on nitrogenase activity in lentils using acetylene reduction assay on intact plants.

5.2.2. Objectives

5.2.2.1. Objective 1- To test whether nitrogenase activity increases under elevated CO_2 (theoretically, more carbohydrates will be available for the nodules, and this may raise nitrogenase activity.

5.2.2.2. Objective 2- To test if there is any difference between the two lentil cultivars in their response to elevated CO_2.

5.2.2.3. Objective 3- To investigate whether incubation time with acetylene affects nitrogenase activity in lentils.

5.2.3. Materials and methods

5.2.3.1. Plant growth

Two Syrian lentil cultivars ILL6995 (commonly known as Idlib 3) and ILL7979 were grown under ambient (400 μmol mol^{-1}) and elevated (809 μmol mol^{-1}) CO_2 in two tightly sealed and ventilated chambers placed in a greenhouse located in Skardon garden, University of Plymouth. Carbon dioxide supplementation was achieved using cylinders of compressed CO_2 (BOC gases) coupled to an IRGA Eurotherm™ controller which constantly coupled the air in the chamber and pulsed CO_2 from the bottled gas to a set point of 750 μmol mol^{-1} (twice ambient). Telaire™ monitors were used to measure CO_2 and temperature in each chamber at 15 minute intervals, and data logged to Hobo™ dataloggers. From these data, daily average CO_2 and temperature were calculated (Figure. 5.1, Figure 5.2). Cylindrical polypropylene pipe pots (21.25 cm high × 10 cm in diameter) were used to grow the plants, and 24 pots (12 of each cultivar) were placed randomly in each chamber.

The seeds were inoculated with *Rhizobium leguminosarum* bacteria, strain ILAR2D (Soya UK Ltd), immediately before sowing into medium-grade horticultural perlite growth medium. Two seeds were sown in each pot and thinned to one plant after

establishment. A complete Hoagland's solution minus nitrogen was irrigated every 10-14 days (100-150 ml/ pot), and similar amounts of tap water supplied in between according to demand. A starting amount of nitrogen (NH_4NO_3) equal to 5 kg N ha^{-1} was added in two identical amounts in the first and third week after sowing.

5.2.3.2. Acetylene reduction assay

Initially the acetylene reduction assay on intact plants was planned to be conducted at two stages, flowering and at three weeks after flowering. However, problems with the ethylene supply and a delay in delivery meant that the second assay was conducted five weeks after flowering.

In preparation for the assay, pots were drained of water 2 hours prior to incubation, and then the base and tops of the pots were tightly sealed using several layers of gas tight tape, and glue was applied around the plant stem above the sealing point (Plate 5.1). The volume of empty pore space was calculated using a displacement method and was estimated at about 1000 ml. A football bladder was used to store and carry the acetylene to the work site. Acetylene (10% v/v) was injected into the pots using a 100 ml disposable syringe, and at the same time an equal volume of air was removed using another needle in order to keep atmospheric pressure constant and the syringe holes made were immediately sealed with tape. Samples of 5 ml were collected after 15 and 30 minutes of incubation using 5 ml sterile disposable syringes, and the needles were inserted in rubber stoppers to prevent any leakage. Syringes were returned to the lab and samples were measured for ethylene concentration directly within a maximum of 3 hours after collection using gas chromatography (GC) by injecting 1 ml sample into the GC.

The roots and nodule fresh weight were recorded, and for dry weight the samples were oven dried for 48 hours at 80°C using a Gallenkamp drying oven.

The gas chromatography used was a Perkin Elmer 8500 fitted with flame ionization detector (FID. The column used in the gas chromatography was a 80/100 porapak Q, 6ft stainless steel, and the oven temperature was 75°C. The flow rate was 30 ml min^{-1}, and the injection temperature was 120 °C. An ethylene concentration of 5ppm was used as a standard for calibration.

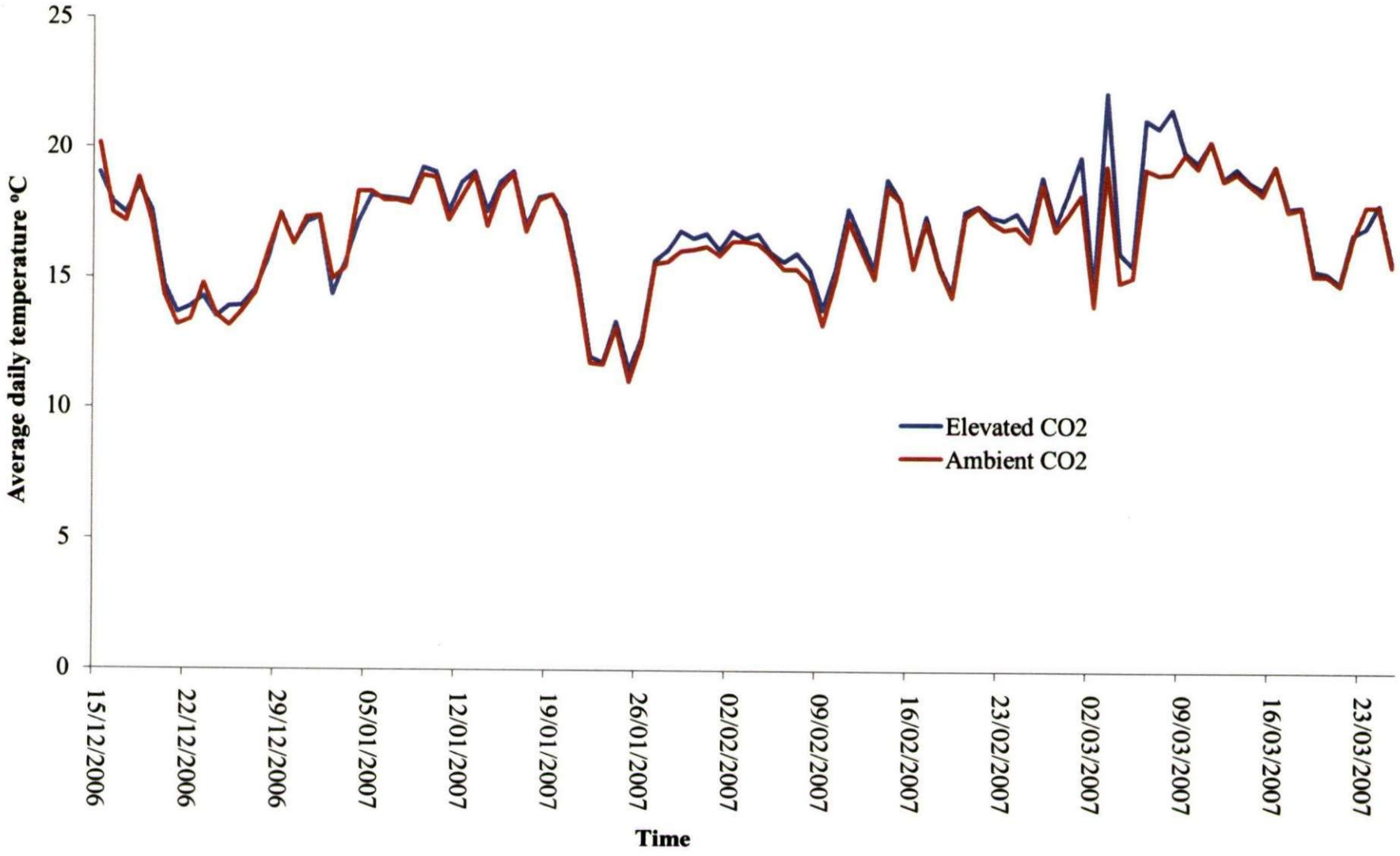


Figure 5.1. Average daily temperature during the growth period of the lentil cultivars.

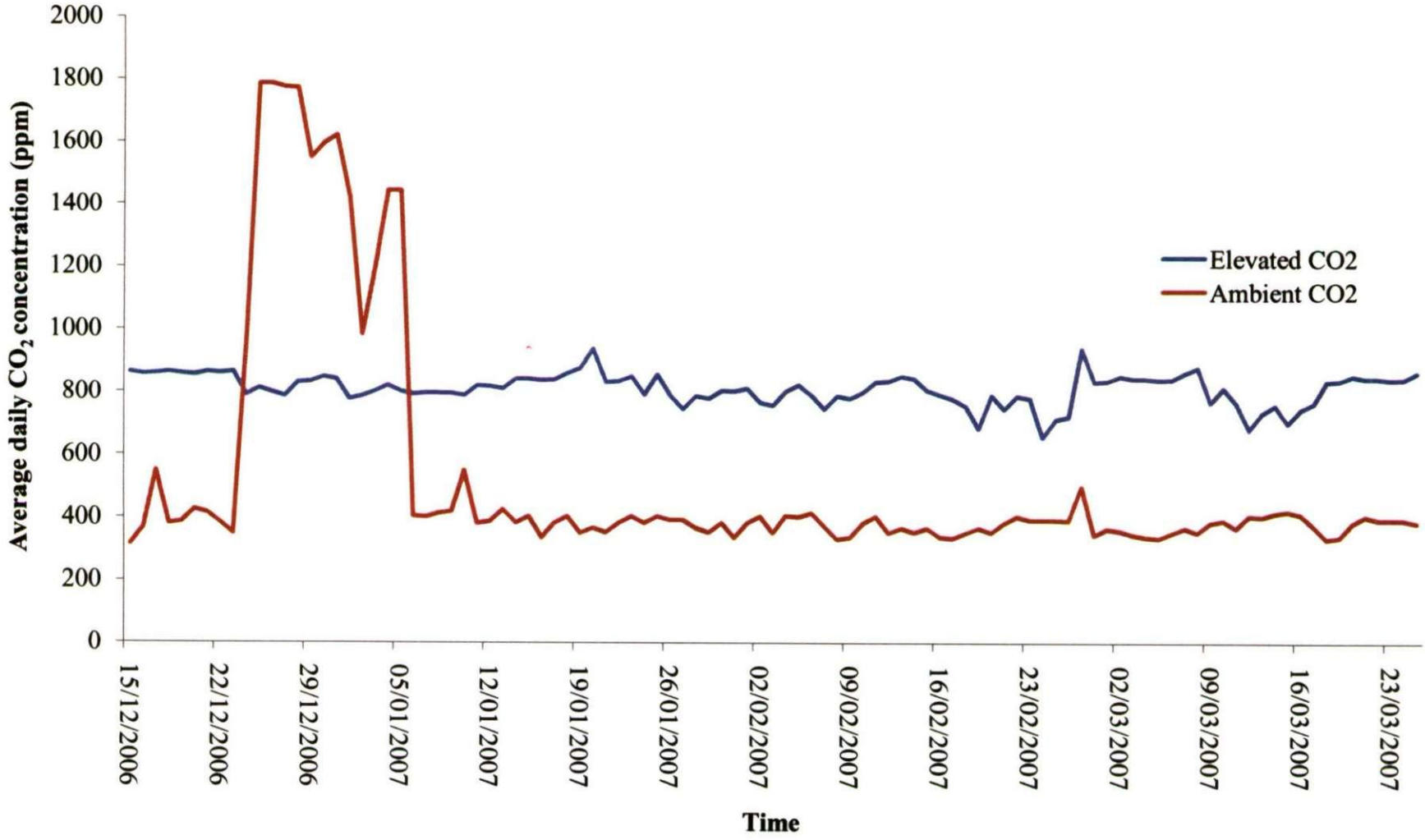


Figure 5.2. Average daily atmospheric CO_2 during the growth period of the lentil cultivars.

Plate 5.1. To show how the pots were sealed in the lentil experiment.

5.3. White clover experiment

5.3.1. Aim: To investigate the effects of elevated CO_2 on nitrogenase activity in white clover.

5.3.2. Objectives

5.3.2.1. Objective 1- Test if nitrogenase activity in white clover increases under CO_2 enrichment conditions.

5.3.2.2. Objective 2- Test the effects of incubation time with acetylene on nitrogenase activity.

5.3.3. Methods and materials

5.3.3.1. Plant growth

The plants of white clover (*Trifolium repens*) (GRM18) (Edwin Tuckers, Ashburton, Devon) were not originally grown for the purpose of this experiment. However the plants were transferred to the greenhouse, where the elevated CO_2 chambers were situated, relatively early in the growth stage. The plants were grown in 152 mm dwarf pots in a growth medium which was a mixture of three parts of a multi purpose compost ('Camelot') produced according to the Peat Producers Code of Practice, and one part of John Innes Potting compost, and there was no additions of any nitrogen fertilizer.

In this experiment, six pots were transferred to the greenhouse, and half of these pots were put in a chamber (as described earlier) where the plants were exposed to CO_2 enrichment conditions, and the other half were put in the greenhouse outside a chamber where the plants were grown under ambient air conditions. The plants of the two treatments received the same amount of water every two to three days, and the plants were exposed to the new growth conditions for more than 6 weeks before the assay was conducted. CO_2 supplementation was achieved in the same way described earlier in the lentil experiment. CO_2 concentrations and temperature were measured using Telaire™ monitors at 15 minute intervals, and data logged to Hobo™ dataloggers (Figure 5.3, Figure 5.4). Unfortunately, because of a faulty logger, data from the ambient air treatment were lost.

5.3.3.2. Acetylene reduction assay

The assay was conducted on portions of nodulated roots, and three samples from each pot were assayed. The roots were dug out of the compost, washed and cleaned from soil particles

and incubated in 60 ml disposable syringes with (5% v/v) acetylene (5 ml of air displaced and replaced by acetylene) for 15 and 45 minutes. Rubber stoppers to prevent any gas leakage were used as described previously. 5% v/v acetylene was used instead of 10% in an attempt to more easily separate acetylene and ethylene from each other in the gas chromatography. 5 ml samples collected using 5 ml disposable syringes which were also sealed using rubber stoppers and were analyzed for ethylene concentration directly after collection by injecting 1 ml into the GC as described earlier.

The roots and nodule fresh weight were recorded, and for dry weight the samples were oven dried for 48 hours at 80°C using a Gallenkamp 250 °C drying oven. Ethylene production was calculated, according to Hardy (1968), from a standard curve (Figure 5.5) of different contents of ethylene. General liner Model in Minitab 15.1 was used to analyse the data.

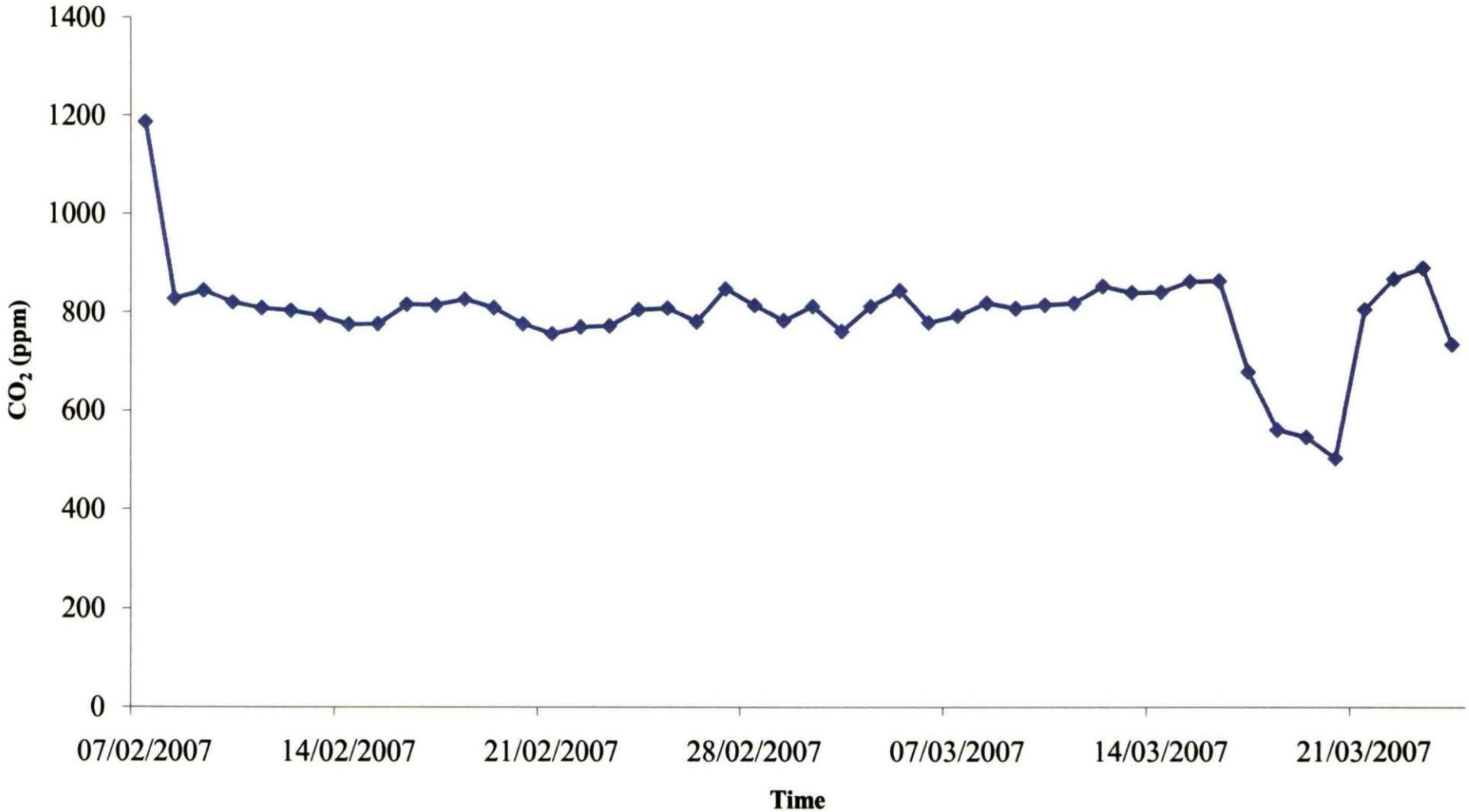


Figure 5.4. Daily CO_2 concentrations in the CO_2 enriched growth chamber during the growth period of white clover plants.

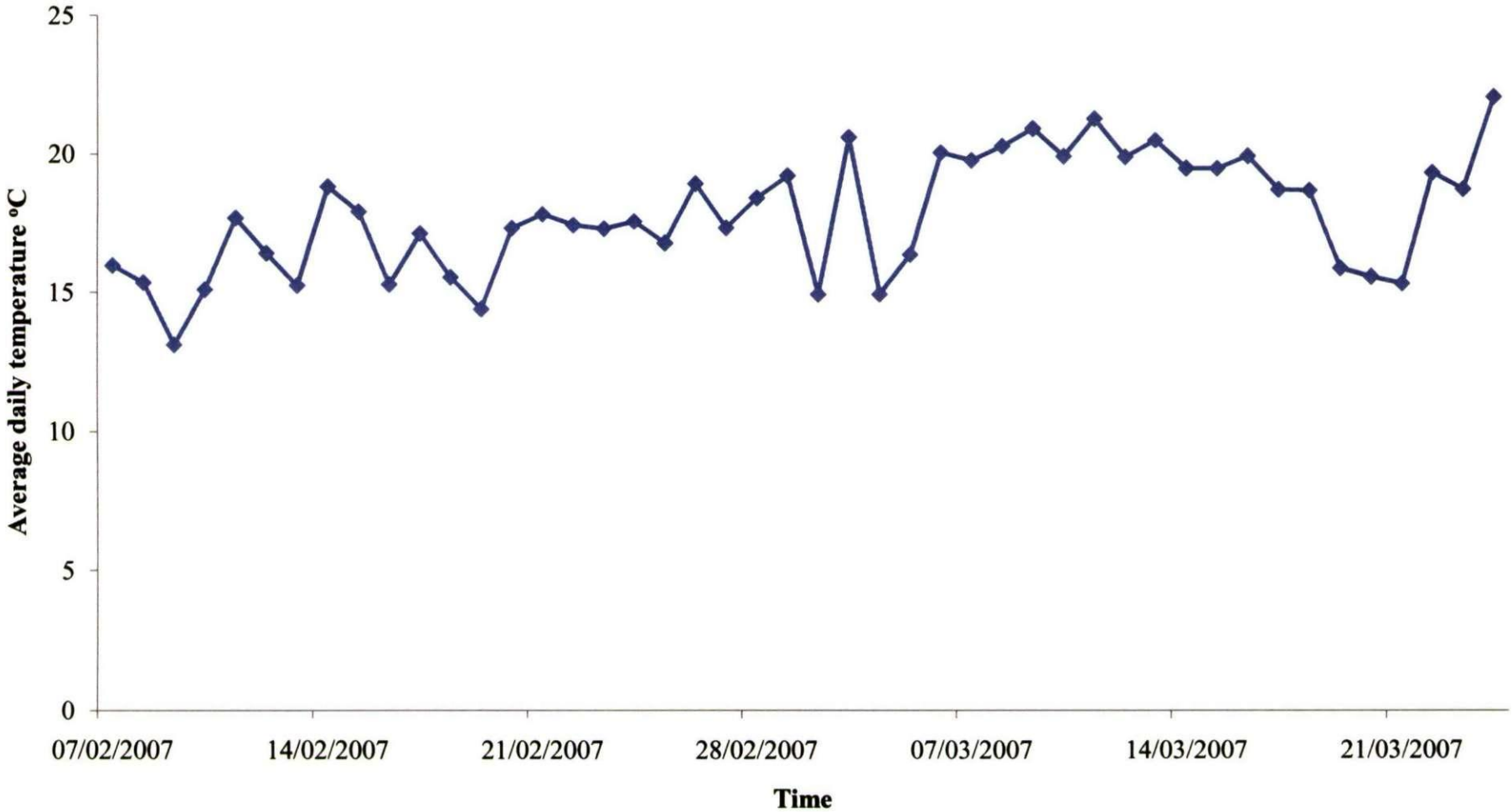


Figure 5.3. Average daily temperature in the CO_2 enriched growth chamber during the growth period of white clover plants.

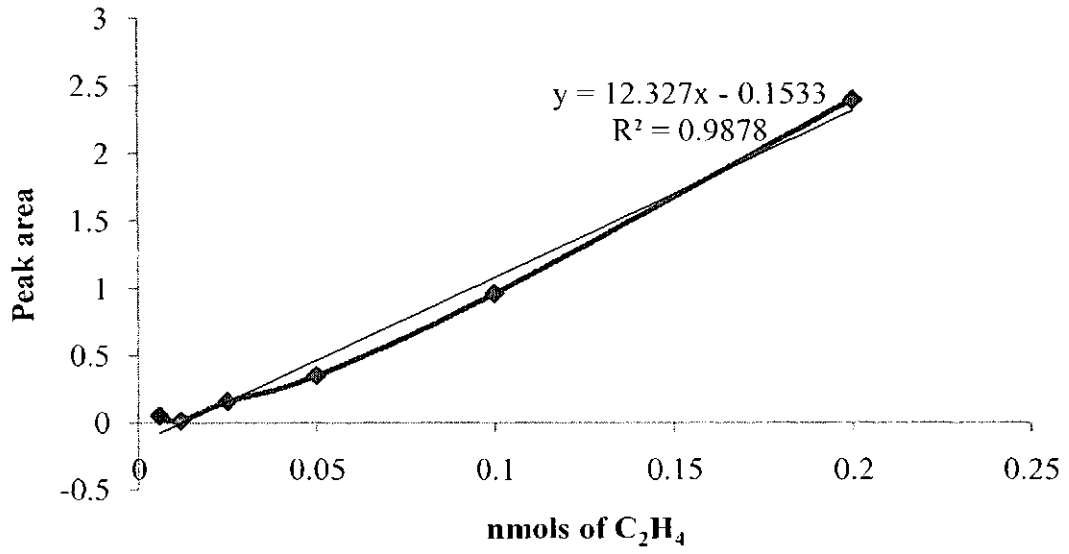


Figure 5.5. The standard curve of peaks areas of C_2H_4 obtained using the GC.

5.4. Results and Discussion

5.4.1. Lentil experiment

The growth of lentil plants was unexpectedly very poor in this experiment, which could be due to a nutrient deficiency as symptoms of manganese deficiency were diagnosed (personal communication Dr. Stuart Lane, University of Plymouth). Some improvement was achieved by adding amounts of manganese to the plants, but the plant growth was still less than normal (see plate 5.1).

The first assay was conducted at flowering (55 days after sowing), and the results were unexpected as ethylene was not detected and print outs of the GC only showed the acetylene peak. When the plants were assayed it appeared that nodulation was very low (2-3 nodules/ plant) and for some plants there were no nodules at all. This low nodule

number can be attributed to the poor plant growth as it is well established that nitrogen fixation or nodulation is dependent on host photosynthesis with the two processes are closely linked (Arnone and Gordon, 1990; Wheeler, 1971). There was also a question mark over the age of the inculating Rhizobium bacteria used which may also have contributed to the low nodulation rate. It was decided to intervene at this point in the experiment and additional new amounts of inoculum were added to the remaining plants. Slightly better nodulation was reported at the next assay point.

The lack of ethylene detection at the first assay point was attributed to a lack of nodules.

As explained earlier, the second assay in this experiment had to be delayed later than was desirable and was conducted at 5 weeks after flowering closer to plant maturity. The GC results of the second assay were also unexpected and ethylene was not detected. Plant growth and nodulation of the second assay point was better to some extent than that at the first sampling, but most of the nodules observed were green in colour which tends to indicate low nitrogenase activity (active nodules are usually identified by their pink, red and sometimes light brown colour showing leghemoglobin activity (Somasegaran and Hoben, 1994). Despite the low number of active nodules ethylene detectation was expected even if in very small amounts and at this point of the experiment attention was directed towards the GC conditions with the suggestion that they were not ideal to separate the two gases (acetylene and ethylene) from each other and an intense period of lab assay optimisation followed. Different regimes of different oven temperatures (60, 50 and 45°C), flow rates (30, 25 and 15 ml min^{-1}) and injection temperature (120, 45°C) were tested (at least each regime was tested for about 50 times) and still sufficient separation was not achieved. Disappointingly, therefore, it was not possible to obtain any reliable measurements for ethylene production from the acetylene

production assay on intact plants of lentils, and efforts were later diverted to test the effects of elevated CO_2 on nitrogenase activity of well nodulated white clover plants instead.

5.4.2. White clover experiment

Half way through the lentil experiment, it was clear that plant growth was not as normal as expected with very poor growth observed under both elevated and ambient CO_2, with few any visual differences between the two treatments (in previous experiments observable effects were obvious). Therefore it was expected that if the plant growth did not recover, it was possible that valuable results would not be obtained. Therefore, since the purpose of this experiment was to investigate the effect of elevated CO_2 on nitrogenase activity, it was decided to use well grown white clover plants in addition to the lentils in order to be able to obtain some comparative results between the two treatments.

From visual observation, the white clover plants grown in the CO_2 enriched chamber were clearly bigger than those grown under ambient conditions indicating a positive growth response to elevated CO_2.

The GC measurements with the white clover were carried out after the intense optimization lab tests with the GC. Two peaks were recorded by the GC (Plate 5.3), and after calculating the ethylene production, the results showed that the rates of nitrogenase activity or specific nitrogenase activity were slightly higher under elevated CO_2 at both measurement times but not significantly ($p=0.174$) (Figure 5.6). The ethylene production was significantly greater after 45 minute incubation than after 15 minutes ($p=0.042$), and there was not a significant interaction between CO_2 treatment and

incubation time. It is necessary to stress that the shape of the peaks were unusual and they were not similar to typical peak shape reported in other literature (e.g. in Hardy 1968; Bergersen, 1980). Additionally, the results obtained were rather high, especially that obtained after 45 minutes of incubation (Figure 5.7), as the average of nitrogenase activity for legumes reported by Hardy *et al,* (1973) was 0.20 nmol.min^{-1} mg-1 nodule fresh weight, and values in white clover reported by Halliday and Pate (1976) were close to this average. Some doubt is therefore cast on these results and it is very likely that the peaks recorded by the GC may not be accurate and therefore the results may not be robust.

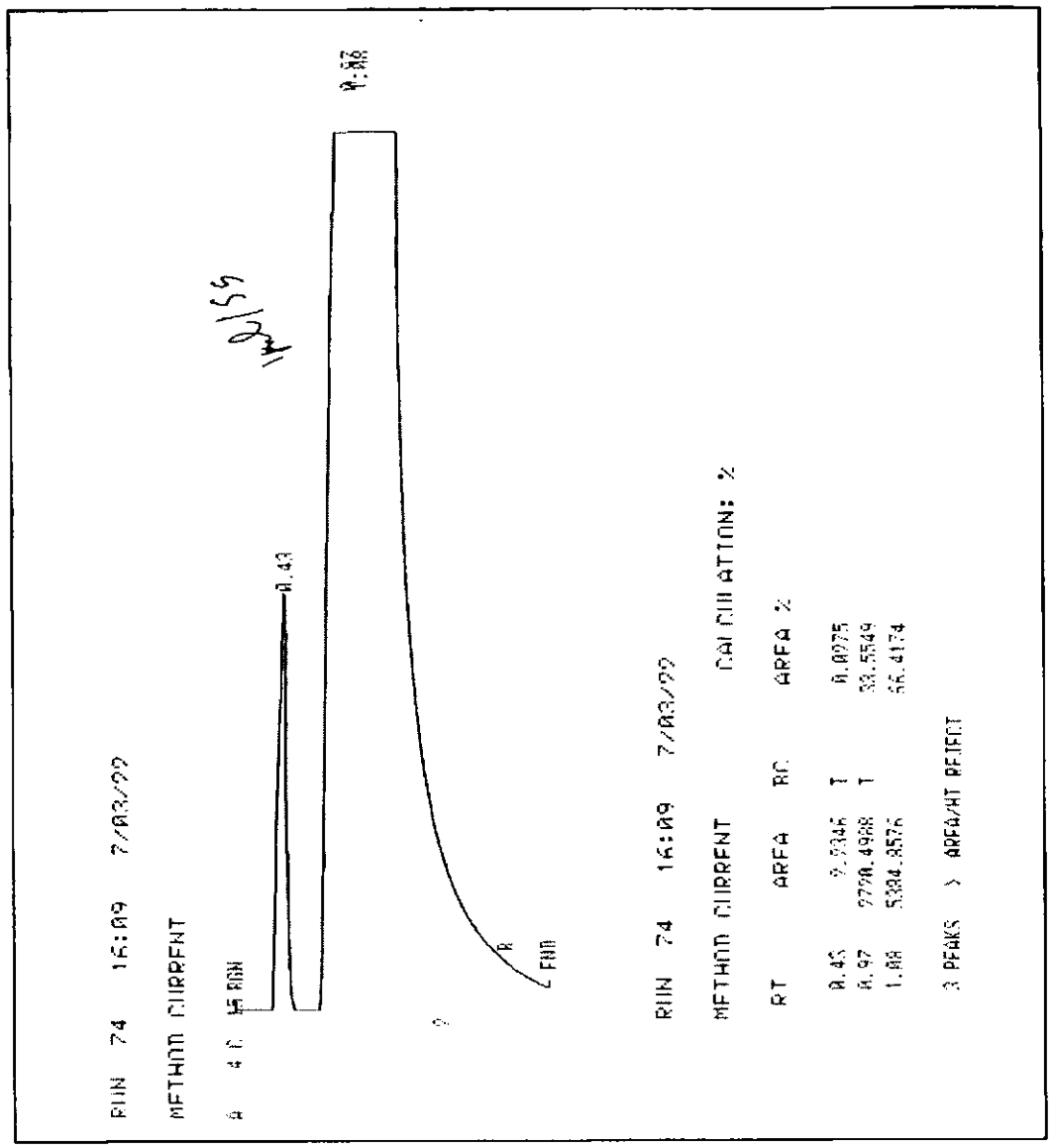


Figure 5.6. An example of peaks recorded by the GC of a gas sample from the white clover assay.

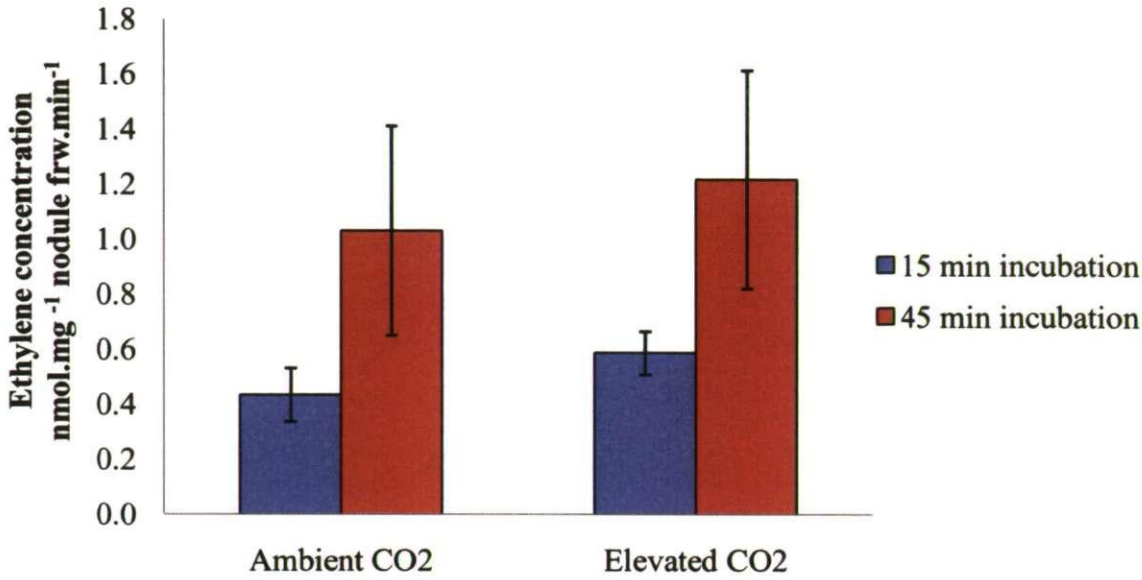


Figure 5.7. The effect of elevated CO_2 on the rates of nitrogenase activity (ethylene production) in nodules of white clover after 15 and 45 min of incubation with acetylene (5% v/v). (Vertical bars are +/- 1 se).

Notwithstanding this caveat, the results of this experiment indicated that elevated CO_2 had no significant effect on specific nitrogenase activity in white clover, and this agrees with other studies conducted on different crops under conditions of high levels of atmospheric carbon dioxide. Norby (1987), in a study on the seedlings of three nitrogen-fixing tree species (*Robinia pseudoacacia* L. and the actinorhizal species, *Alnus glutinosa* (L.) Gaertn. and *Elaeagnus angustifolia* L), indicated that high levels of atmospheric CO_2 (700 μL L^{-1}) did not have a significant effect on specific nitrogenase activity although whole plant nitrogenase activity was significantly higher (whole plant activity being a function of specific activity and total nodule volume). Similar conclusions were reported by Temperton *et al,* (2003) on nitrogen-fixing trees of common alder *Alnus glutinosa* (L.) Gaertn. Further investigations on different legume crops such as soybean (Finn and Brun, 1982), pea (Philips *et al.,* 1976; Masterson and

Sherwood, 1978), and white clover (Masterson and Sherwood, 1978) also stressed the same conclusion.

Results from the current investigation also showed that ethylene production after 45 minutes incubation with acetylene was higher than that after 15 minutes of incubation ($p=0.042$), which indicates that nitrogenase activity, was not reduced with the longer incubation period (particularly the shoots were removed and the roots were shaken before the incubation), but it continued to increase instead. These results contradict the literature (Hardy, 1968; Hardy 1973; Minich *et al.*, 1986; Herdina and Silsbury, 1990; Singh and Wright, 2002; Singh and Wright, 2003), which in general indicates that nitrogenase activity decline with increased incubation time especially when the roots are removed from the shoots and when physical disturbance to the nodules occur (e.g. root shaking, as the carbohydrate supply to the fixing bacteria is reduced over time. For example, Minichin *et al.* (1983) reported that ethylene production decreased by 40 -60% when nodulated roots of some legumes are exposed to acetylene during a 30 minutes assay period, and Herdina and Slisbury (1990) reported that nitrogenase activity in faba bean during the reproductive stage was reduced by about 20% after 40 minutes incubation. In general, the length of time before the inhibition of nitrogense activity occurs is depending on the carbohydrate status of the nodules and the root tissues at the time of the assay (Walsh *et al.*, 1987).

In fact, little confidence in the GC used was generated as it showed a wide variation of values even when injecting standard acetylene or ethylene, and it might be that the detector was faulty and was giving inaccurate results. Therefore the conclusions derived from these results are at best, tentative.

5.5. Critique of experimental conditions

As mentioned earlier, uncertainty cast on the results obtained from this experiment, and probably, the main reason could be the use of the wrong column in the gas chromatography used for the analysis. Choosing the right column to be used in the assay is crucial to the success of the experiment, but in fact, several reasons attributed for this fatal error to occur. First, attempts made to gain some training on conducting the acetylene reduction assay in a laboratory in the south west, unfortunately were unsuccessful.

Second, with no previous personal experience in using the gas chromatography, help was sought from the technician in charge of using the GC in the university, who too was completely unfamiliar with the assay as most of the work conducted using the GC is mainly on liquid samples, and gaseous measurements are very rarely conducted. The possibility of conducting the assay was discussed with the technician and a list of columns used in previous experiments reported by Hardy *et al.* (1973) was shown to her. At that time she wanted to check which column fits best with the GC, and after that she recommended the column used later in the experiment. Samples of ethylene and acetylene were injected into the GC and tests confirmed that it was possible to detect each of the two gases by the GC using the column chosen. Therefore, at that stage, it was assumed that the column chosen was suitable to conduct the assay.

Third, the unexpected low growth of lentil plants and the very low nodulation rate were a major constraint to the experiment, and the assumption made was that the very low nodulation (2 or 3 nodules per plant) was the main reason for not detecting any ethylene

by the GC, and that diverted any attention away from the GC conditions or the column suitability.

Fourth, by the time it was clear that probably, an unsuitable column had been chosen for the test, it was very difficult to re-conduct the experiment using a new column, within the time limitation (being heavily pregnant).

A question was also made whether the time between sampling and measurements, which was relatively long due to the inconvenience of the greenhouse location and that of the GC, could have also affected the results obtained as it was previously reported that longer times between sampling and measurements significantly reduce the values of nitrogenase activity and affect the ability to detect any differences between treatments (Singh and Wright, 2003). Furthermore, in this experiment, the gas samples were kept in the plastic syringes before injecting them into the GC (1-3 hours in the lentils experiment), and this procedure could have led to the loss of ethylene, as it has been reported that using disposable plastic syringes for storage could cause loss of ethylene by adsorption or diffusion, and the rate of the loss varies depending on the ethylene concentration and the syringes used (Vessey, 1994).

Therefore, different factors may have contributed to the uncertainty of the outcome of the current experiment, and more attention to the details should have been taken.

5.6. Conclusions

Unfortunately, because of the poor growth and nodulation of lentil plants, and because of the technical difficulties in detecting ethylene and separating it from acetylene using the gas chromatography, it was difficult to obtain any measurements for nitrogenase activity in lentils. Results from the back-up experiment of the effects of elevated CO_2 on nitrogenase activity in white clover showed that nitrogenase activity (as ethylene production) was slightly higher under elevated CO_2 at the two incubation times with acetylene (15 and 45 minutes) than that at ambient, but not significantly so. Furthermore, ethylene production from samples incubated for 45 minutes with acetylene were significantly higher than those from 15 minutes incubation. Combined with the technical difficulties encountered using the GC that was explained earlier, the unusual results could be because of a faulty detector and therefore, doubts are cast on the results obtained.

Not withstanding the lack of conclusive results from this experiment it is clear from earlier experiments that nodule number and biomass increase in lentils grown at elevated CO_2. Thus, the plant total nitrogenase activity identified by nitrogen budgeting under elevated CO_2 is unlikely to be as a result of specific nitrogenase activity, but more as a result of increased nodule biomass.

6. Chapter 6

General Discussion

This study investigated the effects of elevated CO_2 and its interaction with other environmental factors (drought and nitrogen fertilizer) on the growth, production and nodulation of the leguminous crop lentils (*Lens culinaris* Medic). The main findings are summarised below and then discussed in the context of the current published literature.

6.1. The interaction of elevated CO_2 and drought

- Although non-significantly, above ground dry weight in lentils in the second experiment increased by an average of 12% by elevated CO_2 and seed yield was marginally significantly increased by an average 19%, and this increase was relatively greater in water stressed plants (20%) than in fully irrigated plants (17%).
- The effect of drought was more noticeable than the effect of CO_2, and production was reduced by up to 59%.
- Root weight was increased although non-significantly by an average of 17% by elevated CO_2, and root to shoot ratio was not affected.
- Although non-significantly, both nodule number per plant and nodule fresh weight were increased by an average of 38% and 45% respectively under elevated CO_2 and by contrast, were significantly reduced by drought.
- Nitrogen concentration in the seeds and roots was not affected by elevated CO_2 ($p= 0.372$ and $p=0.387$ for seeds and roots respectively) unlike that in the shoots which was significantly reduced (0.004).
- Phosphorus concentration in the seeds was not significantly affected by elevated CO_2 ($p=0.257$), while that in the shoots was significantly reduced ($p=0.009$) and

the concentration in the roots was marginally significantly lower (p=0.083). The cultivar Idlib 3 showed greater yield results under all conditions than the cultivar ILL7979, although the relative increase in the latter was sometimes greater than the former.

6.2. The interaction of elevated CO_2 and nitrogen fertilizer

- Biomass production and seed yield of lentil cultivar Idlib 3 under all nitrogen treatments increased significantly under elevated CO_2 (p= 0.032 and p= 0.003 respectively) with an average increase of 35% for above ground dry weight and 53% for seed yield
- All production parameters increased significantly (p< 0.001) with the increasing levels of nitrogen.
- Although not statistically significant, nodule number per plant under all nitrogen treatments were greater under elevated CO_2 by an average of +52% than that at ambient CO_2, with the highest number observed at 50 kg N ha^{-1} under ambient CO_2 and at 75 kg N ha^{-1} under elevated CO_2 .
- Root dry weight increased by an average of 37% under elevated CO_2, but this increase however was marginally significant at 10 level (p= 0.078).
- Root to shoot ratio was not significantly increased (p= 0.847).
- Nitrogen and phosphorus concentrations in the dry matter were lower but not significantly.

6.3. Contradicting results

- Above ground dry weight at anthesis in the first drought interaction experiment was unaffected by elevated CO_2, unlike that in the second drought experiment where the increase was significant at 10% level.
- There was a significant reduction in nitrogen concentration of the shoots and leaves under elevated CO_2 in the second drought interaction experiment ($p= 0.004$), whereas, the reduction in the nitrogen interaction experiment was not significant.
- Similarly there was a significant reduction in phosphorus concentration of the shoots and leaves under elevated CO_2 in the second drought interaction experiment ($p= 0.009$), whereas, the reduction in the nitrogen interaction experiment was not significant.

6.4. Less robust results

- Total nitrogenase activity increased as plant size increased (as a result of either elevated CO_2 or nitrogen fertilization)
- Specific nitrogenase activity in the nodules of white clover plants was not affected by elevated CO_2.
- Nitrogenase activity continued to increase in detached nodules of white clover even after 45 minutes of incubation with acetylene.

6.5. No results

- Because of the poor growth and nodulation of lentil plants and technical problems encountered using the GC, it was difficult to obtain any results of the effects of elevated CO_2 on specific nitrogenase activity in lentils.

Parameter studied	CO_2 × D (Exp 1)				CO_2 × D (Exp 2)				CO_2 × N				
	Idlib 3		ILL7979		Idlib 3		ILL7979		Idlib 3				
	+ H_2O	-H_2O	+ H_2O	- H_2O	+ H_2O	-H_2O	+ H_2O	- H_2O	5 kg N ha^{-1}	25 kg N ha^{-1}	50 kg N ha^{-1}	75 kg N ha^{-1}	100 kg N ha^{-1}
LAI	+33	+0.07	+6	-23	+21	+8	+5	-13	+57	0	+19	+20	+81
Aboveground dry weight at anthesis	+16	+3	+2	+17	+33	+58	+40	+27	+67	+16	+43	+48	+63
Belowground dry weight at anthesis					+34	+27	+20	+15	+23	+71	+10	+51	-2
Aboveground dry weight at maturity	+31	+2	+33	+17	+12	+9	+13	+12	+66	+21	+25	+39	+53
Belowground dry weight at maturity					+12	+14	+36	+12	+30	+6	+63	+26	+25
Nodule number					+17	+72	+47	+29	+23	+66	+13	+59	+20
Nodule fresh weight					+52	+120	+32	+22					
Seed yield	+41	+6	+29	+0.4	+16	+11	+17	+33	+101	+30	+16	+58	+59
Harvest Index	+7	+3	+3	-20	+2	+4	+5	+23	+16	+8	-7	+14	+5
Nitrogen concentration (seeds)					-8	0	-2	-7	+51	+31	-9	-6	-5
Phosphorus concentration (seeds)					-8	-11	-3	-8	-11	+3	-1	-7	-9
Nitrogen uptake					+11	+9	+9	+10	+130	+59	+8	+42	+47
Phosphorus uptake					+4	-8	0	+8	+46	+36	+9	+30	+13

Table 6.1. A Summary of the percentage increase in the parameters studied in response to elevated CO_2 compared to ambient CO_2.

6.6. Discussion

6.6.1. Comparison of the results between experiments

Results from this study clearly showed that productivity in lentils increased in response to the exposure of the plants to levels of atmospheric CO_2 higher than current ambient levels. The percentage increase and the level of significance however varied between the experiments and that can be attributed to differences in the microclimate conditions for each experiment as each experiment was conducted in a different location (necessitated by the relocation of the faculty to Plymouth and the glasshouses on the Plymouth campus) and in a different season and for a different purpose. It is not unusual that the response of the same species to elevated CO_2 differs between experiments. For example, Poorter and Navas (2002) indicated in their review that for both wheat (*Triticum aestivum*) and soybean (*Glycine max*), which are the most studied species, there were increases and decreases in seed mass in their response to elevated CO_2.

A stronger significant response was observed from the nitrogen interaction experiment compared to the response from the drought interaction experiments where the response of seed yield was marginally significant ($p=0.059$).This is in fact a comprehensible outcome as the availability of nitrogen in the former, particularly the higher levels (75 and 100 kg N ha^{-1}), boosted the productivity in the plants grown in CO_2 enriched conditions. These results are not unusual, and a greater response to elevated CO_2 is usually reported in well fertilized plants (Stitt and Krapp, 1999: Conroy *et al.*, 1992: Arp *et al.*, 1998). Furthermore, unlike plants from the second drought interaction experiment where a spider mite infection was reported and at a drop of pH occurred for a short period after flowering, plants from the nitrogen interaction experiment were free

from pests and diseases and no infestation was recorded, which emphasises that optimizing the growth conditions of the plants helps lead to greater yield responses under elevated CO_2. The absence of abiotic and biotic stresses has previously been reported to enhance the response of grain legumes to elevated CO_2 (Prasad *et al.*, 2005).

Between the two drought interaction experiments, although the average yields in the second experiment was higher than that in the first (see Table 3.6, chapter 3), relatively, the average increase of yield in response to elevated CO_2 under full irrigation conditions was higher in the first experiment. This may be because of the higher average temperature (22.12 °C compared to 20.60 °C in the second experiment), which is well known to further enhance photosynthesis and growth under elevated CO_2, especially considering that these temperatures are not above the optimum for photosynthesis and growth (Sage and Sharky, 1987; Ainsworth and Long, 2004; Prasad *et al.*, 2005). However this experiment was limited by low irradiance levels, which varied between the chambers in a range of 180-280 μmol m^{-2} s^{-1}, and it is likely that full photosynthetic capacity was not achieved, and therefore the absolute yields were lower than those reported in the second experiment which had the advantage of natural light. In fact, seeing these differences between the experiments, which varied in their microclimatic conditions, stresses the fact that plants' response to elevated CO_2 is largely dependent on other environmental factors such as light, temperature, moisture and nutrients, and their interaction with elevated CO_2 (Baker and Allen, 1994; Rogers *et al.*, 1994; Shaw *et al.*, 2005:), and lentils in this respect are no different.

It was difficult to explain some contradicting results between the experiments other than that caused by differences in the experimental conditions.

6.6.2. Comparing results with the reviewed literature

6.6.2.1.The effect of elevated CO_2 on productivity and carbon partitioning

To date, there has been a huge interest in investigating the effects of elevated CO_2 and its interaction with other environmental factors on vegetation and terrestrial ecosystems (e.g. Idso and Idso, 1994; Clark *et al.*, 1999; Kimball *et al.*, 2002) and different global vegetation models have been developed and used for future projections (Cramer *et al.*, 2001; Luo *et al.*, 2008). Plants respond differently to different levels of CO_2 depending on the species and other interacting environmental factors (Rogers *et al.*, 1994). Most certain however, is that in C_3 plant species, photosynthesis and transpiration are directly affected by CO_2 levels above ambient and net photosynthesis per unit leaf area is increased due to reduced photorespiration and increased substrate availability. Transpiration is reduced as a result of reduced stomatal conductance (Poorter, 1993). These combine to lead to enhancement of up to 75% of the net assimilation rate under doubling levels of CO_2 (Shaw *et al.*, 2005), and stomatal conductance is reduced by an average of 40% (Rogers *et al.*, 1984; Morison, 1985). Water use efficiency (WUE) is improved as a consequence, and Kimball and Idso (1983) concluded that under doubling CO_2, WUE could be doubled. Therefore, growth and productivity are expected to increase and this response is widely documented (Kimball, 1983; Rogers *et al.*, 1983a; Dahlman *et al.*, 1985; Poorter, 1993; Idso and Idso, 1994; Drake *et al.*, 1996; Gannoum *et al.*, 1997; : Miller *et al.*, 1998:;: Wand *et al.*, 1999; Jablonski *et al.*, 2002; Tubiello *et al.*, 2007). Rogers & Dahlman (1993) indicated that almost all studies dealing with the direct effects of elevated CO_2 showed a positive response to CO_2 enrichment. Vegetation models suggest that the increased levels of CO_2 have contributed to the increase in vegetation cover and leaf area over the 20th century (Cramer *et al.*, 2001). Furthermore, as

a result of the enhanced water use efficiency by elevated CO_2, biomass production in areas where rainfall is low will also increase (Chaves and Pereira, 1992). In fact, a considerable number of studies have indicated that the severity of drought can be partially mitigated by elevated CO_2 and in many cases a greater response to elevated CO_2 under drought has been reported (when expressed on a % basis) (Gifford, 1979; Kimball *et al.*, 1993; Kimball *et al.*, 2002:). For example Manderscheid & Weigel (2007) reported that CO_2 enrichment increased biomass and grain yield in wheat by less than 10% under well watered conditions and by 44% under drought conditions. Similar conclusions were also found in cotton (Kimball & Mauney, 1993). This expected positive effect for elevated CO_2 is greatly important in areas like the eastern Mediterranean, where productivity is largely reduced by drought and extreme heat (Kaddour & Fuller, 2004), and particularly when according to the IPCC projections, a decrease in annual precipitation and an increase in mean temperature in the summer in this area is very likely (Christensen *et al.*, 2007). Lentil is an important crop grown in this region, and therefore a great part of the current investigations aimed at investigating the implications of the interaction of elevated CO_2 and drought on the growth and productivity of this crop.

In fact, lentils acted in a similar manner to the majority of plant species reported in the reviewed literature and results showed that productivity was enhanced although marginally significantly (at 10% level) in the second drought experiment as a result of CO_2 enrichment.

Results from the first drought experiment showed that seed yield was significantly enhanced under elevated CO_2, but there was a significant interaction between drought and elevated CO_2 treatments, and drought affected the response to elevated CO_2 and resulted in seed yield being not affected by elevated CO_2 in the water stressed plants

(see Appendix E). In the second drought experiment seed yield was marginally significantly (p=0.059) increased by elevated CO_2, and the interaction between drought and elevated CO_2 was not significant and the response to elevated CO_2 was similar in both drought and full irrigation treatments.. However, because of the technical difficulties encountered in the first experiment, which mainly led to the control chambers having higher CO_2 levels than originally planned and close to that in the elevated chambers up until the flowering stage, it was difficult to be completely conclusive that lentil is less responsive to CO_2 enrichment when water is limited. Probably, the early maturation in the droughted plants which shortened the exposure time to the high levels of CO_2 applied after flowering in the elevated chambers led to the plants not being able to fully exploit the benefits of the CO_2 enriched conditions. The general problem of "terminal drought" in crop production is clearly applicable to lentils. In fact, due to these problems and to uncertainties regarding the statistical analysis, it is preferred to consider the results from the second experiment as the more robust, and therefore, it is probably better to some extent to neglect the outcomes from the first experiment particularly those concerning the response under drought conditions. As referred to earlier, seed yield in both cultivars under both drought and full irrigation conditions in the second experiment was increased by an overall average of 19% (p=0.059) even though there was not a significant interaction between drought and elevated CO_2, , it was noticed that the relative increase particularly in the cultivar ILL7979 was greater when water was limited than that in the fully irrigated plants. The average enhancement of elevated CO_2 under drought conditions was 20% which is slightly higher than the 17% enhancement observed in fully irrigated plants. This increase can be attributed to the increase in net assimilation rate and the enhanced water use efficiency under elevated CO_2 since the plants in each drought treatment received

the same amount of water. The significance of drought in this study was very noticeable under both ambient and elevated CO_2 and the yields more than halved as a result of drought. Elevated CO_2 contributed to some extent in alleviating the effect of drought by an average improvement of about 20%, which overall was marginally significant (at the 10% level).

As explained earlier in chapter 3, it is believed that failing to achieve significance at 5% level is probably due to a combination of few reasons which might have affected the magnitude of growth response to elevated CO_2. Such reasons include the occurence of a spider might infection and a short drop in pH levels in the hydroponics and although it was quickly corrected, this could have affected nutrient absorbance. The limitation of replication in the experimental design could also have contributed.

Although most of the literature indicated significant enhancement of plant growth in response to elevated CO_2 particularly in legumes (Rogers & Dahlman, 1993; Jablonski *et al.*, 2002), there are some reports which showed that the level of enhancement was not always significant. (Navas *et al.*, 1997; Lawson *et al.*, 2000; Mayagi *et al.*, 2007)

Part of the study aimed at investigating the interacting effects of elevated CO_2 and nitrogen fertilizer on lentils. This aspect is also very important particularly in the long term, where production under high levels of CO_2 will probably be increasingly restricted by nutrient limitations, especially nitrogen (Wong, 1979; Bazzaz, 1990; Newton, 1991). It is likely that the response to elevated CO_2 in nitrogen fixing legumes is expected to be greater than that in non-fixing species as nitrogen can be reduced as a limiting factor (Poorter, 1993; Soussana & Hartwing, 1996; Clark *et al.*, 1997; Reich *et*

al., 2001:), and in fact many reviews indicated so (Jablonski *et al.*, 2002; Poorter and Navas, 2003) Moreover, nodules also represent a strong sink for carbon, and it has been reported that strong carbon sinks are essential if plants are to benefit from long term exposure to elevated CO_2 (Lewis *et al.*, 1994; Díaz, 1996). The inhibition effect of exogenous nitrogen to the process of nitrogen fixation is well known (Herdina and Salisbury, 1989; Buttery *et al.*, 1990; Walsh, 1995) and therefore it is necessary to optimize the level of nitrogen fertilizer for future cultivation of lentils to a level at which seed yield is increased and nitrogen fixation is not affected.

In general, increased yields under CO_2 enrichment conditions have been previously reported over a wide range of nutrient availability with plants showing greater response at higher levels of nitrogen e.g. in wheat (Sionit *et al.*, 1981; Fangmeier *et al.*, 1996), soybean (Sionit, 1983), tomato (Li *et al.*, 2007), and cotton (Kimball *et al.*, 1993; Reddy *et al.*, 2004).

To some extent, this was the case in the current nitrogen interaction experiment where biomass production and seed yield both increased at all nitrogen treatments (equal to 5, 25, 50, 75 and 100 kg N ha^{-1}) under elevated CO_2 and this increase was mostly stronger when nitrogen levels were higher. The current work however, also showed that the relative increase in above ground dry weight and seed yield at the lowest nitrogen level was the strongest. It is probable that the large amount of nitrogen fixed was the reason for this large enhancement of the growth. Meier and Fuhrer (1997) also reported a greater response to elevated CO_2 at low nitrogen availability in red clover. Kimball *et al.*, (2002) in their review of previous FACE experiments conducted on different agricultural crops, concluded that growth stimulation at low nitrogen availability was reduced in non legume species, whereas in the clover legume the enhancement was

strong at both ample and low nitrogen availability. Other studies however, reported no effect for elevated CO_2 when nitrogen was limited (Goudriaan and de Ruiter, 1983; Arp *et al.*, 1998; Newman *et al.*, 2006). The effect of nitrogen availability on the growth and production of the lentil cultivar Idlib 3 was also very noticeable and the yield always increased with the increasing levels of nitrogen. However, this increase at the higher levels of nitrogen was stronger under elevated CO_2, and less effective under ambient CO_2 conditions. This can be explained by an improved net assimilation rate and hence growth rate under elevated CO_2 creating a nitrogen demand and therefore the higher amounts of nitrogen were advantageous and more usable in this treatment. In contrast, under ambient CO_2 where photosynthesis is still below saturation levels the extra amounts of nitrogen were not as beneficial.

With respect to harvest index (HI), plant response to CO_2 enrichment was variable. Whilst the majority of previous record reports a reduction (Baker *et al.*, 1989; Vivekanandan *et al.*, 1999) or a non significant effect (Monje and Bugbee, 1998; Pleijel *et al.*, 2000), there are a limited number of reports where increased values has been reported e.g. in wheat (Wu *et al.*, 2004), and some cultivars of rice (Ziska and Teramura, 1992). Mauney *et al.*, (1994) reported a slight increase in the woody perennial, cotton, and a similar response was also found in grape (Pinter *et al.*, 1996).

In the current investigations, harvest index values were slightly increased by elevated CO_2 in all drought and nitrogen interaction experiments, however, this increase was not statistically significant.

It is very common and widely reported that the increases in above ground dry weight under elevated CO_2 is accompanied by increases in below ground dry weight (Finn and Brun, 1982; Jones *et al.*, 1984; Chaudhuri *et al.*, 1990; Rogers *et al.*, 1992; Rogers *et*

al., 1994; Stirling *et al.*, 1998). Stimulated root growth is a particularly important characteristic in plants grown in dry areas as water capture and water use efficiency will be improved as a consequence (Idso and Kimball, 1992; Bhattacharya, 1993). Rogers *et al.*, (1994) in a review of 167 studies on the response of roots to atmospheric CO_2 enrichment, found that root dry weight increased in 87% of the studies that included this measurement and 77% of the studies showed longer or more numerous roots. From FACE experiments, Kimball *et al.*, (2002) reported greater root stimulation than shoots. In general, the response of root: shoot ratio to elevated CO_2 is very variable depending on species and the environmental conditions (Rogers *et al.*, 1996). For example Chu *et al.*, (1992) reported no effect for elevated CO_2 in wild radish, and a similar response was also reported in cotton (Prior *et al.*, 2005) and soybean (Ainsworth *et al.*, 2002). Morison and Gifford, (1984) indicated that from 18 species studied, the root: shoot ratio in 16 species was unaffected by elevated CO_2. Additionally, while some studies reported a reduced root: shoot ratio (Tolley and Strain, 1984; Lekkerkerk *et al.*, 1990), the majority of the studies have shown increased root: shoot ratio in response to high concentrations of atmospheric CO_2 (Whipps, 1985; Rogers *et al.*, 1994; Rogers *et al.*, 1996; Rogers *et al.*, 1999). In lentils, results from the drought experiment showed that although not significant, root growth was increased under elevated CO_2 by an average of 17%, whereas in the nitrogen experiment, the enhancement was greater with an average increase of 37% ($p=0.078$). This relatively modest enhancement in root growth particularly in the drought experiment is probably due to the use of small pots, which is well known to physically restrict root growth under elevated CO_2 (Arp, 1991; Gifford *et al.*, 2000). Usually, restricted root growth, which is considered as a carbon sink can cause a decrease in shoot growth in response to the feedback inhibition from the restricted sink (Arp, 1991; Gifford *et al.*, 2000). However, results from both drought

and nitrogen experiments suggest otherwise. It was clear from the nitrogen experiment that the enhancement in seed yield (average of 53%) was greater than that of the roots as the increase was significant at 1% (p=0.003). Similarly, but to a lesser extent, the enhancement in seed yield in the drought experiment (significant at 10% level) was also greater than the roots. This suggests that probably the effect of pot size in these experiments was only on the morphology and did affect the yield response to elevated CO_2 as water and nutrients were always available to the plants as planned to be. Other studies also reported that the growth response to elevated CO_2 was not related to pot size (Reekie and Bazzaz, 1991). McConnaughay *et al.*, (1993) also concluded that growth response to elevated CO_2 was greater in pots with high nutrient concentrations compared with low concentrations, regardless of total nutrient content or pot size.

Because of relatively lesser enhancement in root growth, root to shoot ratios in both experiment were unaffected by elevated CO_2, which indicate that carbohydrate partitioning between the roots and the shoots was not significantly changed. . Accurate root: shoot ratios are frequently limited by experimental conditions using soil based growing media where total root recovery is difficult. The current investigations utilised a Perlite rooting medium which facilitated good root recovery but this can be criticised as being "non-normal" growing conditions. It is also necessary to indicate that usually in pot experiments as root growth is restricted, root: shoot ratio under elevated CO_2 can be largely affected as it has been reported that while root: shoot ratio in field experiments increased, it leads to decreases in small pot experiments (Arp, 1991).

6.6.2.2.The effect of elevated CO_2 on nodulation and nitrogen fixation

The process of nitrogen fixation gives legumes a highly important role in agriculture, where they can be grown with minimum inputs of fertilizer (Bohlool *et al.*, 1994). Furthermore, legumes can also be grown in rotations with other crops to improve soil quality and increase the amount of nitrogen available for following crops (van Kessel, 1994). How elevated CO_2 affects nitrogen fixation in N fixing species, has been investigated in a considerable number of studies (Masterson and Sherwood, 1978; Temperton *et al.*, 2003; Cen and Layzell, 2004). Usually, under elevated CO_2, increased plant growth will be translated to increased nitrogen demand and as a consequence increased nitrogen fixation rate per plant (Soussana and Hartwig, 1996). In fact, increased nodule biomass and enhanced nitrogen fixation under elevated CO_2 conditions is frequently reported (Philips *et al.*, 1976; Masterson and Sherwood, 1978; Finn and Brun, 1982; Norby, 1987; Temperton et al., 2003; Cen and Layzell, 2004). Results from current investigations on lentils showed that although there was not a significant difference, and nodule number per plant, in both drought and nitrogen interaction experiments and under all treatments applied, was greater under elevated CO_2 conditions by an average of 38% and 34% in the drought and nitrogen experiments respectively. Additionally, when nodule fresh weight was recorded in the second drought experiment, the response to elevated CO_2 was similar to that of nodule number, and weights although not significantly, were always higher under elevated CO_2 conditions by an average of about 45%. Clearly these results were not unusual and were predicted since the increase in carbohydrates assimilated in plants exposed to CO_2 enrichment conditions provides the nodules, which are considered as strong carbon sinks, with additional amounts of carbon and leading eventually to greater nodule

number and biomass. Moreover, the increase in nodulation under elevated CO_2 was associated with an increase in total nitrogen content, although not statistically significant, and since plants in the same treatments received the same amount of exogenous nitrogen, it is feasible to conclude that the extra amounts accumulated in the CO_2 enriched plants came from enhanced nitrogen fixation.

It is also well known that drought reduces nodulation (Wahab and Abd-Allam, 1995; Serraj *et al.*, 1998; Serraj and Sinclair, 1998), and under elevated CO_2, the severity of drought has been reported to be alleviated (Serraj *et al.*, 1998). Results from the current drought experiment on lentil showed that although the main effect of elevated CO_2 was not statically significant, there was a clear enhancement in nodule number and fresh weight in water stressed plants under elevated CO_2 and on average nodule biomass was more than doubled, and this was a relatively greater response than that in the fully irrigated plants (40%).

Nitrogen fixation is a process that demands a high metabolic cost from the plants, and therefore, the additions of combined nitrogen that can be readily absorbed by the plants usually suppress nodulation (Herdina and Salisbury, 1989; Walsh, 1995). Under elevated CO_2, it can be assumed that the increased demand for nitrogen could partly mitigate the inhibition effect of N_2 fixation at certain levels of combined nitrogen (Soussan and Hartwig, 1996). Thomas *et al.*, (2000) concluded that at levels of substrate nitrogen that usually inhibit nitrogen fixation in the nodules of a tropical N-fixing tree (*Gliricidia sepium*), the increased availability of C to nodules under elevated CO_2 partly alleviated the inhibition effect of the substrate nitrogen. In the current nitrogen interaction experiment on lentils, the highest nodule numbers were obtained under moderate additions of nitrogen (25-50 kg N ha^{-1} at ambient CO_2, and 25- 75 kg N

ha^{-1} at elevated CO_2). The negative effect of combined nitrogen on nodulation was only observed from levels equal to 75 kg N ha^{-1} under ambient CO_2 conditions, while that under elevated CO_2, the effect was only noticeable at higher levels equal to 100 kg N ha^{-1}, and by contrast the 75 kg N ha^{-1} levels showed the highest nodule number indicating that elevated CO_2, although not significantly, partly offset the negative effects of combined nitrogen on nodulation. Other studies however, indicated that the inhibition effect of combined nitrogen was not affected by elevated CO_2 (Tobita *et al.*, 2005).

In the current nitrogen interaction experiment, it was also concluded that further investigations including measurements of nodule weight and nitrogenase activity are needed, since nodule number on its own may not be enough to reflect the actual efficiency of nitrogen fixation. Calculations of nitrogen budget showed that minimum amounts of nitrogen fixed were not always associated with the highest nodule number, although the actual amounts fixed could be higher.

Overall, the results from the current investigations showed that, although not significantly elevated CO_2 improved nodule biomass and this must have helped improving the process of nitrogen fixation, and furthermore, it partially mitigated the negative effects of either drought or the addition of combined nitrogen on nodulation. However, whether specific nitrogenase activity was affected in response to elevated CO_2 is still unclear, and because of difficulties explained in chapter 5, the attempt to study the effects of elevated CO_2 on nitrogenase activity in lentils were unfortunately unsuccessful, and this aspect could be an interesting subject to be further explored in future work.

6.6.2.3.The effect of elevated CO_2 on nitrogen and phosphorus content

In general, the literature reports that nitrogen and other nutrient concentrations usually decrease under elevated CO_2 (Wong, 1979; Baxter *et al.*, 1994; Reich *et al.*, 2001; Fangmeier *et al.*, 2002; Taub *et al.*, 2008). Results from current investigations were slightly mixed between the experiments, and while nitrogen concentration in the seeds and roots were always unaffected by elevated CO_2, that in the shoots was only significantly reduced in the drought interaction experiment. This contradiction to the general trend is interpreted as due to positive interaction of the enhanced photosynthesis under elevated CO_2 enhancing the process of nitrogen fixation (Although, the increase in nodule biomass was not statistically significant) which provides the plants with the additional amounts of nitrogen essentially needed to sustain the growth. Therefore, in spite of the significant increase in biomass particularly in the nitrogen experiment (the increase was marginally significant at 10% level in the second drought experiment), the expected N dilution effect in CO_2 exposed plants was not significant for lentils. This is supported by other work with other legumes such as soybean which showed a similar response where protein levels were unaffected by elevated CO_2 (Rogers *et al.*, 1983). Jablonski *et al.*, (2002) in their meta-analysis of reports on 79 crops and wide species concluded that nitrogen content in the seeds was not affected by elevated CO_2 in legumes, but it was significantly reduced in non-legumes. However, some other crops such as wheat have also shown a similar response when plants are well fertilized (Havelka *et al.*, 1984), and a reduction in nitrogen concentrations in response to elevated CO_2 is not necessarily always the case (Stitt and Krapp, 1999).

The reduction in one part of the plants, (the shoots in this case) and not in the others indicates that probably there was a shift in nitrogen distribution between the plant parts

when plants are grown under CO_2 enrichment conditions suggesting effects on partitioning. Newton (1991) similarly stated that in most pasture species grown under elevated CO_2, there was a reduction in nitrogen concentration in the shoots but not in the seeds. Furthermore, Lutze and Gifford (1998) showed that there was a reduction in shoot nitrogen to root nitrogen of Wallaby Grass (*Danthonia richardsonii*) plants that was exposed to high levels of CO_2.

When phosphorus content is concerned, some workers reported that P concentration in plants tissues are usually less affected by elevated CO_2 than most other minerals (Norby *et al.*, 1986; Overdieck and Reining, 1986; Overdieck, 1993). For example, Norby *et al.*, (1986) suggested that the increased uptake of P from a nutrient poor soil under elevated CO_2 might be as a result of increased propagation of fine roots and mycorrhizae and rhizosphere bacteria which stimulate P mineralization. Results from the nitrogen interaction experiment fall into this part of the reported literature, where P concentration in plant tissue was lower but not significantly. In the drought interaction experiment, P concentration in the seeds was not significantly affected by elevated CO_2, but it was significantly reduced in the shoots, and the reduction in the roots was marginally significant ($p=0.083$). These outcomes stress again that the response of plants to elevated CO_2 can be largely influenced by other environmental factors (Rogers *et al.*, 1994; Shaw *et al.*, 2005).

6.7. Critiques of the experimental approach

The current investigations were all semi-controlled chamber based pot experiments conducted either in open-top chambers which were artificially lit, or in enclosed chambers located in a sunlit greenhouse. This approach was followed because it was the most appropriate for the purpose of the study within the resources available. Despite the fact that it is possible to grow lentils in the fields of the south east of England and achieve high yields (Andrews *et al.*, 2001), it is still not a valuable option to grow Syrian lentils in open-top chambers in the field since it is impossible to control or study the drought interaction effect, which is the main aim of the study, in the wet environment of the south west of England. Moreover, even when drought treatment is not concerned, it is still very inconvenient to grow lentils in the field when only one environmental factor (in this case nitrogen availability) is studied as it is impossible to control any other interaction that might occur. Additionally, field based experiments place time constraints on the research which would be difficult for a time restricted PhD research programme.

In fact, numerous experiments that have investigated the effect of elevated CO_2 on vegetation have been conducted in a variety of controlled environments (Poorter, 1993; Stulen and Den Hertog, 1993) varying from highly accurate computer controlled chambers (Payer *et al.*, 1993) to other relatively less accurate systems such as semi-controlled green houses (Körner and Arnone, 1992; Körner *et al.*, 1993), portable temperature gradient tunnels (Gifford and Rawson, 1993), and solar domes (Cotrufo and Ineson, 1996).

Undoubtedly, experimental pot conditions are completely different to those complex plant systems in the field and plants can act differently under each set of conditions. Therefore, the transferability of the results from the former to the latter can be put to question, especially since some studies at the ecosystem levels have shown that outcomes could be different (Körner *et al.*, 1993). For example, other studies have indicated that nutrient availability and competition for light will largely restrict the possible responses of plants to elevated CO_2 (Overdieck, 1986; Grulke *et al.*, 1990; Körner and Arnon, 1992; Norby *et al.*, 1992). More recently, some studies have emerged arguing that results from chamber based experiments (enclosed system or open-top chambers) were overestimating the response of plants to elevated CO_2 (Ainsworth and Long, 2005; Long *et al.*, 2005), and results from free-air CO_2 enrichment (FACE) studies showed that the enhancement of growth under elevated CO_2 could be as much as 50% less than previously anticipated by chamber studies (Long *et al.*, 2006). On this basis, these workers have suggested that projections for world food supply for the 21st century made by the IPCC (2001) could be too optimistic, and a requirement of downward revision is needed. However, these conclusions have come under severe criticism, and an article by Tubiello *et al.*, (2007) indicated that these results are incorrect and explained in minute details the reasons why finally concluding that the degree to which plants respond to elevated CO_2 from either FACE and non-FACE studies is very similar. Furthermore, Kimball *et al.*, (2002), studying the response of agricultural crops to FACE and comparing the response to that from enclosure studies also concluded that the results from both types of studies were consistent lending confidence to both types of experimentation. Ziska and Bunce (2007) in their review which compared results from FACE and non-FACE enclosure methodologies on three globally important crops (wheat, rice, and soybean), found that

the results from both approaches are quantitatively consistent. Therefore, when regarding the results obtained by the current investigations on lentils, it can be concluded that these results are robust and can be applied for future projections for the growth of lentils. At the same time the possibility that the responses reported could be an overestimation should not be eliminated.

Overall, this study attempted to contribute to the increasing body of experimentation that is dealing with the effects of climate change on plant growth and yield. With the availability of different scenarios of future climate and society, conducting this type of experimentation is essential for developing crop production models that project the response of crops to the changing climate and this in turn will help in adapting the strategies and practices that help secure food supply worldwide.

6.8. Future work

In this study, the effect of elevated CO_2 on the growth, production and nodulation of lentils was investigated in a combination with two factors, drought and nitrogen availability, as both growth and nodulation of lentils are largely affected by those factors. These effects were only tested either on one or two cultivars of lentils using one strain of rhizobium bacteria, and therefore, further testing using more cultivars (mainly drought tolerant) and probably different strains or a tested super strain of the rhizobium bacteria in the light of precipitation scenarios predicted by the IPCC will be important to achieve high yielding crops in the drier climate projected for the eastern Mediterranean region.

As lentil in Syria is mostly grown in the marginal dry land, the interaction with drought, or other factors (e.g. nutrient availability) could be investigated using open top chambers in the field in Syria (although FACE is very costly to construct) which is, in spite of the chamber effect, still an environment closer to field conditions than glasshouse pot experiments.

This investigation concluded that nitrogen fixation was significantly increased under elevated CO_2 and this increase was associated with an increase in nodule number and nodule fresh weight, but due to difficulties explained earlier, it was difficult to conclude whether specific nitrogenase activity was also increased in response, and this aspect remains an interesting aspect to be investigated further.

Other environmental factors particularly temperature which is expected to increase in pararrel with the increase in CO_2 levels especially in the Mediterranean basin (Christensen et al., 2007), can greatly affect lentil growth in this area, and therefore testing the CO_2 – temperature interaction effect with different cultivars and probably at different sowing times could be essential for future cultivation of lentils. Investigating the interaction of elevated CO_2 and other abiotic stresses such as soil salinity and acidity, which affect the growth of the crop and the nitrogen fixing bacteria, could also be an important aspect to be explored in the future.

The increase in atmospheric CO_2 concentrations will also be accompanied by an increase in the ozone levels which can have an adverse effect on plant growth and productivity (IPCC, 2007). Therefore, investigating the interaction effect of elevated ozone and elevated CO_2 on lentil will also be crucially important to accurately predict the response of lentil to the changing climate. Finding varieties more adaptive to these new conditions is projected as a priority. In fact, breeding for cultivars that are adaptive to the changing climate conditions with higher CO_2 levels but at the same time warmer, drier and with higher concentration of ozone, should be the main aim of future investigations on lentils and other strategic crops in the region.

6.9. Conclusions

This investigation confirmed that lentil responds positively to elevated CO_2 and this response is highly dependent on the environmental condition as the increase in seed yield was only marginally significant in the drought experiment (p=0.059) by an average of 19%, whereas the increase was clearer in the nitrogen experiment (p=0.003) with an average of 53%.When drought was imposed, results showed that although not always significantly, biomass production, seed yield and nodulation were less affected by drought in plants grown in CO_2 enriched conditions. When additions of nitrogen fertilizer were made, the yield increased with the increasing levels of nitrogen and this increase was always greater under elevated CO_2. Furthermore, although not significantly, elevated CO_2 alleviated the inhibition effect of exogenous nitrogen on nodulation and nodule number under elevated CO_2 still increased at levels which were inhibitory under ambient CO_2 conditions. The root enhancement under elevated averaged 17% and 37% in the drought and nitrogen experiments respectively, but this enhancement was not significant in the former and marginally significant in the latter (p=0.078), and this could probably be due to the small pot size used in the investigations. Root to shoot ratios were always unaffected by elevated CO_2, and harvest indices were slightly higher but not significantly.

In spite of the significant increase in biomass under elevated CO_2 (marginally significant in the second drought experiment), nitrogen concentration in most plant parts was not significantly lower. There was an example where nitrogen concentration was significantly reduced in the shoot, which means that there was a possible shifting in nitrogen partitioning under elevated CO_2 conditions. Phosphorus concentrations were lower but not always significantly.

Data collected from these experiments could be incorporated into a crop growth models, and along with precipitation scenarios predicted by the IPCC for the East Mediterranean area, a clearer picture for the future production of lentils in the region could be predicted.

In the last five years (2003-2007), lentil production in Syria has ranged between 125300 to 165000 tonne with an average yield ranging between 918-1138 kg ha^{-1} depending mainly on annual rainfall. According to the results from current investigation, the response of lentils to doubling CO_2 levels is highly dependent on other environmental factors, and the increase in the yield could be between 11-59%, which means that the range of increase could be between 13783 to 97350 tonnes.

Since elevated CO_2 only partially alleviated the effect of drought, adopting strategies to improve lentil productivity under drought stress conditions should still be the main aim of future research, but this should be studied in the light of the anticipated increase in atmospheric CO_2.

Appendices

Appendix A

An example of the Minitab output for a split plot analysis

General Linear Model: seed yield versus Replicate, CO2, Drought, cv (second drought exp).

Factor	Type	Levels	Values
Replicate	random	4	1, 2, 3, 4
CO2	fixed	2	1, 2
Drought	fixed	2	1, 2
cv	fixed	2	1, 2

Analysis of Variance for seed yield, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P	
Replicate	3	227543	227543	75848	1.07	0.478	
CO2	1	623565	623565	623565	8.82	0.059	
Replicate*CO2	3	212103	212103	70701	0.77	0.512	**(main plot error)**
Drought	1	16052474	16052474	16052474	175.86	0.000	
cv	1	942047	942047	942047	10.32	0.002	
Drought*cv	1	176904	176904	176904	1.94	0.168	
CO2*cv	1	6212	6212	6212	0.07	0.795	
CO2*Drought	1	67359	67359	67359	0.74	0.393	
CO2*Drought*cv	1	18552	18552	18552	0.20	0.653	
Error	82	7485049	7485049	91281	**(sub-plot error)**		
Total	95	25811808					

Appendix B

General Linear Model: Seed yield versus Rep, CO2, drought, variety

Factor	Type	Levels	Values
Rep	fixed	5	1, 2, 3, 4, 5
CO2	fixed	2	1, 2
drought	fixed	2	1, 2
variety	fixed	2	1, 2

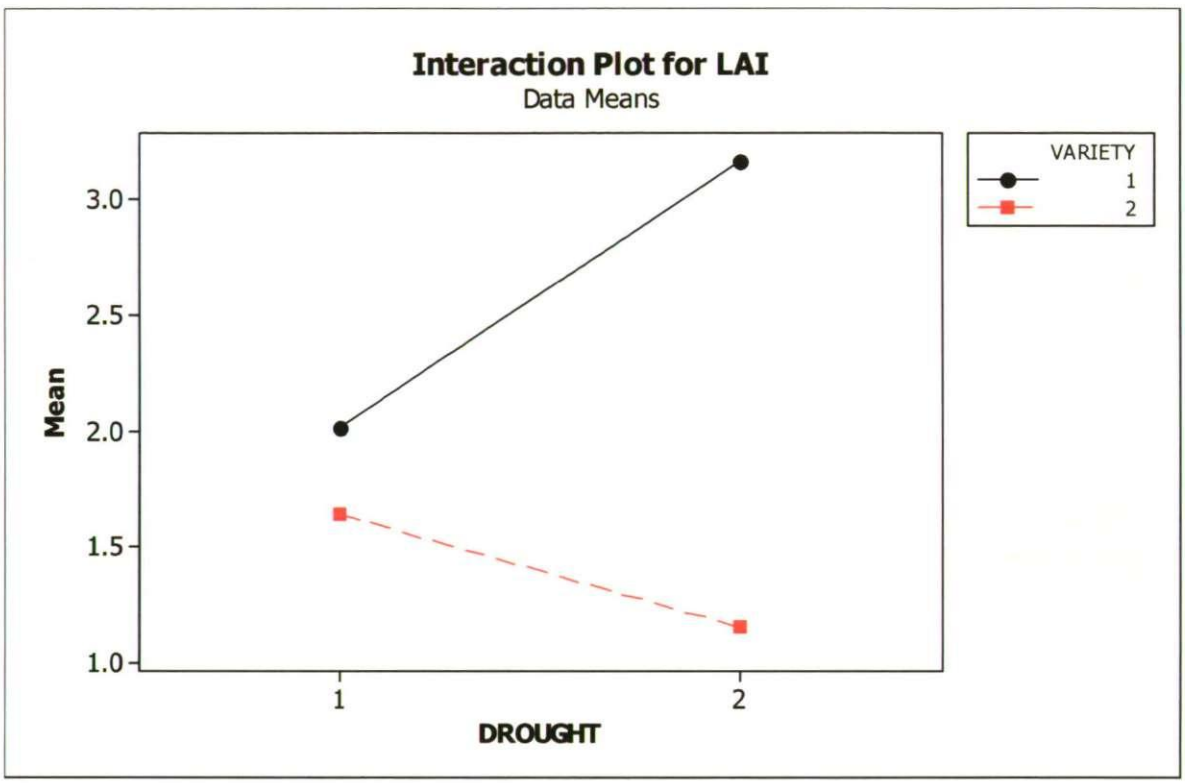
Analysis of Variance for Seed yield, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Rep	4	13.258	12.355	3.089	1.65	0.175
CO2	1	10.157	10.157	10.157	5.44	0.024
drought	1	146.047	157.642	157.642	84.46	0.000
variety	1	36.845	38.648	38.648	20.71	0.000
CO2*drought	1	11.678	11.678	11.678	6.26	0.016
CO2*variety	1	1.845	1.845	1.845	0.99	0.325
drought*variety	1	8.585	9.192	9.192	4.93	0.031
CO2*drought*variety	1	0.608	0.608	0.608	0.33	0.571
Error	52	97.055	97.055	1.866		
Total	63	326.077				

S = 1.36618 R-Sq = 70.24% R-Sq(adj) = 63.94%

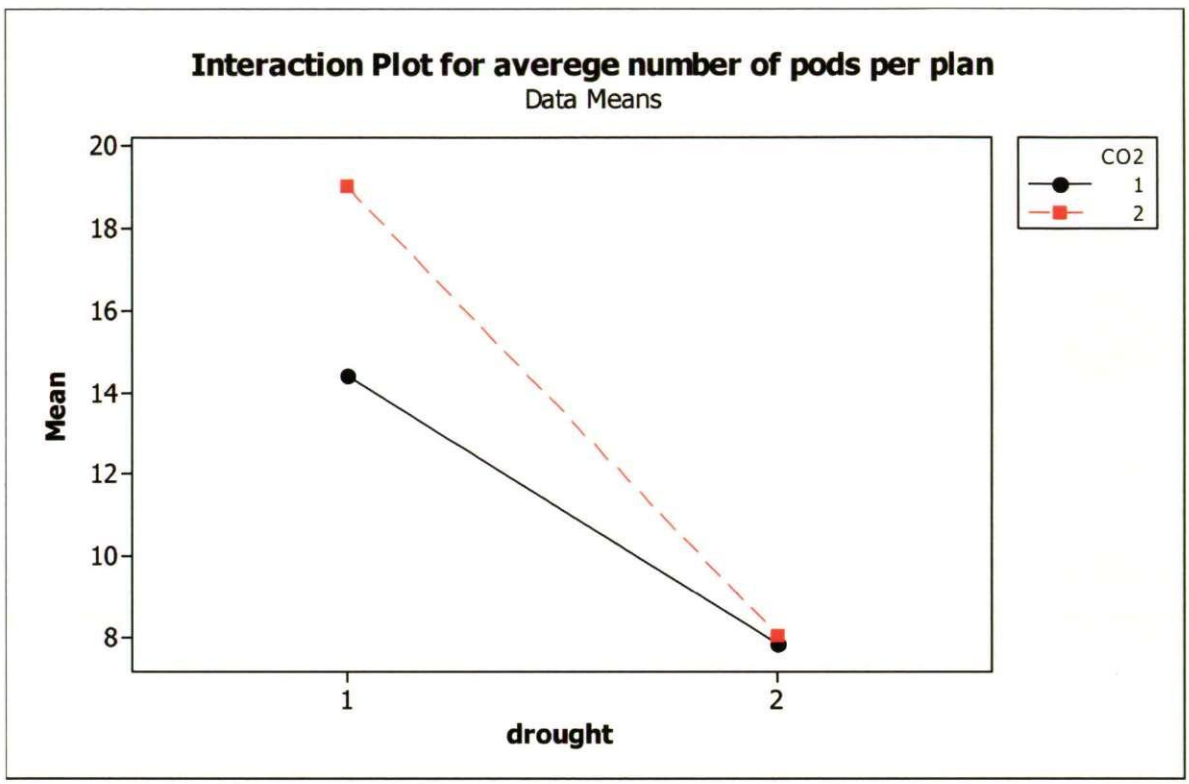
Appendix C

The interaction plot for LAI at anthesis between varieties (1= Idlib 3, 2= IL7979) and drought (1= full irrigation, 2= water stress).



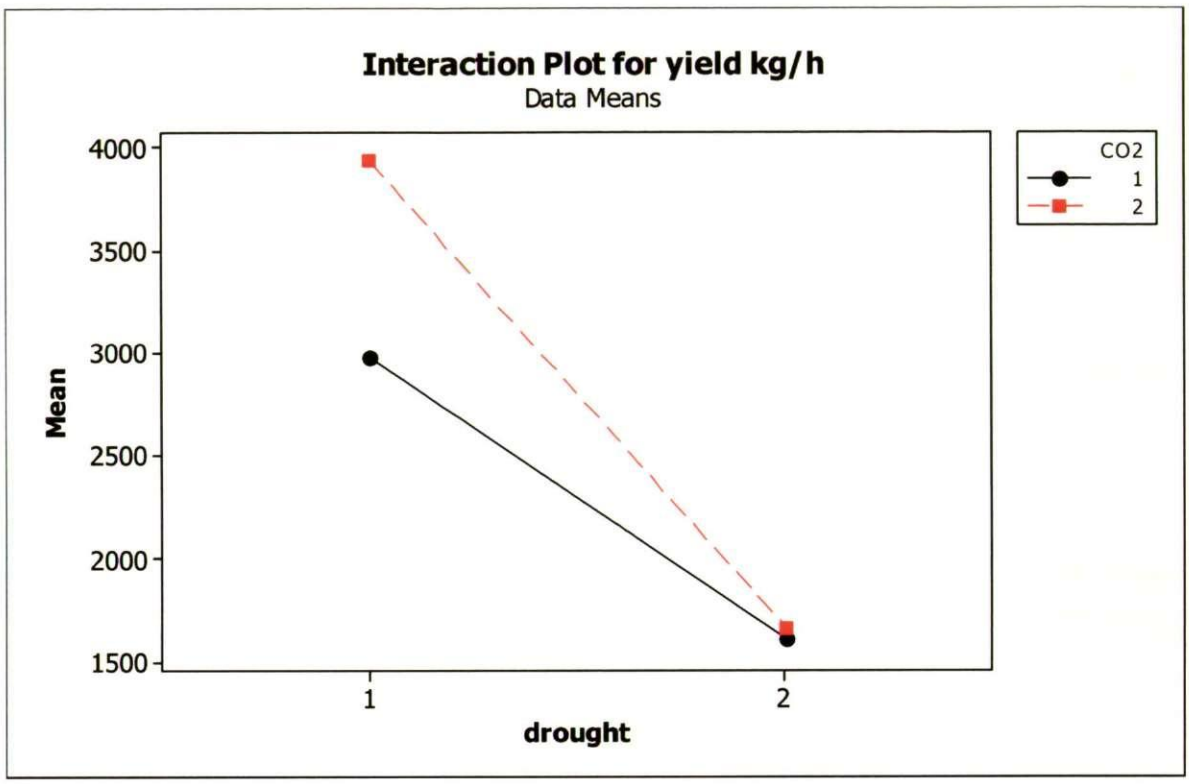
Appendix D

The interaction plot for pod number per plant between CO_2 treatment (1= ambient CO_2, 2=elevated CO_2) and drought (1= full irrigation, 2= water stress).



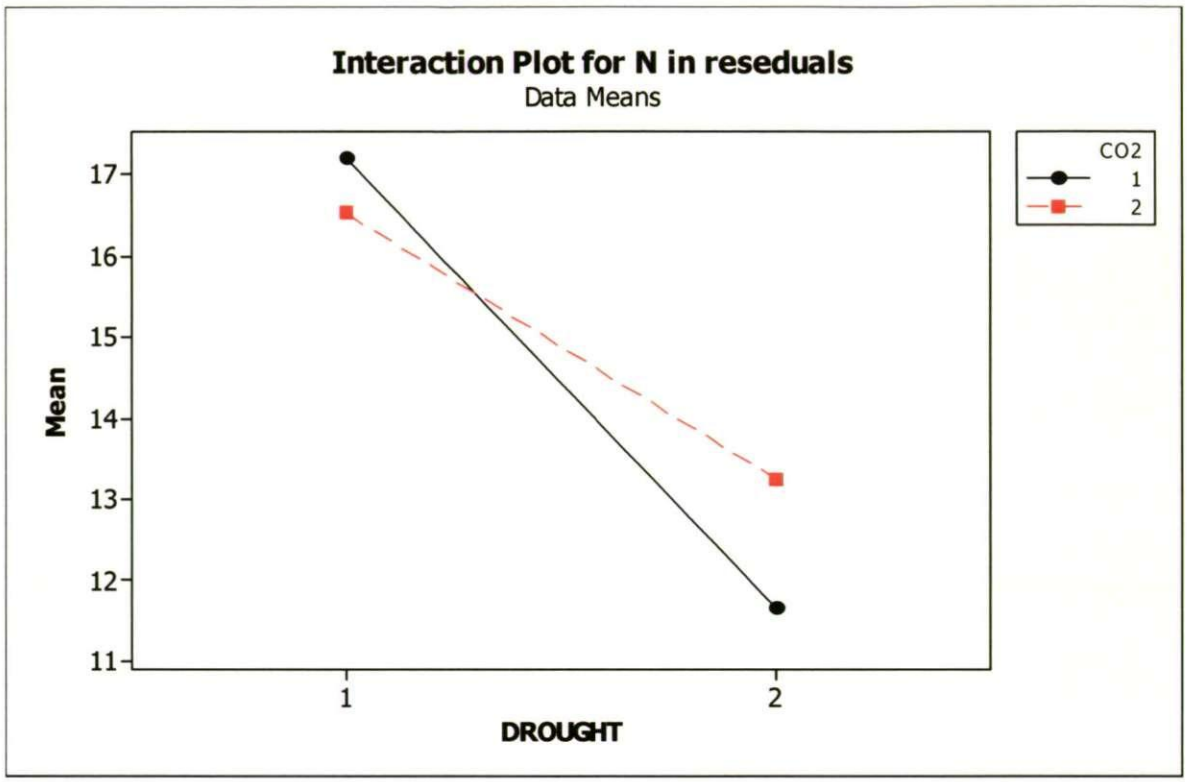
Appendix E

The interaction plot for seed yield between CO_2 treatment (1= ambient CO_2, 2=elevated CO_2) and drought (1= full irrigation, 2= water stress).



Appendix F

The interaction plot for nitrogen left in the residual water between elevated CO_2 (1= Ambient CO_2, 2= Elevated CO_2) and drought (1= drought, 2= Full irrigation) treatments in the second drought interaction experiment.



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