Polyphenolic, tannin and chemical compositional changes in leaves of sub-tropical grasses and fynbos shrubs at elevated atmospheric CO_2 concentrations

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Abstract

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The effects of elevated atmospheric CO_2 concentrations on plant polyphenolic concentration, tannin concentration and chemical composition were investigated in leaves of sub-tropical grass species and fynbos shrubs. The working hypothesis was based on predictions that carbon based secondary compounds (polyphenolics and tannins) would increase when carbon in excess of growth requirements accumulate in plant leaves under nutrient imbalanced conditions. This imbalance would arise due to an increase in atmospheric CO_2 level. Furthermore, empirical evidence suggests that nutrient paucity would enhance carbon-based secondary compound production.

This hypothesis was tested in two different systems involving plants with differential photosynthetic mechanisms and growth strategies.

- Polyphenolics, tannins and chemical composition (N, P, C and TNC) were quantified in grass species from a natural, C_4 dominated, sub-tropical grassland in KwaZulu/Natal. Three plots were subjected to different free-air CO_2 enrichment treatments, i.e. elevated (550-800 ppm), intermediate

(no more than 400 ppm) and ambient CO_2 (currently at 365 ppm). One of the seven grass species, *Alloteropsis semialata*, had C_3 photosynthetic mechanism.

- Polyphenolics, tannins and chemical composition (N, P, C and TNC) were quantified in three fynbos species grown in open-top chambers under controlled greenhouse conditions. The plants were grown under ambient (360 ppm) and ambient + 350 ppm CO_2 in typical low nutrient acid sands of the fynbos biome.

This study shows that despite some of the grasses having the capacity to produce tannin-like substances, polyphenolics and tannins do not increase in the grass species studied. Polyphenolic and tannin concentrations were increased only in *Leucadendron laureolum* amongst the fynbos species. Its sister species *Leucadendron xanthoconus* did not show any change in phenolic or tannin concentrations.

Chemical composition in grasses were largely unaffected by elevated CO_2, however, some species-specific responses were observed. The C_3 *A. semialata* showed a decrease in P concentration and a consequent increase in C:P ratio at elevated CO_2. Only *L. laureolum* showed a response in chemical composition at elevated CO_2, whereas its sister species did not show any response except a decrease in N concentration.

In conclusion, fast growing grass species invest extra carbon into growth rather than polyphenolics and tannins and show small species-specific chemical changes at elevated atmospheric CO_2 concentrations. Increased investment into

phenolics and tannins as well as changes in chemical composition in fynbos species were species-specific even within plants from the same genus. Thus generalizations about plant responses to elevated CO_2 based on theoretical principles cannot be directly applied. This is especially true in complex natural environments where ecophysiological processes may dictate phytochemical responses.

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DECLARATION

I declare that *Polyphenolic, tannin and chemical compositional changes in leaves of sub-tropical grasses and fynbos shrubs at elevated atmospheric CO_2 concentrations* is my own work, that it has not been submitted for any degree or examination in any other university, and that all the sources I have used or quoted have been indicated and acknowledged by complete references.
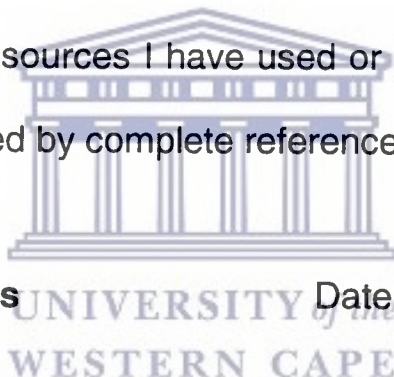
Full name: **Dawood Hattas**   Date: **October 2002**

Signed: …………………………..

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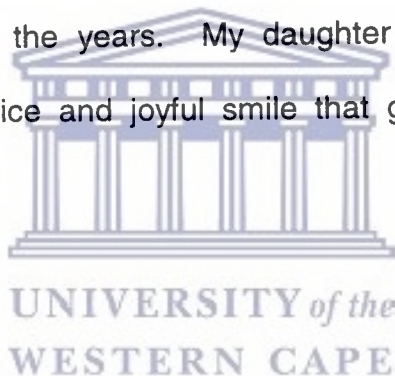
Mr Guy Clegg is thanked for allowing the grassland experiment to be set up on his farm Pleasant View. Mr Timothy Lesch is acknowledged for performing the C analysis. I also acknowledge Dr Richard Madsen for his

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CHAPTER 1 - Introduction

1.1. Contextualisation of study

The increase in CO_2 concentration since the start of the industrial revolution has produced a rise in the concentration of CO_2 gas in the atmosphere. The pre-industrial CO_2 concentration of 280 ppm (in 1750) had reached 367 by 1999, and subject to no intervention, is expected to rise to between 540 and 970 ppm by 2100 (Albritton *et al.* 2001).

A rise in CO_2 concentration is not a new phenomenon. Carbon dioxide concentrations have fluctuated between 160 and 300 ppm over the last 160000 years and may have approached 1000 ppm 50 million years ago (Bowes 1993). However, these changes have occurred over thousands to millions of years. The atmospheric CO_2 concentration has increased by about 31% since 1750 (Albritton *et al.* 2001) to the current 367 ppm.

The rise in atmospheric CO_2 concentration has positive effects in increasing biomass yield, especially in C_3 plants that are not operating at their optimum photosynthetic potential at the current CO_2 regime (Bowes 1993, Salisbury & Ross 1992). The increase is brought about by a stimulation of photosynthesis through an elevation of substrate availability (i.e. CO_2). This increase in substrate increases the activity of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco - the enzyme

that catalyses the first step of photosynthesis, *viz.* the combination of atmospheric CO_2 with RuBP (ribulose-1,5-bisphosphate)), thereby enhancing CO_2 assimilation.

With the demand for food increasing with an increase in world human population (currently about 6 billion), the increase in atmospheric CO_2 concentration and consequently biomass yield would be welcomed. However, an increase in atmospheric CO_2 concentration may affect plants negatively by affecting carbon allocation and chemical composition in plants, which could feedback on other ecosystem properties that influence productivity. These include possible changes in biodiversity (Scholes & Bailey 1996) and litter quality that affects decomposition (Peñuelas & Estiarte 1998, Stock & Midgley 1995).

Higher CO_2 concentrations have been reported to decrease N concentrations in plants (Poorter *et al.* 1997, Saxe *et al.* 1998, Stock & Midgley 1995). This decrease in N might limit protein synthesis and may thus promote the production of carbon based secondary compounds (especially condensed tannins) in plant leaves (Peñuelas & Estiarte 1998). An increase in polyphenolics, condensed tannins and a decrease in N concentrations at elevated CO_2 levels may affect forage quality and influence herbivore feeding behaviour (Bazzaz 1990, Peñuelas & Estiarte 1998, Tognetti & Johnson 1999).

A knowledge of changes in plant chemical composition under elevated CO_2 levels is required if we are to develop a comprehensive

understanding of growth responses at elevated CO_2 (Poorter *et al.* 1997). Any change in total non-structural carbohydrate (TNC), N and P concentration may affect growth physiology in plants. A mechanistic understanding of these changes and their role in carbon allocation would assist in developing this understanding.

In this study we investigated how elevated atmospheric CO_2 affects polyphenolic, tannin and chemical composition in plant leaves of different growth form (grasses and shrubs) from different biomes. The investigation was undertaken in a free-air CO_2 enrichment experiment in a sub-tropical grassland, and on three fynbos species grown in controlled open top chambers in a greenhouse in Cape Town.

1.2. Historical perspective of global climate change

Predicting changes in natural ecosystems as a result of atmospheric change is one of the major challenges in modern ecology (Norton, Firbank & Blum 1999). Any change in global temperature and weather patterns, and hence the global climate will have a direct impact on human livelihood by affecting agricultural food and fibre production (Reddy & Hodges 2000; Stock & Midgley 1995). Global climate change and factors contributing towards it may also change key ecosystem processes by altering plant species diversity, composition, and distribution of natural vegetation (Ojima, Galvin & Turner 1994).

Fluctuations in global temperature are not uncommon. In the past billion years, mean global temperature has been about 13 °C warmer and about 5 °C cooler than at present. More recently, during the 100000-year Pleistocene glacial/interglacial cycle, global temperature was cooler varying by about 5 °C, with some local temperature changes as great as 10 to 15 °C in high latitude regions. Since the last glaciation, about 10000 years before present, there have been marginal changes of probably less than 2 °C (relative to the current global average temperature) in global mean temperature. The past 1000 years have seen the so called "medieval warm period" from about the 10th to the 12th century, with summer temperatures 1 °C warmer in western Europe, Iceland and Greenland. In contrast, the "Little Ice Age", which lasted approximately from 1450 to the mid-19th century, saw the earth's mean temperature at 1 °C less than that of today. These extreme events saw vineyards in western and central Europe extend as much as 5 ° latitude further north than today (during the medieval optimum), and the freezing over of the river Thames in London in the Little Ice Age (Mearns 2000).

Since the 19th century, the earth's surface temperature has become about 0.6 °C warmer, and 1998 was the warmest year since 1861 (Albritton *et al.* 2001). However, this effect was non-uniform, as some regions experienced cooling in the 20th century (Mearns 2000).

Tide gauge data show that global mean sea level rose between 10 and 20 cm in the 20th century. This was primarily due to thermal

expansion of the oceans and melting of glaciers and ice caps. Land-based average precipitation rates have also displayed a slight increase during the 20th century, with heavy precipitation rates strongly influenced (Albritton *et al.* 2001). Furthermore, escalation in the percentage of the globe experiencing severe drought and extreme moisture surplus has been recorded since the late 1970s (Mearns 2000).

Despite the evidence of a changing global climate and its effects, the debate on the causes of global warming is still unresolved. Many authors ascribe global warming to anthropogenically induced increases in the concentration of atmospheric gases (Melillo, Hall & Ågren 1996, Peñuelas & Estiarte 1998, Perry & Borchers 1990), while others argue that the increase in global temperature as observed in the 20th century is a recovery from the Little Ice Age (Mearns 2000). However, there is new and stronger evidence that most of the warming observed over the last 50 years is a consequence of anthropogenic activities (Albritton *et al.* 2001).

1.3. Factors contributing to increase in atmospheric CO_2 concentration (c_a)

Fluctuations in CO_2 concentration over many millions of years have shown a steady shift in equilibrium that occurred over thousands to millions of years. This apparent equilibrium has been disturbed by the burning of fossil fuel (Bowes 1993, Tilman 1993), changes in land use and land management, specifically tropical deforestation (Albritton *et al.* 2001, Perry & Borchers 1990, Schimel 1995, Scholes & Van der Merwe 1996).

Terrestrial photosynthesis and oceanic dissolution account for an estimated 120 and 115 Giga tons (Gt) C yr^{-1} respectively, but this is virtually balanced by CO_2 evolution from respiration, decomposition, fires, and oceanic release. Anthropogenically produced gases from fossil fuel burning, cement manufacture, and land use add 5-8 Gt C yr^{-1} to the atmosphere. These man-made carbon sources represent a small fraction of the global carbon budget, but are the major factor in destabilising the atmospheric CO_2 compensation point (Bowes 1993).

Changes in land use and land management have modified entire landscapes and altered plant and animal communities in many ecosystems throughout the world. The driving force behind these changes was agricultural production and forestry, which resulted in

removal of indigenous species and introduction of exotic species. Land management such as fire, grazing, and tillage affect ecosystem composition, nutrient cycling, and organic matter distribution. Primarily land conversion occurred in temperate forests and grassland areas, but more recent alterations are concentrated in tropical areas. Over the last 120 years, land conversion to cropland has resulted in soil carbon loss of about 180 Gt (Bowes 1993), presumably contributing to the ever-increasing c_a.

1.4. Recent changes in atmospheric CO_2 concentrations

Since the advent of the industrial revolution the concentrations of atmospheric gases such as carbon dioxide (CO_2), methane (CH_4), nitrous oxide (N_2O), nitric oxides (NOx), carbon monoxide (CO), chlorofluorocarbons (CFCs), and water vapour have all increased. Carbon dioxide, CH_4, N_2O, CFCs and water vapour are greenhouse gases. Methane, NOx and CO reacts in the troposphere to produce ozone (O_3), which is both a greenhouse gas and a pollutant (Scholes & Van der Merwe 1996). These greenhouse gases are practically transparent to all short-wave radiation, which is absorbed by the earth's surface. The earth's surface, in turn, re-radiates some of this energy in the form of long-wave (infrared) radiation. The atmosphere is more effective in absorbing this long-wave radiation, which is then both emitted

upward to space and downward towards the earth. This enhanced warming due to re-radiated long-wave radiation from the atmosphere is known as the greenhouse effect (Mearns 2000, Salisbury & Ross 1992). The extent of the greenhouse effect is governed by the concentration of greenhouse gases in the atmosphere. Therefore, the magnitude of the greenhouse effect is a function of the concentration of greenhouse gases in the atmosphere.

Carbon dioxide is the most abundant greenhouse gas in the atmosphere. Its concentration has increased by 31% since 1750, and concentrations have increased from around 280 ppm then, to 367 ppm by 1999 (Albritton *et al.* 2001, Peñuelas & Estiarte 1998). The global annual rate of increase in CO_2 concentration has been 1.5 ppm over the past two decades, and subject to no intervention, carbon cycle models project atmospheric CO_2 concentrations of 540 to 970 ppm by 2100 (Albritton *et al.* 2001). Major sources of CO_2 emissions include burning of fossil fuels (which contributed three quarters of the anthropogenic emissions of CO_2 in the last 20 years) and cement production; while tropical deforestation and land use changes contribute to CO_2 concentration increases by removing carbon sinks (Albritton *et al.* 2001, Bowes 1993, Mearns 2000, Melillo *et al.* 1996, Schimel 1995) and by enhancing soil respiration rates.

The human population has increased more than fivefold in the last century to 6 billion (Cheikh, Miller & Kishore 2000). This growing population exerts an increased pressure on the terrestrial ecosystem as

demands increase for resources such as food, fuel, fibre and water (Ojima *et al.* 1994). These increased demands for life sustaining resources and its consequential contribution to global change by changing the composition of atmospheric gases, has led to the signing of the United Nations Framework Convention on Climate Change (UNFCCC) by more than 150 nations. South Africa signed this agreement in 1993 (Scholes & Van der Merwe 1996) and it was ratified in 1997 (Department of Environmental Affairs and Tourism 1997).

In signing and ratifying the agreement, countries are required to: take account of the greenhouse gas emissions from their terrestrial area; embark on assessments of the possible impact of climate change; and to shape greenhouse gas control plans. South Africa and other developing countries have been given a grace period to achieve these objectives (Department of Environmental Affairs and Tourism 1997, Scholes & Van der Merwe 1996, United Nations Framework Convention on Climate Change 1997).

Over the past six years in South Africa, CO_2 emissions showed a mean annual increase of 2%, and subject to no intervention, are expected to increase by 3% per year for the next 20 years. Coal mining, electricity generation, automobile fuel use, and municipal and household fuel use are expected to increase by a minimum of 3% per sector per year for the next 20 years. These growth rates are based mainly on

population and economic growth, and urbanization (Scholes & Van der Merwe 1996).

While South Africa adds only 1.2% to the additional radiation load on the global atmosphere, this amount is disproportionately large relative to the national fraction of the global economy and population. Hence, South Africa is placed among the top thirty countries in the world, with respect to the tons of C emitted per capita per year (Scholes & Van der Merwe 1996).

Following intense negotiations at the third meeting of the Conference of the Parties (Conference of the Parties to the Convention, COP-3), the Kyoto Protocol (Conference of the Parties, Third session 1997), which contains legally binding emission targets for developed countries was agreed upon. The target is to reduce collective emissions of six key greenhouse gasses (CO_2, CH_4, N_2O, SF_6, Hydrofluorocarbons and Perfluorocarbons) to at least 5% below 1990 levels by the period 2008 to 2012. However, to enter into force, the protocol has to be ratified by at least 55 Parties to the Convention, incorporating developed countries (listed as Annex I countries in the Protocol) that account in total for at least 55% of the total CO_2 emissions for 1990 from that group. As at 25 September 2002, 84 Parties have signed and 95 countries have ratified or acceded to the Protocol (UNFCCC). South Africa ratified the Protocol on 3 May 2002 (Department of Environmental Affairs and Tourism, South Africa). However, of the Annex I countries only 24

countries representing 37.1% of the 1990 CO_2 emissions have ratified the Protocol, 17.9% short of the total needed for the Protocol to enter into force.

In ratifying the Protocol South Africa has to: implement climate change policies; enhance energy efficiency; reduce and/or limit emissions in the waste and transport sectors; protect sinks for greenhouse gases; phase out market instruments that are counter productive to the aims of the Protocol; and promote sustainable forms of agriculture and relevant research. This has obvious implications for the South African economy, especially the energy sector that produced 75% of the countries primary energy from coal in 1997 (White Paper on the Energy Policy of the Republic of South Africa 1998).

Anticipating impending international pressure on developing countries to contribute in the fight against global warming, the South African government formulated policy objectives that pave the way for producing relatively cleaner energy through its White Paper on Energy Policy. Through this policy, the South African government commits itself to making clean, affordable and appropriate energy available to all sectors of the population (White Paper on the Energy Policy of the Republic of South Africa 1998). The policy further states that:

> *Government will monitor international developments and will participate in negotiations around response strategies to global climate change, in order to*

progressively balance its environmental responsibilities

and development interests, along with health related local

issues, in these processes.

The Department of Mineral and Energy will

follow a 'no regrets' approach in the energy sector with

regard to the potential global environmental impacts of

energy activities. (White Paper on Energy Policy, RSA

1998)

Where 'no regrets' is defined as that which decreases and minimises

environmental impacts commensurate with cost effectiveness and

positive cash flow.
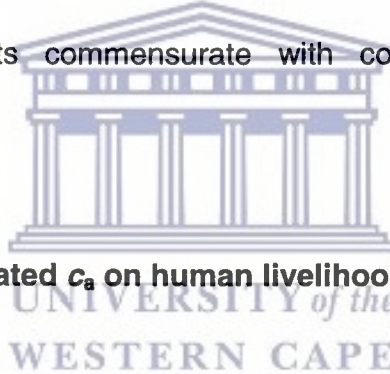
1.5. Effects of elevated c_a on human livelihood

Global increases in c_a may affect terrestrial ecosystem function by

changing physiological growth processes in plants. These growth

processes are dictated by the photosynthetic mechanism of species,

though the specific environment is as important, and sometimes the

primary determining factor. Mechanistic theory of photosynthesis

suggest that C_3 species will be positively affected by elevated CO_2, while

C_4 species should not show any alteration in growth due to elevated CO_2

(Bazzaz 1990, Bowes 1993). The difference in physiological response of

C_3 and C_4 species lies in the different photosynthetic mechanisms by which these species fix CO_2 from the atmosphere (see 1.6 below).

Society will surely be affected as elevated c_a impart changes on agricultural food and fibre production. Due to differences in climatic conditions, some regions are likely to be affected negatively, while other regions may benefit (Reddy & Hodges 2000). Developed regions may be buffered from serious impact because they possess the available resources to cope with changes in climate. Conversely, developing countries will be vulnerable to even moderate climate shifts or further land degradation because of a lack in economic and technological capacity to effectively regulate the impact (Ojima et al. 1994).

1.6. Effects of elevated CO_2 concentration on photosynthesis

Carbon dioxide and water are the basic raw materials that plants use in photosynthesis to convert solar energy into food, fibre, and other forms of biomass. The present atmospheric CO_2/O_2 concentration ratio, along with photorespiration and Rubisco limitations, restricts C_3 vegetation to only 60-70% of its photosynthetic potential. The projected doubling of the c_a by 2100 would therefore increase the rate of photosynthesis in C_3 species. This increase will have negligible effects on C_4 species, as C_4 photosynthesis is saturated at a CO_2 concentration well below the current ambient level (Young & Long 2000). This prediction has generally been

supported by growth data (Bowes 1993), however, a more recent meta-analysis showed that C_4 species will respond positively to both photosynthesis and biomass accumulation at elevated c_a (Wand *et al.* 1999).

1.6.1. C3 photosynthetic mechanism

Species that produce 3-phosphoglyceric acid (3-PGA) as the initial assimilation product of CO_2 are commonly called C_3 species. The carbon assimilates are photosynthetically reduced via the Calvin cycle which takes place in the stroma of the chloroplast. In C_3 species, CO_2 is the substrate for Rubisco, and the current Rubisco K_m (CO_2) value (denoted as K_c) for terrestrial C_3 species is 8-25 μM. In the present-day atmosphere, the C_3 chloroplast stroma (where photosynthesis takes place) contains 5 μM CO_2. This is less than the K_c for Rubisco, while the value for dissolved O_2 is about 240 μM. The problem of low substrate is therefore exacerbated by the restraining effect of O_2, and together they inhibit C_3 photosynthesis. The predicted increase in c_a should therefore increase the CO_2/O_2 concentration, and hence Rubisco activity in leaves of C_3 plants through increases in substrate availability. At high temperature CO_2 is less soluble in water of the chloroplast, an effect that lowers the rate of photosynthesis in C_3 plants (Bowes 1993). Thus future

carbon assimilation will be hampered by increases in mean maximum temperatures as anticipated by global warming.

1.6.2. C_4 photosynthetic mechanism

Plant species that yield four-carbon acids as the major initial CO_2 fixation products are universally referred to as C_4 species. In the C_4 pathway CO_2 is fixed into C-4 acids malate and aspartate through the action of PEPc (phosphoenolpyruvate carboxylase). This reaction takes place in the mesophyll cells and the malate and aspartate are then transported to the bundle sheath cells, where both are decarboxylated. The liberated CO_2 is then used in the bundle sheath chloroplast via the Calvin cycle to produce 3-PGA, sucrose and starch. Carbon dioxide and therefore Rubisco only exist in the bundle sheath cells, as do most Calvin cycle enzymes, and hence the complete Calvin cycle occurs in the bundle sheath. Plants having the C_4 mechanism are much less restricted by CO_2 levels because they effectively pump CO_2 into the bundle sheath cells when they transport malic and aspartic acid into these cells.

There are three biochemical variants of the C_4 pathway. Those exhibiting NADP-ME (nicotinamide adenine dinucleotide phosphate – malate enzyme) photosynthetic type are called 'malate formers' and C_4 grasses with this pathway reach their maximum abundance in mesic areas. This sub-type occurs most commonly in C_4 Panicoideae of which

supertribe Andropogonodae appear to be exclusively NADP-ME. The NAD-ME (nicotinamide adenine dinucleotide – malate enzyme) and PCK (phosphoenolpyruvate carboxykinase) photosynthetic sub-types are 'aspartate formers', reaching their maximum abundance in arid regions. These photosynthetic sub-types are concentrated in the Chloridoideae where the NADP-ME type is absent, but they also occur in the Paniceae (Gibbs Russel *et al.* 1991).

Photorespiration does not occur in C_4 species, which affords it potentially higher rates of net photosynthesis at high temperature. However, compared to C_3 species, C_4 species require two additional ATP molecules to assimilate one molecule of CO_2 increasing their photon requirement. This additional energy requirement may become irrelevant at high irradiation since light will be in excess of requirements. Furthermore, in C_3 species at 30 °C, the amount of energy diverted into photorespiration in photosynthesis will be substantially more than the additional energy required for CO_2 assimilation in C_4 species (Young & Long 2000). Therefore temperature appears to be an important factor in photosynthetic response. Indeed, the energy used in photorespiration as a proportion of photosynthesis increases with temperature. At 25 °C and below, the energy needed for net assimilation of one molecule of CO_2 is higher in C_4 than in C_3 photosynthesis, but above 25 °C the situation is reversed (Young & Long 2000). It is therefore not surprising that temperature is the dominant climatic parameter in explaining C_3 and C_4

distribution across all geographic regions (Ehleringer, Cerling & Helliker 1997).

1.6.3. Downregulation

Downregulation or acclimation is defined as a change in photosynthetic CO_2 assimilation when plants grown at elevated CO_2 concentration are transferred to an ambient CO_2 environment (Janssens, Mousseau & Ceulemans 2000).

Elevated CO_2 concentrations permits plants to increase carbon fixation rates, reduce water loss by stomatal regulation, and/or possibly increase nitrogen use-efficiency by reallocating nitrogen from Rubisco. Depending on environmental conditions, a particular combination of these options may be most advantageous. Hence, when plants are grown in elevated CO_2 concentration conditions, under optimal water and nutrient supply, optimising photosynthesis may be expected to be a priority (Arp 1991).

Results of downregulation to elevated CO_2 concentration are so diverse and conflicting that no generalizations can be made. Arp (1991) found a strong correlation between pot size and photosynthetic capacity in C_3 plants. Experiments conducted in pots with a capacity of 3 dm^3, 3-12.5 dm^3, and large containers or in the field, were found to reduce photosynthetic capacity strongly, moderately, or not at all, respectively.

Davey *et al.* (1999) showed that *Agrostis capillaris, Lolium perenne* and *Trifolium repens* exhibited photosynthetic downregulation at elevated CO_2 concentration in 12 dm^3 pots, in open top chambers after two years. Tognetti & Johnson (1999) found no photosynthetic downregulation in oak seedling grown in large containers, in open-top chambers and light saturation for six months.

The most plausible explanation for the different findings appears to lie in an extension of the definition of downregulation, i.e. resource availability, coupled to sink availability, may govern downregulation. However, the short time period of the experiment by Tognetti & Johnson (1999), raises uncertainty in this postulation, especially since seedlings may be more responsive to any type of enrichment, including elevated CO_2. At this phenologically vulnerable stage, and in an attempt to rise past litter and competitors, seedlings (especially those of trees) grow more like weeds than like adults of their species. The relative growth rate of tree seedlings is therefore characteristically much higher than that of adults. This will result in a strong downregulation response in seedlings when they get larger, but that this effect is a consequence of size-related changes due to growth strategy (Loehle 1995). However, this does not imply that adult trees will not be stimulated by atmospheric elevated CO_2 concentrations. In fact, Saxe, Ellsworth & Heath (1998) reported a significant positive correlation between photosynthetic rate and exposure time in coniferous and deciduous trees.

1.7. Effects of elevated CO_2 concentration on primary production

The enrichment of greenhouse atmospheres with CO_2 has become routine in improving the output, quality and value of vegetables, cut flowers and ornamental plants (Hunt *et al.* 1991). Most elevated CO_2 studies have been conducted with crops, but the majority of these crops have been bred to grow rapidly, and to respond to nutrient enrichment. Thus, it is not unexpected that most crops have shown accelerated growth under elevated CO_2 concentrations, as apposed to inconsistent responses among naturally occurring plants (Loehle 1995).

A number of grass species, including maize, have been found to show increased growth rate under elevated CO_2 concentrations, whereas other grass species show no effect, except when increased CO_2 was accompanied by drought stress (Young & Long 2000). However, other frequently studied crop species appear to be CO_2-saturated at present c_a (Wand *et al.* 1999).

Although empirical evidence by Wilsey, Coleman & McNaughton (1997) suggests that biomass production and productivity may be governed by ecosystem processes, there is still debate as to whether increased c_a directly enhances plant growth in C_4 species. Most of the experiments to date were conducted under controlled environments, and even less is known of the effects of elevated CO_2 on natural occurring grasslands. However, the limited empirical data for naturally occurring

plants show growth response varying from nil, to negative, to positive response at elevated atmospheric CO_2 concentration (e.g. Norton *et al.* 1999*a*, Norton *et al.* 1999*b*, Wand *et al.* 1999). Therefore, growth response to elevated CO_2 appears to be a function of species, environment, and the interaction between these two variables.

As a consequence of their physiological differences, it is predicted that C_3 grasses would have a competitive advantage over C_4 grasses under CO_2 fertilization (Long & Jones 1992).

1.8. The effects of CO_2 on nutrient balance in plants

It is generally accepted that biomass will increase as a result of increased photosynthesis at atmospheric CO_2 fertilization in plants (Hunt *et al.* 1991, Salisbury & Ross 1992, Ting 1982). Knowledge of the effects of elevated CO_2 on primary production may go far in gaining a perspective of species response to elevated CO_2, but to attain a more comprehensive understanding of growth responses, it is necessary to know what changes in chemical composition will occur (Poorter *et al.* 1997). Any change in nutrient balance may influence carbon cycling by influencing decomposition, and plant-herbivore interaction by affecting forage quality, and consequently herbivore feeding behaviour (Díaz *et al.* 1998, Peñuelas & Estiarte 1998, Tilman 1993, Tognetti & Johnson 1999).

Furthermore, when nutrients and water are limited at elevated CO_2, there is a general increase in carbon allocation to roots (Bazzaz 1990).

1.8.1 Nitrogen and Phosphorus

Minerals such as nitrogen and phosphorus may influence the magnitude of carbon metabolism directly, via growth and morphogenesis. Bazzaz (1990) reported that several studies have shown that the enhancing effect of elevated CO_2 concentration disappears when nitrogen and phosphorus are limited. Nitrogen is an essential constituent of proteins (such as Rubisco) and chlorophyll and is required for the formation of thylakoids. Orthophosphate is incorporated in high-energy compounds, such as ATP, triose-, pentose-, and hexosephosphates. The availability of inorganic phosphates plays a crucial role in the Calvin cycle and the transport of metabolites and assimilates. Phosphate deficiency results in accumulation of unphosphorylated carbon compounds (starch, sucrose and glucose) and may increase both structural and non-structural carbohydrates (Terry & Rao 1991) in the chloroplasts and thus depress photosynthesis even under otherwise favourable conditions (Larcher 1995).

Taiz & Zeiger (1998) reported that the relative concentration of leaf orthophosphate and triose phosphate control whether photosynthetically fixed carbon is partitioned as starch in the chloroplast or as sucrose in

the cytosol. A low concentration of orthophosphate in the cytosol limits the export of triose phosphate from the chloroplast, thereby promoting starch synthesis. Conversely, unlimited orthophosphate in the cytosol inhibit starch synthesis in the chloroplast and promotes the export of triose phosphate to the cytosol, where it is converted to sucrose.

Low P supply impaired the export of newly fixed carbon (from the chloroplast to the cytosol) more than it reduced photosynthesis per unit leaf area so that photosynthates accumulated in sugar beet leaves (Terry & Rao 1991). Sugar phosphates and RuBP regeneration is also impaired as a result of increase in starch concentration, which was manifested by an increase in enzymes involved specifically in starch synthesis. Furthermore, phosphorus deficiency clearly increased the pool sizes of unphosphorylated carbon compounds (starch, sucrose and glucose) and may have increased both structural and non-structural carbohydrates in soybean and wheat. These results suggest that P deficiency increased the enzymatic capacities for both starch and sucrose synthesis (Terry & Rao 1991) and that synthesis of either starch and/or sugar is governed by heritable traits (Nakamura et al. 1997).

Nitrogen is an important limiting factor in the environment, and in fast growing vegetation, nitrogen will rapidly become deficient. Consequently, sugar repression of photosynthesis has wide-ranging physiological significance even prior to the expected doubling of CO_2 levels in the next century (Paul & Driscoll 1997).

Growth limited by nitrogen results in decreased concentrations of Rubisco and a corresponding decrease in carboxylation efficiency. However, a concomitant change in RuBP regeneration capacity has been found in many species. This suggests an optimisation of resources in the chloroplast so that neither active Rubisco nor the apparatus for RuBP regeneration is in excess. If optimal distribution of resources between components of the photosynthetic apparatus is a ubiquitous phenomenon, then this adaption might also be expected in plants grown at elevated c_a. If inter cellular CO_2 concentration (c_i) increases proportionately, then limitation would be shifted away from carboxylation to RuBP regeneration. Given that a considerable investment of energy and nitrogen in Rubisco, up to 25% of leaf nitrogen, it is likely that Rubisco will be subject to selective regulation (Long, Osborne & Humphries 1996).

The theoretical nitrogen requirement in C_4 species is less than that of C_3 species as a result of the higher CO_2 concentration at the site of Rubisco in C_4 species. The CO_2 concentration at the site of Rubisco in C_4 plants is 10 to 100 times that found in C_3 plants, which results in a C_4 leaf at 30 °C requiring only 13.4 to 19.8% of the Rubisco in a C_3 leaf to achieve the same saturation in CO_2 assimilation. The lower nitrogen requirement in Rubisco will be partially offset by the requirement of nitrogen for the enzymes in the photosynthetic C_4 carboxylation cycle, in particular PEPc. However, due to its ten times higher maximum catalytic

rates of carboxylation, much lower amounts of PEPc are required, relative to Rubisco. Together, Rubisco and PEPc in C_4 plants constitute less than half the concentration of nitrogen invested in Rubisco in C_3 plants (Young & Long 2000).

A decrease in leaf nitrogen and an increase in photosynthetic rates in C_4 species result in a photosynthetic nitrogen use efficiency (NUE - lower leaf nitrogen contents sustain significantly higher rates of carbon uptake) that is about twice that of C_3 species (Davey et al. 1999). Furthermore, on nitrogen poor soils NUE of C_4 crops can be twice that of C_3 (Young & Long 2000). A decrease in the Rubisco requirements in C_3 species at elevated CO_2 levels may reduce the NUE advantage of C_4 species. Because of limited available data, there is still a lack of information about changes in NUE in C_3 and C_4 graminoids at elevated CO_2 (Wand et al. 1999).

Leaf nitrogen decreased in trees grown at elevated CO_2 concentration even after nitrogen content was corrected for starch and soluble sugars accumulation due to elevated CO_2, implying that the 'C-dilution effect' does not entirely account for this observation (Saxe et al. 1998). In fact, it is possible that nitrogen concentration is reduced due to increased nitrogen-use efficiency. The response of Pinus ponderosa was consistent with this prediction (Johnson, Ball & Walker 1995), since leaf nitrogen decreased while other mineral concentrations were unchanged (Saxe et al. 1998).

Wilsey *et al.* (1997) showed that C_3 grasses, irrespective of ecosystem, showed a decrease in nitrogen concentration when grown under elevated CO_2 conditions. Furthermore, C_4 species showed no change in nitrogen concentration at elevated CO_2. These results suggest that nitrogen response to elevated CO_2 is controlled by photosynthetic mechanism rather than ecosystem. This was confirmed by Wand *et al.* (1999) who showed a mean N decrease of 21% in C_3 grasses, whereas decreases in C_4 grasses were not significantly affected at elevated CO_2.

Plant responses to CO_2 enrichment, possibly mediated by low nutrient supply, could affect leaf-level carbon allocation patterns by altering C:N and C:P ratios (Stock & Midgley 1995). Decreases in leaf nitrogen concentration in response to elevated CO_2 are widely reported (Peñuelas & Estiarte 1998), while the effects on phosphorus concentration are not well studied.

1.8.2. C:N and C:P ratio

Decreases in plant tissue nitrogen concentration and the consequent increase in C:N ratio due to elevated CO_2 concentration are widely reported (Peñuelas & Estiarte 1998). Carbon dioxide-induced changes in C:N ratio may induce a feedback effect by influencing nutrient cycling within ecosystems and affect the rate of nutrient mineralization (Stock & Midgley 1995). Litter high in C:N ratio will have lower decay rate (Bazzaz

1990, Saxe *et al.* 1998, Stock & Midgley 1995), which may lead to decreased availability of nitrogen. Furthermore, elevated CO_2 induced changes in litter C:N ratios could produce major shifts in nitrogen dynamics. The rate of nitrogen supply, in turn, could influence community structure in the long-term (Tilman 1993).

An increase in C:N ratio may have similar feedback effects on plant-herbivore interactions (Bazzaz 1990). Herbivores might shift their food preferences to plants with lower C:N ratios (high protein content) or suffer population reduction due to the poor quality food source. A decrease in herbivory of species with high C:N ratio (low protein content) would afford it an advantage in their interactions with other plant species. Such shifts in herbivory could further complicate the effects of elevated CO_2 on competitive interactions and community structure (Tilman 1993).

Stock & Midgley (1995) reported that C_3 plants in particular show an increase in foliar C:N ratios with CO_2 enrichment. This was confirmed by Saxe *et al.* (1998) in a review of elevated CO_2 effects on tree and forest functioning. Wilsey *et al.* (1997) showed that C_4 species showed no change in nitrogen concentration or biomass at elevated CO_2 concentration, which implies that C:N ratio may also be unchanged.

Despite showing strong control of plant CO_2 response, phosphorus paucity has not enjoyed the same attention as nitrogen paucity in CO_2 fertilization studies (Midgley, Stock & Juritz 1995). Any change in phosphorus concentration and consequently C:P ratio may

thus affect plant response to elevated CO_2. Bazzaz (1990) reported that phosphorus decreased with increasing atmospheric CO_2 concentration in trees. Consequently, this should result in an increase in C:P ratio in these plants. The effects of elevated CO_2 on C:P ratio is however poorly studied.

1.8.3. Total non-structural carbohydrates (TNCs)

The most significant change in chemical composition of plants grown at elevated CO_2 is an increase in non-structural carbohydrates. Indeed, an increase in TNC (which include starch, fructans, sucrose, etc.) concentration has been found to be one of the most consistent responses to elevated CO_2 concentration (Poorter *et al.* 1997, Saxe *et al.* 1998, Tognetti & Johnson 1999). However, this response is not universal and there is substantial interspecific variability in the magnitude of the response and the carbohydrates involved, which might contribute to the species variability in CO_2 responses (Saxe *et al.* 1998).

Poorter *et al.* (1997) found substantial variation among leaves of 27 C_3 species (24 wild and agricultural herbaceous species and three tree seedlings) with TNC increases ranging from almost zero to over a hundred percent. However, the experimental growth conditions for all the species were different, which may be the cause of the observed variation among species. An increase in starch concentration commonly made the

largest contribution to TNC in C_3 plants, however, soluble sugar levels may also be affected (Saxe *et al.*1998). Nakamura *et al.* (1997) showed that allocation to starch and/or sugar appears to be regulated by heritable traits, when they found that wheat accumulated a large amount of sugar while soybean accumulated large amounts of starch.

Wand *et al.* (1999) reported that C_3 and C_4 plants showed differential accumulation in TNC content. They further showed that mean TNC content increased by 37% in C_3 species, whereas TNC accumulation in C_4 species was insignificantly increased at elevated CO_2. From the available data it is evident that increases in starch and TNC content are ubiquitous in nutrient limited plants (Poorter & Pérez-Soba 2001).
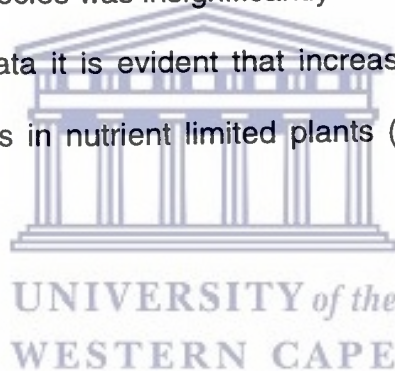
1.8.4. Mycorrhizae

Plant nutrient balance is largely dependent on root ability to absorb water and mineral elements from the soil. Some plants rely on mutualistic associations with mycorrhizae (a root fungus) to accomplish this uptake. A mycorrhiza is a symbiotic and mutualistic association between a non-pathogenic or weakly pathogenic fungus and the roots. The fungi are dependent on the host plant for carbon (Rillig, Field & Allen 1999), but may improve plant water and mineral absorption, particularly phosphate, NH_4^+, K^+ and NO_3^- (Salisbury & Ross 1992). Theory suggests that under

nutrient limited conditions elevated CO_2 may indirectly enhance the association of tree roots with mycorrhizal symbionts, since available photosynthate can be allocated to mycorrhizas and serve to increase exploitation of nutrients that limit further growth (Saxe *et al.* 1998). Indeed, recent data suggests that mycorrhizal colonisation is increased with CO_2 fertilisation in nutrient poor soils (Lewis, Thomas & Strain 1994). The presence of mycorrhizae under nutrient poor soils and elevated CO_2 may therefore influence plant carbon and nutrient allocation.

1.9. The effects of elevated CO_2 concentration on phenols and tannins

Besides 'primary' compounds, which are intermediates of the biochemical pathway, structural components of membranes, light capturing pigments, etc., plants produce a vast array of secondary carbon compounds. Phenolics and tannins are among these compounds that are predicted to increase as a result of elevated CO_2, and may affect plant-herbivore interactions by reducing *in vivo* digestibility of plant matter, and litter decomposition by affecting litter quality (Lambers 1993). The synthesis of phenolics and tannins differ in C_3 and C_4 plants. Polyphenols are synthesised via the common shikimate pathway that produces tyrosine in C_4 and phenylalanine in C_3 plants (Hrazdina 1992). Tyrosine is the precursor for flavonoids and ultimately tannin synthesis in C_4 plants

whereas phenylalanine is the precursor in C_3 plants (Figure 1.1) (Salisbury & Ross 1992, Ting 1982). The synthesis of polyphenolics and tannins in C_3 plants involves the phenylpropanoid segment that produces cinnamic acid directly from phenylalanine (Hrazdina 1992). In C_4 plants the common *p*-coumaric acid is synthesised directly from tyrosine. Both mechanisms then follow a common path for condensed tannin synthesis.

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Photosynthesis

↓

Carbohydrates

↓

Shikimic acid pathway

Tyrosine (C_4)   Phenylalanine (C_3)

Amino acids

p-coumaric acid ← Cinnamic acid

Box 1. Low molecular
weight phenolics   Flavan-3-ols/Flavan-3,4-diols

↓
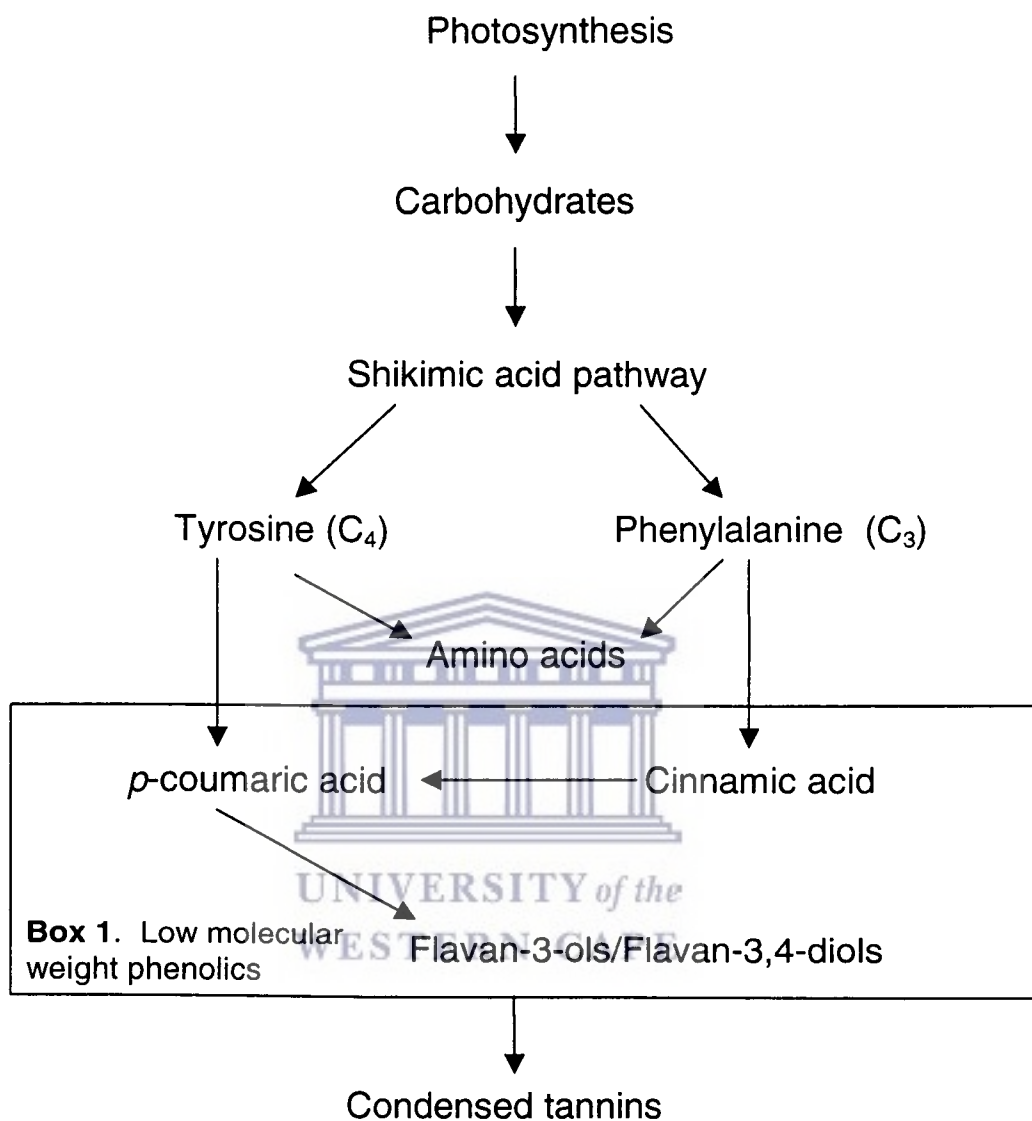
Condensed tannins

Figure 1.1 A simple scheme for the biosynthesis of condensed tannins from tyrosine (in C_4 plants) and phenylalanine (in C_3 plants). The boxed area (Box 1) indicates the region of low molecular weight phenolic synthesis. Polymerisation of phenolic compounds flavan-3-ol and flavan-3,4-diols produces insoluble condensed tannins.

The extensively reported reduction in plant tissue nitrogen concentration and the consequent increases in C:N ratios under increased atmospheric CO_2 concentration, encouraged some authors to forecast a vast increase in carbon-based secondary or structural compounds (CBSSC) in plant tissues in response to elevated CO_2 concentrations (Peñuelas & Estiarte 1998). The roots of these predictions lie in the carbon-nutrient balance hypothesis (Bryant, Chapin & Klein 1983), which directly relates plant CBSSC concentration to the balance between carbon and nitrogen in the plant and its immediate environment, and on the growth-differentiation balance hypothesis (Herms & Mattson 1992), which propose that growth is inversely related to differentiation processes in the plant. The growth differentiation hypothesis, which includes and extends the nutrient-balance hypothesis, considers that any environmental alteration that affects photosynthesis (carbon source) and growth (carbon sink) with varying intensities, will influence the relative carbon pool available for carbon-based secondary compounds. Hence, these hypotheses are in reality source-sink hypotheses (Peñuelas & Estiarte 1998), a summary of which is given in Figure 1.2.
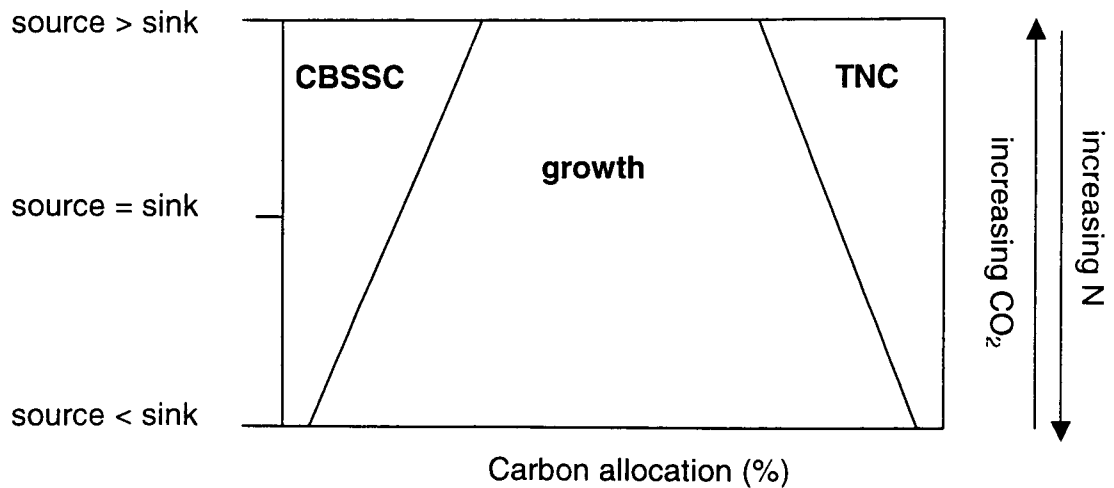
Figure 1.2 Carbon based secondary or structural compounds (CBSSC) and the source-sink hypotheses. The carbon nutrient-balance hypothesis and the growth-differentiation hypothesis propose that variations in source-sink relationships are accompanied by alterations in the relative partitioning of carbon to growth, Total Non-structural Carbohydrates (TNC) and CBSSC. Changes in relative partitioning due to increased CO_2 and decreased nitrogen availability are summarized in the diagram. A gradient of carbon source-sink relationship is displayed on the *y*-axis while the *x*-axis shows the carbon allocation to growth, TNC and CBSSC. When carbon source exceeds carbon sink, TNC and CBSSC would be relatively over-invested. Carbon dioxide has greater positive effect on the strength of carbon source whereas nutrient stress has greater negative effect on the strength of the carbon sink, however, both are expected to increase plant tissue concentrations of CBSSC (Peñuelas & Estiarte 1998).

High CO_2, which increases carbon supply, or, nutrient stress, which increases demand for carbon, promotes a relative increase in carbon availability and the accumulation of TNCs in source leaves. This accumulation of carbon exceeding growth requirements results in carbon levels that allow increases in CBSSC. In fact, nitrogen limitations that lead to increased source:sink ratio have been shown to be activators of the phenylpropanoid (phenolic) metabolism by increased transcription of the relevant genes (Peñuelas & Estiarte 1998).

The synthesis of some amino acids (tyrosine in C_4, and phenylalanine in C_3 plants) and the production of phenolic compounds both occur via the shikimate pathway. When nitrogen supply and the rates of protein synthesis in plants are high, tyrosine and phenylalanine are rapidly incorporated into protein, thereby limiting the production of phenolics. In contrast, at low nitrogen supply, when the rate of protein synthesis is constrained and less tyrosine and phenylalanine are incorporated into protein, the capacity of the phenylpropanoid pathway is sufficient to produce phenolics (Lambers 1993). This implies that the incorporation of carbon into secondary compounds is governed by the demand for amino acids that are required for protein synthesis. Thus, the effects of elevated CO_2 on secondary compound production might be an indirect effect if growth is stronger than nitrogen uptake, so that growth becomes limited by nitrogen (Lambers 1993).

The vast majority of elevated CO_2 studies investigate its effects on primary production (photosynthesis and biomass) and its interactions with nitrogen (e.g. Lutze & Gifford 1998; Blum, Hendrey & Nösberger 1997; Nakamura *et al.* 1997; Vivin, Martin & Guehl 1996). Very few consider the effects of elevated CO_2 on secondary carbon compounds such as phenolics and tannins. However, in the few studies to date, similar to most other responses, phenolics and tannins have been found to exhibit variable responses to elevated CO_2. However, some of these studies are biased by high nutrient levels (e.g. Poorter et al. 1997). Bazzaz (1990) reported that the concentration of carbon-based secondary compounds show no change in leaves for CO_2 enriched plants, whereas Arp (1991) reported variable response in foliar phenolic concentration, and Poorter *et al.* (1997) found an increase in soluble phenolics. However, more recently, Peñuelas & Estiarte (1998) noted that accumulating evidence strongly indicates that elevated CO_2 effects include increases in soluble phenolics and condensed tannins, but that this trend was not prevalent for other carbon-based secondary chemicals such as lignins, structural carbohydrates and terpenes. They further show that the extent of the effect (nil, negative or positive) is dependant on plant species and experimental conditions.

Studies of the effects of elevated CO_2 on polyphenolic and tannin concentrations are limited to C_3 shrubs and tree species (e.g. Kuokkanen *et al.* 2001, Poorter *et al.* 1997, Tognetti & Johnson 1999). This is in part

due to models of plant functional type (Bryant *et al.* 1983) that predict that fast growing plants will use the extra carbon for growth (Díaz *et al.* 1998). Thus, not much is known of the effects of elevated CO_2 on polyphenolic and tannin concentrations in grass species.

The presence of tannin-like substances has been reported in epidermal cells of leaf blades in the Poaceae (grass) family (Ellis 1990). These substances are most commonly found in Andropogoneae, Arundinelleae, Panicoideae, but rarely in Paniceae and Chloridoideae subfamilies. Ellis (1990) further showed that grasses with C_3 and C_4 pathways were apparently capable of forming tannin-like substances, but that these substances were most commonly found in C_4 grasses with the NADP-ME photosynthetic type. However, a limited number of grasses with NAD-ME and PCK have also shown the presence of tannin-like substances (Ellis 1990). These tannin-like substances have been confirmed as condensed tannins in *Eulalia villosa* (Du Toit, Wolfson & Ellis 1991) and as leucoanthocyanidins, which have condensed tannin-like properties, in *Hyparrhenia filipendula, H. hirta, Imperata cylindrica, Themeda triandra* and *Bothriochloa insculpta* (O'Connor & Bredenkamp 1997). Hence, some grasses have the capacity to produce tannins and tannin-like substances, and the production levels of these substances may be enhanced under elevated CO_2 conditions.

1.10. Study objectives and questions

This study was initiated to gain an ecophysiological perspective of the potential effects of global change, more specifically elevated atmospheric CO_2 concentration on plants from a C_4 dominated sub-tropical grassland and C_3 fynbos shrubland.

1.10.1. Objectives
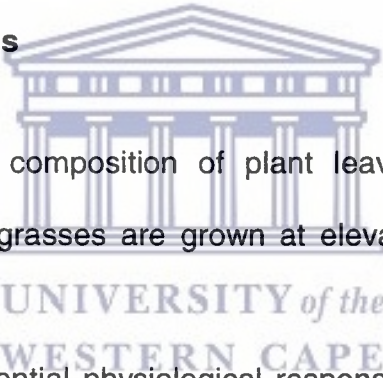
The experiment was designed to test the hypothesis that elevated atmospheric CO_2 concentrations will create an state of excess photosynthates in plant leaves which would be allocated to carbon based secondary compounds, particularly polyphenolics and tannins. This hypothesis finds its roots in the source-sink balance hypothesis (Peñuelas & Estiarte 1998), which propose that sink limited plants will invest surplus photosynthates into the production of carbon based secondary compounds.

We chose to test this hypothesis in species with well-known physiological differences in photosynthetic mechanism and growth strategy. The C_4 grass species are inherently fast growing, investing assimilated carbon into growth, whereas C_3 species are relatively slower growing. However, recent evidence showed that contrary to theoretical principles of C_4 photosynthetic mechanism, fast growing C_4 grass species

would exploit excess carbon at elevated CO_2 to enhance biomass (Wand *et al.* 1999).

In light of the ubiquitous decrease in nitrogen concentration with CO_2 fertilization, the inherently slower growing C_3 plants are hypothesized to accumulate carbon in excess of growth requirements that may be allocated to the production of carbon based secondary compounds (Peñuelas & Estiarte 1998, Saxe *et al.* 1998). This effect may be exacerbated when nutrients are limiting.

1.10.2.   Questions

- Does chemical composition of plant leaves change when C_3 shrubs and C_4 grasses are grown at elevated atmospheric CO_2 concentrations?
- Is there a differential physiological response to the production of polyphenolic compounds in the leaves of C_3 and C_4 species at elevated atmospheric CO_2 concentration?
- Do plants from the same genus respond similarly under CO_2 fertilization?

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CHAPTER 2 - The effects of elevated CO_2 on leaf polyphenol, tannin and chemical composition of field grown grass species

2.1.　Introduction

Grass species are representatives of the most important plant family on earth (Poaceae), in terms of individual numbers, biomass, area covered, habitat diversity and value to humans (Gibbs Russel *et al.* 1991). Humans consume cereals (wheat, rice, maize, etc.) directly as part of the daily diet, or indirectly after conversion into meat, milk, eggs and other animal products (Langer 1972). Grasses also have an important role in ameliorating the greenhouse effect by sequestering carbon from the atmosphere via photosynthesis (Long & Jones 1992). Any change in growth physiology as a result of elevated c_a may therefore have profound ecological, social and economic ramifications.

Grasslands are predominantly used as fodder for grazing animals for the production of meat, milk, wool and other animal products (Langer 1972). A changing environment brought about the anticipated increase in atmospheric c_a may affect grassland growth physiology and biodiversity. However, our knowledge of c_a effects on natural C_4 grasslands is limited (Poorter & Pérez-Soba 2001, Wand 1999).

The economic importance of grasses in agriculture has meant that past research has focussed on how elevated c_a affects C_3 and C_4 crop species (e.g. Horie *et al.* 2000, Lawlor & Mitchell 2000, Young & Long

2000). Consequently not much has been done on elevated c_a effects on wild graminaceous species (Bowler & Press 1993, Wand *et al.* 1999). However, the limited empirical evidence of non-crop grass species, show responses to elevated c_a amongst C_3 and C_4 grasses to be species specific (e.g. Bowler & Press 1993; Norton, Firbank & Blum 1999; Wand 1999; Wand *et al.* 1999).

In a meta-analysis of the available data, Wand *et al.* (1999) showed that biomass in C_4 grass species is positively affected by an increase in c_a, however, to attain an understanding of the underlying processes that give rise to these changes, it is necessary to know what chemical changes will occur (Poorter *et al.* 1997). An increase in c_a that alters the supply of carbon to plants (Peñuelas & Estiarte 1998), may affect the quality of forage, thereby potentially modifying herbivore consumption and fitness (Bazzaz 1990), and indirectly plant community composition (Díaz *et al.* 1998).

Nitrogen concentration in C_4 grasses has been reported to show little or no change at elevated c_a (Lambers 1993). Wilsey *et al.* (1997) also reported that C_4 tropical grasslands would be less affected by a decrease in N concentration at elevated c_a than C_3 species. This was confirmed by Wand *et al.* (1999) in a meta-analysis of available data that showed little change in TNC and N concentration in C_4 species under elevated CO_2. However, most of these findings are based on data derived from controlled laboratory studies. To gain a perspective of how

applicable these findings are in extrapolating across ecosystems, it is essential to test these results in natural field environments over longer time periods than has been done for most laboratory studies.

This study set out to test the source-sink balance hypothesis under increased c_a and to ascertain how leaf chemical composition, and hence herbage quality for grazers, will be affected in seven wild sub-tropical grassland species growing under field conditions.

2.2. Materials and Methods

2.2.1. Site description and experimental design

The study area falls in the sub-tropical grassland of South Africa. The grassland is dominated by grasses with the C_4 photosynthetic pathway, and the grasses exhibit strong seasonality of growth, reaching a peak in late summer, after which senescence sets in (Wand 1999).

The study was carried out in the area of Bongwan near Port Shepstone (approximately 30°40'S 30°00'E) in southern KwaZulu/Natal. Underlying the area of Bongwan is an 80 km long gas fault which produce 97% pure CO_2 (Harris, Stock & Lanham 1997). The CO_2 gas is thought to be a product of acid groundwater reacting with carbonate rock at depth producing natural CO_2 springs in the area. The grassland was thus subjected to natural free-air CO_2 enrichment, which is reported to

represent an 'acid test' in natural environments of knowledge derived form smaller-scale chamber and glasshouse studies (Saxe *et al*. 1998). Free-air CO_2 enrichment studies have the advantage of providing minimal alterations to plant micro-environment, where natural climate cycles and biotic interactions occur, and minimizing artifacts such as soil disturbances and artificial soil (Saxe *et al*. 1998).

The study site is located on the farm Pleasant View, adjacent to a pipe inserted into a CO_2 spring on the farm. The site is about 650 m above sea level and the mean annual rainfall is high (800 mm), which causes leaching, and consequently results in low nutrient soil (total N of about 0.15%, Wand 1999). The mean minimum and maximum temperatures are 13.7 °C and 22.6 °C respectively (Table 2.1). The loamy sand is acidic with pH ranging from 4.2 to 4.8 (Stock *et al*. submitted). The grassy slope that is surrounded by sugar cane plantations, was used for cattle grazing prior to 1992, rested for five years and annually burnt as a fire precaution (Wand 1999).

Table 2.1 Mean maximum and minimum temperature and average monthly precipitation (1961 to 1990) at Paddock (South Africa Weather Service).

Month	Average maximum temperature (°C)	Average minimum temperature (°C)	Precipitation (mm)
January	24.8	17.1	158
February	25.0	17.2	148
March	24.8	16.6	150
April	23.4	14.4	73
May	22.0	12.4	52
June	20.4	10.2	22
July	20.4	10.2	42
August	21.0	10.7	62
September	21.4	12.0	106
October	21.4	13.0	163
November	22.3	14.3	150
December	24.1	16.0	145
Annual	22.6	13.7	1271

Three 7 m x 7 m plots down a slope of about 15 ° were selected on the southerly side of the CO_2 vent at 20, 40 and 80 m from the vent. The plots were subdivided into 25 x 1 m² subplots in a 5 x 5 layout with 40 cm buffer zones in between (Stock *et al.* submitted). Since July 1996, when the experiment was initiated, the grass was exposed to three CO_2 concentrations: *viz.* elevated (600 to 800 ppm), no more than 400 ppm (hereafter referred to as intermediate) and ambient (about 360 ppm). The treated plot was situated at the bottom and the ambient plot at the

top of the slope. Carbon dioxide was piped to the treated site, constantly receiving between 550 and over 700 ppm at a hotspot in the middle of the site. These values were calculated from $\delta^{13}C$ isotopic data for *T. triandra* (annually from 1996 to 2000) growing in the treated plot relative to the 80 m control plot (Stock *et al.* submitted) and the intermediate plot received wind dispersed CO_2. Grass species *Alloteropsis semialata* (R.Br.) Hitchc. Subsp. e*ckloniana* (Nees), *Andropogon appendiculatis* Nees, *Digitaria diagonalis*, *Eragrostis plana* Nees, *Sporobolus pyrmidalis* Beauv., *Themeda triandra* Forssk. and *Tristachya leucothrix* Nees were harvested in July 1998. All these species have C_4 photosynthetic mechanisms except *A. semialata* that is C_3 (Gibbs Russel 1991). The identity and photosynthetic mechanism of *A. semialata* (two sub-species of *A. semialata* exist, i.e. *semialata* which is C_4 and *eckloniana* which is C_3) was confirmed by ^{13}C isotope determination (Wand 1999).

Five bulk samples of each species were harvested from plots in each of the three CO_2 treatments. After drying at 70 °C samples were ground in a Wiley mill to pass through a sieve with a mesh size of 0.5 mm and stored in tightly capped pill vials before chemical analyses.

2.2.2. Chemical analysis

Ground leaf material was analysed for total nitrogen (N), total phosphorus (P), total carbon (C), total non-structural carbohydrates (TNC), total

polyphenols, condensed tannins and protein-precipitating tannins using methods described below.

2.2.2.1. Nitrogen and Phosphorus

2.2.2.1.1. Acid digestion procedure

Samples weighing about 0.3 g were digested with 4 ml of hot H_2SO_4-H_2O_2 solution to convert the bound nitrogen into ammonium ions. The acid mixture was prepared by adding 350 ml H_2O_2 (30%) to 0.42 g Se and 14 g $Li_2SO_4.H_2O$, while 420 ml H_2SO_4 was added slowly whilst mixing in an ice-water bath (Allen 1989).

Samples were digested in a digestion block, starting at 150 °C and raising the temperature at 30 min intervals to 220, 250, 280, 300 and finally to 350 °C for 1 h, yielding a straw-yellow coloured solution. The samples were then allowed to cool for 10 min and 20 ml water was added to prevent crystallization. Digests were filtered through Whatman No. 44 filter paper into 100 ml volumetric flasks and made up to volume with distilled water.

2.2.2.1.2. Nitrogen

The Kjeldhal method was used to determine total nitrogen. Twenty-five millilitre aliquots were transferred to a distillation tube and made basic by

the addition of excess 10 M NaOH solution. The liberated ammonia was steam distilled in a Büchi distillation unit (Büchi 320 N2 Distillation Unit, Büchi Laboratories, Switzerland). The distillate was collected in 5 ml 2% aqueous H_3BO_3 solution, which retained the ammonia. The reasonably stable dihydrogen borate was titrated with 0.01 M HCl solution using Bromocresol green as indicator. Anhydrous $(NH_4)_2SO_4$ solution in the range of 0 to 2.5 mg N was used as standard.

2.2.2.1.3.   Phosphorus

Phosphorus concentration was determined colorimetrically by the Molybdate blue assay (Murphy & Riley 1962). Four ml aliquots of the digest solutions were transferred into 100 ml volumetric flasks containing 38 ml water. Murphy & Riley solution (8 ml) was added and intermittently swirled. The absorbance was measured at 882 nm (Novaspec visible spectrometer, LKB Biochrom Ltd., England) after allowing colour development for 1 h. Potassium dihydrogen orthophosphate solution was used to prepare standards in the 0 to 30 μg P concentration range.

2.2.2.2.   Total Non-structural Carbohydrates (TNCs)

Total non-structural carbohydrates were extracted using the Weinmann method and analysed by employing the Shaeffer-Somogyi Copper-iodometric titration method as modified by Smith (1981).

Buffer solution: Buffer solution pH 4.5, was prepared by mixing two volumes of 0.2 M CH_3CO_2H solution with three volumes of 0.2 M $NaCO_2CH_3$ solution. The final pH was adjusted with dilute NaOH solution.

Leaf samples weighing about 1.5 g were boiled in an Erlenmeyer flask containing 15 ml distilled water for 5 min with periodic swirling. Sample solutions were cooled down, and 10 ml of buffer solution was added. Oligosaccharides and starch in the sample were then hydrolysed by adding 5 ml aqueous Amyloglucosidase solution (enzyme extracted from *Aspergillus niger*, A3042, Sigma, St Louis, USA), containing 315 enzyme units ml^{-1}. The Erlenmeyer flasks were then covered with a cork stopper and incubated for 24 h at 55 °C. Following the incubation period, the samples were filtered through Whatman No. 1 filter paper into 100 ml volumetric flasks. The filter paper was washed several times with distilled water. After the addition of 2 ml 10% aqueous $Pb(CH_3COO)_2.3H_2O$ (Lead acetate) solution the volumetric flasks was made up to volume with distilled water. The lead acetate formed a complex with protein in the solution that yielded a white precipitate. Solutions were allowed to stand for 1 h whereafter the precipitate was separated from the sample by centrifugation at 3000 g.

Aliquots (5 ml) were made up to 10 ml with distilled water, hydrolysed by the addition of 0.5 M H_2SO_4 and boiled at 100 °C for 15 min. After cooling, samples were neutralised by the addition of 1 M NaOH solution. The samples were then subjected to the quantitative assay for reducing power by the Copper-iodometric titration. A 3 ml glucose solution (containing 1 mg glucose ml^{-1}) was used as standard.

2.2.2.3. Total Carbon

Total carbon was measured in a CNH-auto analyser (Carlo Erba NA 1500 Nitrogen Analyser). Samples weighing about 0.5 mg were sealed in a tin capsule and dropped at pre-set intervals into the instrument. After combustion and separation of components, carbon was quantitatively measured.

2.2.2.4. Total polyphenols and tannin analysis

A vast array of tannin polymers exists in nature. Hagerman & Butler (1991) reported molecular weights of tannins ranging from 500 to 20000, and due to this structural diversity of naturally occurring tannins, no single commercial reference standard is available for tannin quantification.

To determine the absolute tannin concentration in plants, it is necessary to extract tannins from the species under investigation. This

extraction is tedious when dealing with plants with low tannin concentration. To accommodate for this, Hagerman & Butler (1989) proposed the use of the moderately sized *Sorghum* tannin that contain exclusively condensed tannins as an appropriate condensed tannin standard. *Sorghum* tannin was extracted from high tannin *Sorghum* seed and used as standard in the condensed and protein precipitating tannins assays.

2.2.2.4.1. Extraction and partial purification of *Sorghum* tannin from *Sorghum* seed

The extraction procedure was performed as outlined by Hagerman (1995). *Sorghum* seed (SA 423) was obtained from the ARC-Grain Crops Institute, Potchefstroom, South Africa.

Chemicals: 0.05 M Acetate buffer solution (pH 4.0). Glacial acetic acid (2.85 ml) was diluted with 800 ml distilled water. The pH was adjusted to 4.0 with 2 M NaOH solution. The solution was then made up to 1 l with distilled water.

10 mM Ascorbic acid ($C_6H_8O_6$) in absolute ethanol (hereafter referred to as ethanol)

10 mM $C_6H_8O_6$ in methanol (hereafter referred to as methanol)

50% aqueous acetone

Lipophilic Sephadex LH-20 (LH-20-100, Sigma, St Louis, USA)

Two hundred g of *Sorghum* seed were ground at 5 °C using a Waring blender. The ground material was stirred in 600 ml of ethanol for 45 min using an efficient overhead stirrer motor with a propeller spindle. The ethanol suspension was centrifuged to separate the residue from the solution. The supernatant liquid, which was assumed to contain very low molecular weight tannins was discarded. The residue was again subjected to four consecutive extractions in 150 ml of methanol with overhead stirring. The suspension was centrifuged after each extraction and the supernatants containing the tannin were combined and filtered to clarify the solution. The resulting solution was mixed with an equal volume of 0.05 M acetate buffer, yielding a cloudy orange solution. Methanol was completely removed from the solution by rotary evaporation under reduced pressure and room temperature (proteins bind to tannin at temperatures above 30 °C). The remaining aqueous solution was extracted three times with 300 ml ethyl acetate. Subsequent to discarding the ethyl acetate fractions, the volume of the aqueous phase was reduced by rotary evaporation under reduced pressure and room temperature to about 20 ml. Absolute ethanol was added to make up a final volume 80:20 ethanol/water (v/v), and this

solution was mixed with 4 volumes of a sephadex-ethanol slurry. During this step the tannins adsorb onto the sephadex beads imparting a light brown colour to it. The sephadex gel was transferred to a sintered glass funnel and washed repeatedly with ethanol (with slow suction) until the eluate no longer showed absorption in the UV range. The filtrate was discarded. The sephadex gel was then washed with 50% aqueous acetone, which desorbed the tannin from the beads. The acetone filtrate was then subjected to rotary evaporation under reduced pressure and at room temperature. The resulting aqueous solution was lyophilised, yielding a brown, amorphous, flaky, crude tannin powder.

2.2.2.4.2. Extraction procedure

Depending on the amount of polyphenolics and tannins in the plant material, 0.1 to 0.2 g plant material was extracted into 2 ml 70% aqueous acetone (Hagerman 1995) by sonicating in an ice-water bath for 30 min and centrifuging at 2000 g for 10 min. The supernatant was quantitatively analysed for total polyphenols, condensed tannins and protein-precipitating tannins.

2.2.2.4.3.   Total polyphenols

Total polyphenols were analysed using a redox general phenolic assay (Price & Butler 1977) as modified by Hagerman (1995).

Chemicals: 0.10 M NH$_4$Fe(SO$_4$)$_2$.12H$_2$O in 0.1 M HCl (R1)

0.008 M K$_3$Fe(CN)$_6$ (R2)

Depending on the amount of polyphenolics in the plant material, 0.1 to 0.2 ml aliquots of the extract supernatant was transferred into Erlenmeyer flasks containing 50 ml distilled water. Three ml R1 was added to each Erlenmeyer flask at 1 min intervals to all the flasks. Exactly 20 min after the addition of R1, 3 ml R2 was added to each Erlenmeyer flask at 1 min intervals. The solutions were intermittently swirled and exactly 20 min after the addition of R2, the absorbance was measured at 720 nm (Novaspec visible spectrophotometer). Gallic acid solution in the 0 to 0.17 mg range was used as standard.

2.2.2.4.4.   Condensed tannins

Condensed tannins were quantified by using a functional group assay specific for proanthocyanidins (Porter, Hrstich & Chan 1986), as modified by Hagerman (1995).

Chemicals: n-Butanol containing 5% HCl (Acid-butanol)

2% NH$_4$Fe(SO$_4$)$_2$.12H$_2$O in 2 M HCl (R3)

Depending on the amount of tannin in the plant material, 0.1 to 0.2 ml aliquots of the extract supernatant solution were added to boiling tubes containing 6 ml acid-butanol solution. Reagent 3 (0.2 ml) was added to each tube and mixed using a vortex mixer. The tubes were loosely capped to prevent any water contamination, and heated in a water bath at 100 °C for 50 min. After cooling, the absorbance was measured at 550 nm (Novaspec visible spectrophotometer). The assay was standardised against *Sorghum* tannin in the 0 to 0.8 mg range.

2.2.2.4.5.   Protein-precipitating tannins

Protein-precipitating tannins were determined by using a protein-binding bio-assay (Hagerman 1987), as modified by Hagerman (1995).

Chemicals: 0.05 M Acetate buffer (pH 5.0) containing 60 μM $C_6H_8O_6$. Glacial acetic acid (2.85 ml) was diluted with 800 ml distilled water. Ascorbic acid (10.6 mg) was added, and the pH was adjusted to 5.0 with 2 M NaOH solution. The solution was made up to 1 l with distilled water.

Agarose - Type I, Low EEO, gel point 36°C (A-6013, Sigma, St Louis, USA)

0.10 M NH$_4$Fe(SO$_4$)$_2$.12H$_2$O in 0.1 M HCl (R1)

0.008 M K$_3$Fe(CN)$_6$ (R2)

Bovine Serum Albumin (BSA) - Fraction V powder,

96-99% albumin (A-3350, Sigma, St Louis, USA)

Gel plate preparation:

Gel plates were prepared by dissolving 1.0 g agarose per 100 ml buffer solution. The solution was heated with continuous stirring until the agarose dissolved. Whilst continuously stirring, the solution was allowed to cool to 45 °C, and 0.01 g BSA was added per 100 ml solution. The solution was maintained at 45 °C in a water bath, while 10 ml volumes were transferred into matching petri dishes and allowed to cool on a level surface. Gel plates were sealed, wrapped in plastic to prevent any contamination, and stored at 5 °C until required.

Four equidistant 4 mm diameter wells were punched in the agar plates, and each well was filled with 60 μl aliquot of each extract. The gel plates were sealed with polyethylene film (wrapped in plastic to prevent any moisture loss) and incubated at 30 °C on a level surface for 96 h. After incubation the gel plates were carefully removed and unprecipitated protein was removed from the gel by washing in 0.3 M NaCl solution (100 ml per plate) for 1 h. The saline solution was discarded and the gel plates were stained in a 50% R1/R2 (v/v) solution (20 ml per plate) for 3 min. The tannin-containing rings developed a dark blue colour. The

staining solution was discarded and the plates were rinsed in 0.1 *M* HCl solution (50 ml per plate). The diameter of the rings were measured and the area (which is directly equivalent to the protein-precipitating tannin in the sample) was calculated by the formula Area = πr^2, where *r* is half the diameter. The assay was standardised with *Sorghum* tannin in the 0 to 0.12 mg well^{-1} range.

2.2.3. Statistical analysis

Due to the non-normal distributions of proportions and percentages, as well as to account for possible skewed data as a result of small sample size, C:N and C:P ratios were ln-transformed and percentage N, P, TNC and total polyphenols were arcsine-transformed to improve normality and homogenize variance.

Multivariate GLM followed by Tukey's multiple range tests were performed for each response variable to test the significance between species and treatment. To test the effects of treatment within species, One-way ANOVAs followed by Tukey multiple range tests were performed on treatment for species that showed significant results.

2.3. Results

Nitrogen

Nitrogen concentrations were different amongst the species studied (Table 2.2). *Digitaria diagonalis* showed the lowest, whereas *A. semialata* and *T. leucothrix* showed the highest N levels (Figure 2.1). Nitrogen concentrations at intermediate and elevated CO_2 levels were similar and showed a general decrease relative to the ambient CO_2 grown plants ($P < 0.05$). There was interaction between species and CO_2 level (Table 2.2).

Elevated c_a did not affect N concentrations within species, except in *A. semialata*, *S. pyrmidalis* and *T. triandra* (Figure 2.1). When compared to ambient CO_2 treatment, *A. semialata* showed a significant decrease of 31% (Figure 2.1, $F_{2,11} = 6.284$, $P = 0.015$) in N concentration at intermediate CO_2, but was no different from elevated CO_2 treated grass. Grass grown at elevated c_a showed a 16% decrease in N relative to ambient, but was not significantly different from grass grown at intermediate CO_2 in *S. pyrmidalis* (Figure 2.1, $F_{2,12} = 5.436$, $P = 0.021$). In *T. triandra*, N concentration at intermediate CO_2 showed a surprising increase of 27% (Figure 2.1, $F_{2,9} = 4.739$, $P = 0.039$), but elevated CO_2 treated grass was no different from ambient or intermediate treatments. Results in *A. semialata* and *T. triandra* do not show expected theoretical

patterns for N at elevated c_a. These erratic results may be attributed to edaphic factors as the plots were replicated down a slope, and hence, soil and nutrient properties may be different.

Total non-structural carbohydrates

Total non-structural carbohydrates showed different concentrations amongst species studied here (Table 2.2), with *A. appendiculatis* having the lowest, and *T. leucothrix* the highest TNC concentrations (Figure 2.1). Strangely, intermediate CO_2 treated grass showed an overall lower TNC levels than the ambient and elevated CO_2 treatments ($P < 0.05$).

No differences in TNC concentration was detected, except for *E. plana*, which showed an increase of 37% at elevated, but was no different from intermediate CO_2 treated grass (Figure 2.1, $F_{2,11} = 4.219$, $P = 0.044$).

Table 2.2 Multivariate ANOVA testing the effects of CO_2, species and the interaction between these factors on N, TNCs, C:N ratio, P, C:P ratio and total polyphenols in seven different grass species grown at three different CO_2 treatments. Values are F values.

	CO_2 effect	Species effect	CO_2 x species
N	6.019**	19.131***	2.318*
TNCs	3.637*	12.091***	0.875 NS
C:N ratio	7.085**	20.354***	2.344*
P	4.273*	6.190***	1.436 NS
C:P ratio	4.770*	6.875***	1.271 NS
Total polyphenols	4.904**	14.787***	0.979 NS

Significant differences at ***$P < 0.001$; **$P \leq 0.01$; *$P < 0.05$; NS = not significant F values
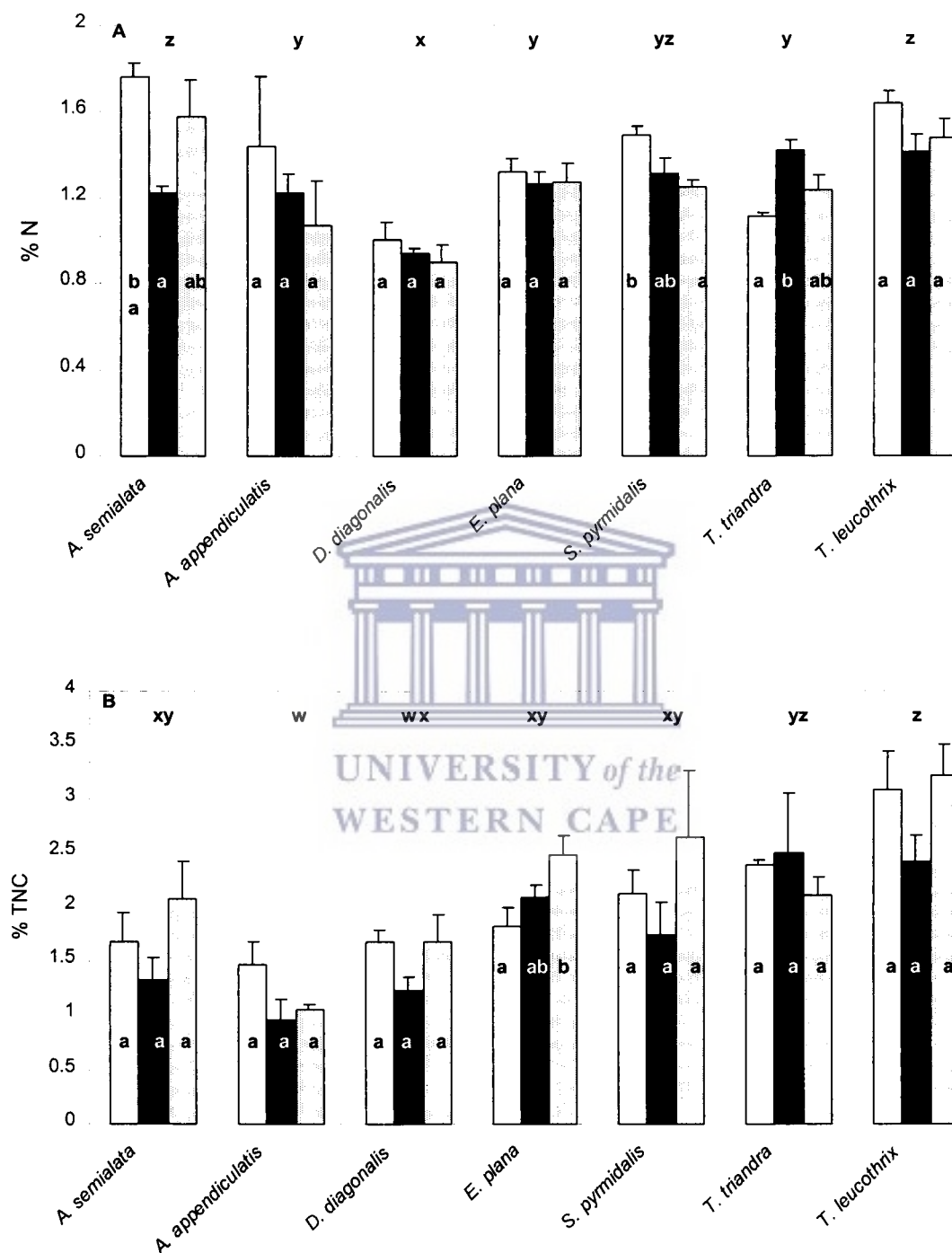
Figure 2.1 The effects of CO_2 level (ambient *open column*, intermediate *shade column* and elevated *dotted column*) on percentage nitrogen (A) and total non structural carbohydrates (B) in the leaves of seven grass species, *A. semialata, A appendiculatis, D. diagonalis, E. plana, S. pyrmidalis, T. leucothrix* and *T. triandra* Values are means ± SE. Different letters (*w, x, y* and *z*) indicate significan differences between species and (*a* and *b*) indicate significant differences withi species ($P < 0.05$).

C:N ratio

Carbon:nitrogen ratios were different amongst species studied here (Table 2.2), with *A. semialata* having the lowest, and *D. diagonalis* the highest ratios (Figure 2.2). As a consequence of N responses within treatment, C:N ratios showed a similar, but inverse pattern, with treated C:N ratios being higher than ambient, but no different from intermediate CO_2 treated grasses ($P < 0.05$). Interaction between species and CO_2 level was found (Table 2.2).

Relative to ambient, C:N ratio in *A. semialata* showed a significant increase of 40% at intermediate CO_2 (Figure 2.2, $F_{2,11} = 5.380$, $P = 0.023$). However, elevated was not significantly different from intermediate or ambient CO_2 grown grass. There was a significant increase of 24% in C:N ratio in *S. pyrmidalis* at elevated CO_2 (Figure 2.2, $F_{2,12} = 7.189$, $P = 0.009$), whereas intermediate showed no significant difference from ambient or elevated CO_2 grown grass. A significant decrease of 27% was observed in C:N ratio of intermediate relative to ambient CO_2 treated *T. triandra* grass (Figure 2.2, $F_{2,9} = 8.865$, $P = 0.007$), but no significant difference was found between ambient and elevated CO_2 treated grass. These erratic patterns are due to the unexpected results found for N concentrations in *A. semialata* and *T. triandra*.

Phosphorus

Phosphorus concentrations were different amongst species studied here (Table 2.2), with *D. diagonalis* having the lowest and *T. leucothrix* the highest levels (Figure 2.2). Unexpectedly, P concentration at intermediate was higher than elevated, which were both no different from ambient CO_2 treated grasses ($P < 0.05$).

No significant differences were found for treatment within species, except in *A. semialata* where P concentration at elevated CO_2 decreased by 60% relative to ambient CO_2 grown grass (Figure 2.2, $F_{2,11} = 5.948$, $P = 0.018$). However, grass exposed to intermediate was no different from either ambient or elevated CO_2 grown grass.

Figure 2.2 The effects of CO_2 level (ambient *open column*, intermediate *shade column* and elevated *dotted column*) on C:N ratio (A) and percentage phosphoru (B) in the leaves of seven grass species, *A. semialata, A. appendiculatis, D diagonalis, E. plana, S. pyrmidalis. T. leucothrix* and *T. triandra.* Values are mean ± SE. Different letters (*w, x, y* and *z*) indicate significant differences betwee species and (*a* and *b*) indicate significant differences within species (*P* < 0.05).

C:P ratio

Carbon:phosphorus ratios were the same in all species, except for *D. diagonalis* that was about two fold that of the other grass species (Table 2.2, Figure 2.3). As a consequence of the results for P concentrations, C:P ratios showed a similar, but inverse pattern, with elevated C:P ratio being higher than intermediate, which were both no different from the C:P ratio in ambient CO_2 treated grasses ($P < 0.05$).

Carbon:phosphorus ratio in *A. semialata* showed an inverse pattern to that found for P concentration. Elevated CO_2 treated grass showed an increase of 122% relative to ambient CO_2 grown grass (Figure 2.3, $F_{2,11} = 8.135$, $P = 0.007$), but were both no different from intermediate CO_2 treated grass.

Total polyphenols

Total polyphenol concentration was different amongst species (Table 2.2), with *A. semialata* and *S. pyrmidalis* having the lowest and *A. appendiculatis* and *E. plana* the highest concentrations (Figure 2.3). No treatment effect was found in all species ($P > 0.05$).
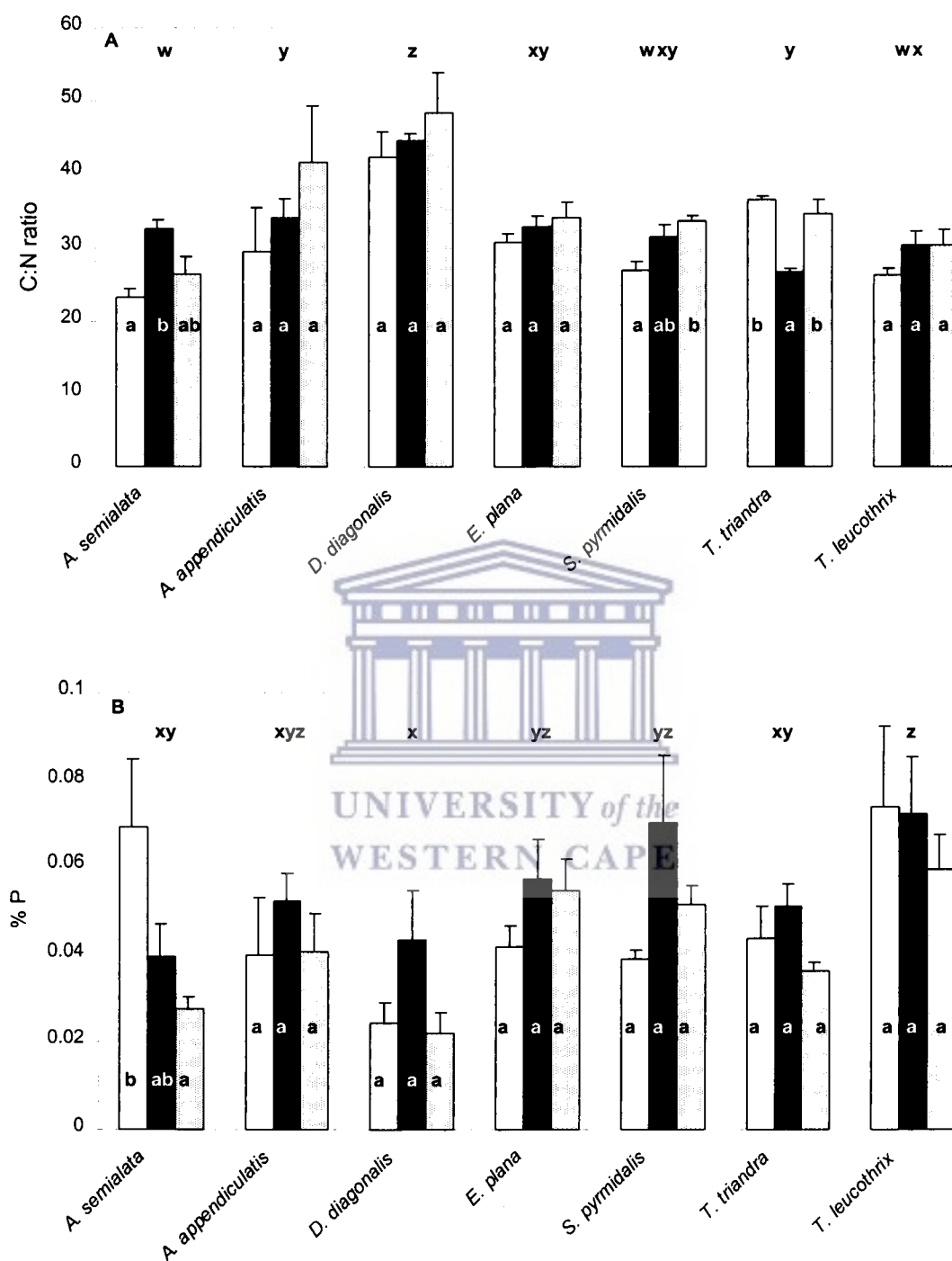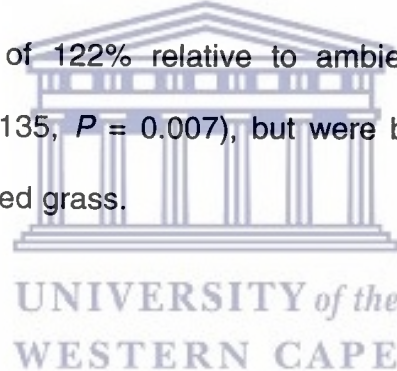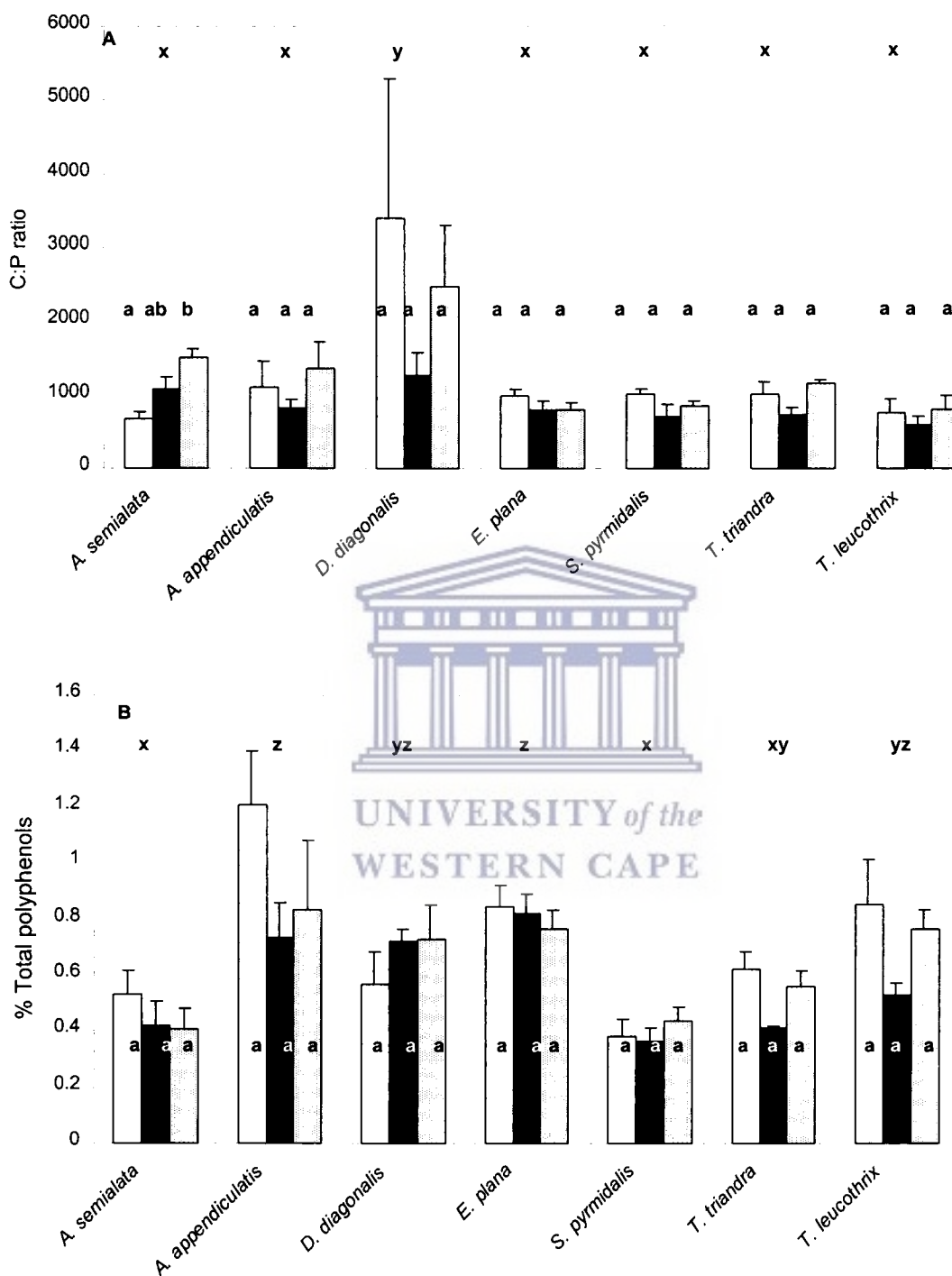
Figure 2.3 The effects of CO_2 level (ambient *open column*, intermediate *shade column* and elevated *dotted column*) on C:P ratio (A) and percentage tota polyphenols (B) in the leaves of seven grass species, *A. semialata, A appendiculatis, D. diagonalis, E. plana, S. pyrmidalis. T. leucothrix* and *T. triandra* Values are means ± SE. Different letters (*x, y* and *z*) indicate significant difference between species and (*a* and *b*) indicate significant differences within species (*P* 0.05).

Condensed tannins

Absence of the characteristic red coloured tannin-Fe complex that indicates the presence of tannins in samples after boiling, proved that none of the samples contained condensed tannins.

Protein precipitating tannins

The assay for the presence of protein precipitating tannins was negative.

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2.4. Discussion

Overall N concentration showed a decrease at CO_2 levels above ambient, whilst still supporting higher biomass production as reported by Stock *et al.* (submitted) in a study of biomass productivity at the same study site. This is contrary to findings by Wand *et al.* (1999) for non-stressed C_4 grasses and that of Wilsey *et al.* (1997) who showed no change in N content in the exclusively C_4 grasslands of the Serengeti (Tanzania, Africa) at elevated CO_2. Nitrogen may be used more efficiently by investing less available N into Rubisco (Arp 1991, Long *et al.* 1996, Stock & Midgley 1995) and more into other compounds that limit growth (Poorter & Pérez-Soba 2001). This was confirmed by Davey *et al.* (1999) who showed a sustained increase in carbon uptake, and hence growth, at lower N content and attributed it to enhanced photosynthetic nitrogen-use efficiency in UK grassland species that included *Agrostis capillaries* and *Lolium perenne*. Alternatively, the observed decrease in N may merely be due to recirculation of N from leaves to roots (a trait of C_4 grass species), thereby directing N to the strongest sink to prevent any part of the plant becoming N deficient (Salisbury & Ross 1992).

Despite not being significant at intermediate CO_2, N concentration showed a decrease with increasing CO_2 level in *S. pyrmidalis*. This is contrary to empirical evidence for C_4 grasses (Lambers 1993) and

findings by Wand (1999) for the species. Wand (1999) further showed a 50% increase in biomass for *S. pyrmidalis*, which suggests that the plant may reallocate N to other processes that may be limiting growth. This finding is consistent with data presented by Davey *et al.* (1999) for C_4 grass species. However, this response was species-specific as all other species studied here showed no change in N concentration within treatment at species level.

As a consequence of the reduction in overall N content with increasing CO_2 level, an overall increase in C:N ratio was observed with increase in CO_2. Similarly, *S. pyrmidalis* showed an increase in C:N ratio with increase in CO_2 level. Again, this was a species-specific response. Empirical evidence suggests that this increase in C:N ratio could retard litter decomposition rates (Bazzaz 1990). However, improved soil water content (as shown by Stock *et al.* submitted) due to improved water use at elevated CO_2 (as shown by Wand *et al.* (1999) for C_4 grass species) may counter-balance decomposition rates by creating a more moist environment that is expected to speed up plant matter decay rates (O'Connor & Bredenkamp 1997). These postulations are based on N concentrations in live plant material, and it is unclear how retranslocation of N in senescence will affect litter quality under elevated CO_2 conditions (Ågren & Hansson 2000).

The increase in TNC concentration in *E. plana* is species-specific and contrary to the limited data of TNC response that show little change

from that of ambient CO_2 grown C_4 grasses (Wand *et al.* 1999). Indeed, this empirical non-response in TNC content was reinforced by findings in the other C_4 species studied here. Thus, the C_4 species studied here appear not to experience any source-sink imbalance at CO_2 levels above ambient.

Phosphorus concentrations decreased with increases in CO_2 level in *A. semialata*, which resulted in similar but inverse effects in C:P ratios. Wand (1999) showed photosynthetic downregulation in greenhouse and field grown *A. semialata* species at elevated CO_2, and attributed it to a similar reduction in carboxylation capacity, despite the latter not being significant. As mentioned earlier, leaching due to high rainfall in Bongwan resulted in low nutrient and consequently low P concentrations in the soil. Low soil P is reportedly associated with a decrease in RuBP and other sugar phosphates (in part due to an increase in phosphatase activity) in the chloroplast of leaves (Terry & Rao 1991). This decrease in RuBP should reduce carboxylation capacity thereby causing photosynthetic downregulation, which is consistent with findings by Wand (1999) for *A. semialata*. Davey *et al.* (1999) also showed that an increase in CO_2 level moved control of photosynthesis away from limitation by Rubisco towards RuBP-regeneration capacity in *Agrostis capillaris* and *Trifolium repens*. If this postulation is valid, then the apparent association between P availability and downregulation may warrant re-evaluation of the current perception that downregulation in C_3

species is due to a decrease in N content (Wand *et al.* 1999). The interaction between P availability at elevated CO_2 and photosynthesis requires further study.

The non-response in polyphenolic concentration is not unexpected since fast growing grass species are predicted to invest surplus carbon into growth rather than carbon secondary compounds (Díaz *et al.* 1998). Furthermore, inherently fast growing grass species have a low capacity to accumulate carbon based secondary plant metabolites (Lambers 1993). The presence of tannin-like substances in leaf epidermal cells of *A. appendiculatis*, *T. triandra* and *T. leucothrix* as shown by Ellis (1990) is therefore interesting. Ellis (1990) further showed that the presence and concentration of these substances are apparently induced by herbivore damage, and that it is occasionally associated with dystrophic soils. These findings indicate the capacity of these grasses to form carbon based secondary compounds at ambient CO_2 levels. So why was an increase in polyphenolic concentration not found at CO_2 levels above ambient? Firstly, the grasses were not grazed; and secondly, as showed by Stock *et al.* (submitted), the assimilated carbon is invested into growth. The presence of tannin-like compounds may be due to impaired soil nutrition (particularly N) that precludes the incorporation of tyrosine into protein, thus making it available for polyphenolic production in the shikimate pathway (Lambers 1993). The occurrence of tannin-like substances in certain grasses may also be an evolutionary adaption that

ensured species survival under severe herbivore pressure. Indeed, tannin-like substances are mostly found in C_4 species with NADP-ME pathways (i.e. they are malate formers) that occur in grassland and savanna communities of South Africa (Ellis 1990) where grasses are the primary food source for grazers.

Despite downregulation at elevated CO_2 (Wand 1999), the C_3 *A. semialata* did not show an increase in TNC, or polyphenol concentration. Thus, the mechanism of downregulation in *A. semialata* appears not to be related to feedback inhibition resulting from TNC accumulation or reduction in N concentration. The decrease in photosynthetic assimilation at elevated CO_2 levels may preclude the species from reaching a state where carbon is assimilated in excess of growth requirements. The non-response in TNC and polyphenolic concentrations in this species may therefore not be surprising.

Generally, chemical composition in grass species studied here showed expected limited response to CO_2 enrichment (Poorter *et al.* 1997). The limited available data for TNC and N content in C_4 grass species confirm this expectation (Wand *et al.* 1999). These findings may be due to an actual lack in response, or as a result of a lack in statistical power owing to low replication (Norton *et al.* 1999). The high degree of variability within data sets may have caused non-significance within treatments that may have resulted in type II errors. This type of variability may be due to soil heterogeneity at the different down slope positions

and edaphic factors that are not accounted for. Furthermore, Stock *et al.* (submitted) showed that the treated site had higher soil moisture (possibly due to a feedback effect of CO_2 on plant water use) relative to the more up-slope positions. This differential soil moisture content might also be a complicating factor that might have masked some of the CO_2 effects. It is also possible that these results may reflect long-term acclimation to enhanced atmospheric CO_2 concentration since the site has been exposed to enhanced CO_2 for more than 70 years. Effects often seen in short-term laboratory based experiments may therefore not be detected.

In conclusion, this data suggests that elevated c_a will induce little change in chemical composition and hence quality in C_4 and C_3 grass species, and where changes do occur they are species-specific. Biomass (in Stock *et al.* submitted) and phytochemistry results indicate that C_4 and C_3 species of the Bongwan grassland do not experience source-sink imbalances imposed by elevated atmospheric CO_2 enrichment. Finally, there are still gaps in our understanding of the effects of elevated CO_2 on *in vivo* mineral nutrition, and on how plant mineral nutrients mediate responses at elevated atmospheric CO_2 concentration.

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CHAPTER 3 - The effects of elevated CO$_2$ on leaf polyphenol and tannin production as well as chemical composition in three fynbos species

3.1. Introduction

It is widely reported that responses to elevated c_a are amplified when other resources, particularly water, nutrients and light are unlimited (Bazzaz 1990). Conversely in nutrient poor environments, an increase in carbon supply could alter carbon allocation patterns especially C:N ratios, C:P ratios and carbon-based secondary compound production (Stock & Midgley 1995). The Fynbos biome is one such nutrient limited environment characterised by acidic soils and low concentrations of soil N and P (Richards, Stock & Cowling 1997).

The Cape Floristic Region, of which the Cape fynbos shrubland forms a considerable part, is amongst the most species rich communities in the world (Cowling & Holmes 1992), and hence its ecological importance. The term fynbos is used to describe six distinct plant communities that include Grassy, Asteraceous, Restiod, Ericaceous, Proteiod and Closed-scrub fynbos (Figure 3.1). Any alterations in atmospheric CO$_2$ concentration (the substrate for photosynthesis), which lead to enhanced growth, allocation and reproduction (Bazzaz 1990), may disrupt the sizes, productivities and balance between the various plant populations comprising this important plant kingdom. Since such

changes could lead to substantial changes in biodiversity, it is important

to understand the physiological responses of representative species to

CO_2 enrichment.

Group   Series

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                                        ┌──────────────────┐
                                ┌───────│   Grassy fynbos   │
                                │       └──────────────────┘
                                │       ┌──────────────────┐      ┌──────────────┐
                                ├───────│ Asteraceous fynbos│──────│  F. aethiopica │
                                │       └──────────────────┘      └──────────────┘
                                │       ┌──────────────────┐
┌──────────────┐               ├───────│   Restioid fynbos │
│ Cape fynbos  │               │       └──────────────────┘
│  shrubland   │───────────────┤       ┌──────────────────┐
└──────────────┘               ├───────│ Ericaceous fynbos │
                                │       └──────────────────┘
                                │       ┌──────────────────┐      ┌──────────────┐
                                ├───────│  Proteoid fynbos  │──────│ L. laureolum │
                                │       └──────────────────┘      │ L. xanthoconus│
                                │       ┌──────────────────┐      └──────────────┘
                                └───────│  Closed scrub     │
                                        │     fynbos        │
                                        └──────────────────┘
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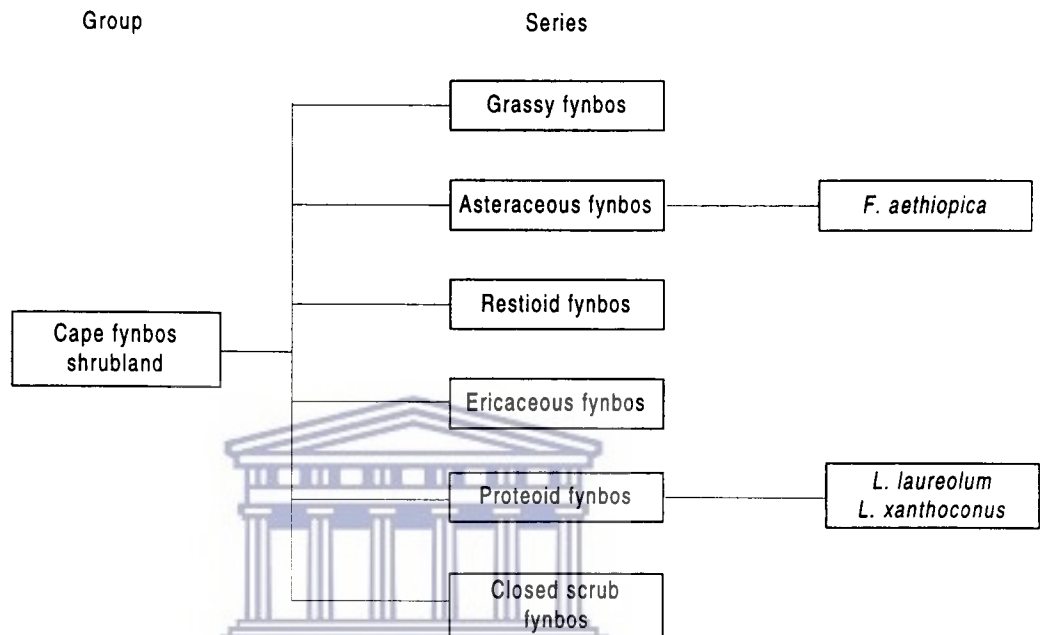
Figure 3.1 Classification of Cape fynbos shrubland based on Campbell (1985) as illustrated in Cowling & Holmes (1992) indicating to which series *Felicia aethiopica*, *Leucadendron laureolum* and *Leucadendron xanthoconus* belong.

The source-sink balance hypothesis which is an extension of the carbon-nutrient balance hypothesis (Bryant *et al.* 1983) proposes that carbon supply in excess of growth requirements could lead to the production of carbon-based secondary compounds in plants (Peñuelas & Estiarte 1998). In a review, Peñuelas & Estiarte (1998) showed that despite negative and non-response to elevated CO_2 in some studies,

there was a general trend towards increasing polyphenol and tannin concentration with increases in c_a, especially under low nutrient and N-limited conditions. These findings are in agreement with the carbon-nutrient balance hypothesis.

The aim of this study was to test the source-sink balance hypothesis under CO_2 enrichment in the nutrient limited Cape Fynbos shrubland by investigating carbon allocation patterns to carbon-based secondary compounds, and to ascertain c_a effects on chemical composition (nitrogen, phosphorus and total non-structural carbohydrates) in three selected fynbos species.

3.2.   Materials and Methods

3.2.1. Species selection and Experimental design

Proteaceae species dominate the Cape Floristic region and are amongst the few indigenous fynbos groups able to form dense overstoreys, typically 1.5 to 2 m in height. The size of these species leads to a dominance of its specific location, limiting light penetration, and hence inhibiting the commonly high plant species diversity of fynbos communities (Midgley *et al.* 1995). If these species should benefit from an increase in CO_2 level, it could have dramatic ramifications for the future of biodiversity in this system. A group of the *Proteaceae* family,

Leucadendron (Figure 3.1), with its wind dispersed seeds and consequent potential for range expansion, could be favoured if its members benefit from CO_2 fertilization. We therefore chose to select two well-studied members of this group, *Leucadendron laureolum* and *Leucadendron xanthoconus*, as well as *Felicia aethiopica*, a member of the *Asteraceae* family (Figure 3.1) as specimens in this study. *Felicia aethiopica* was selected due to its reliance on mycorrhizal symbiosis for nutrient acquisition in its roots, while the two *Leucadendron* species were non-mycorrhizal and formed cluster roots, with rootlets that are heavily covered with long root hairs (Allsopp & Stock 1993).

Eighteen growth chambers containing plastic refuse bins (50 cm diameter, 80 cm high) with removable polyethylene cylinders on top were used in this experiment. Nine of the chambers received ambient (360 ppm) CO_2 whereas the rest were subjected to ambient + 350 ppm CO_2 conditions. Each chamber contained *L. laureolum, L. xanthoconus, F. aethiopica, Salvia africana-lutea* and *Podalyria sericea* which were competitively grown at a constant plant density of 38 plants m^{-2}, i.e. 30 plants per bin in an area of 0.78 m^2. The soil medium was typically low nutrient acid sands of the fynbos biome. Plants were randomly arranged and rotated so that they were at different aspects in different containers. Plants were regularly watered to prevent water stress, and summer temperatures were regulated to not exceed 34 °C. After a two-year experimental period, above and below ground plant parts were harvested

and dried at 70 °C. Before chemical analysis, leaves were finely ground in a Wiley mill to pass through a 0.5 mm mesh sized sieve and stored in tightly capped pill vials.

3.2.2. Chemical analysis

Ground leaf material was analysed for total nitrogen (N), total phosphorus (P), total carbon (C), total non-structural carbohydrates (TNC), total polyphenols, condensed tannins and protein precipitating tannins using methods described in chapter 2.

3.2.3. Statistical analysis

Due to the non-normal distributions of proportions and percentages, as well as to account for possible skewed data as a result of small sample size, C:N and C:P ratios were ln-transformed and percentage N, P, TNC, total polyphenols, condensed tannins and protein precipitating tannins were arcsine-transformed to improve normality and homogenize variance.

Multivariate GLM and Tukey's multiple range tests were performed as indicated in section 2.2.3.

3.3. Results

There was no interaction in all three species between CO_2 effect and chemical variables investigated here (Table 3.1, $P > 0.2$).

Nitrogen

At a species level, *F. aethiopica* showed a significantly higher N concentration than the *Leucadendron* species (Table 3.1, Figure 3.2), which in turn had similar N concentrations (Figure 3.2). Nitrogen showed a general decrease with CO_2 fertilization (Table 3.1). Nitrogen concentration in *L. xanthoconus* and *L. laureolum* showed a reduction at elevated CO_2 concentration (Table 3.1, Figure 3.2, 23%, $F_{1,29} = 12.952$, $P = 0.001$ and 32% $F_{1,29} = 8.476$, $P = 0.007$ respectively). Nitrogen concentration in *F. aethiopica* decreased by 14% and approached significance ($P = 0.057$).
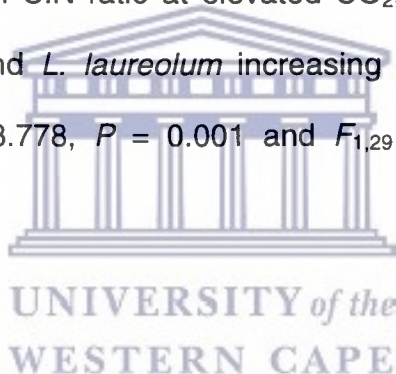
TNC

The *Leucadendron* species had significantly higher TNC concentrations than *F. aethiopica* (Table 3.1, Figure 3.2). Elevated CO_2 resulted in a general increase in TNC concentration (Table 3.1). Total non-structural carbohydrates showed a non-significant increase of 33% in *L. xanthoconus*, and a significant increase of 50% in *L. laureolum* (Table

3.1, Figure 3.2, $F_{1,29}$ = 1.543, P = 0.221 and $F_{1,29}$ = 6.200, P = 0.019 respectively).

C:N ratio

Owing to the low N concentration in the *Leucadendron* species, C:N ratio in these species was considerably higher than that of *F. aethiopica* (Table 3.1, Figure 3.2). Higher CO_2 concentrations resulted in an overall increase in C:N ratio (Table 3.1). Both *Leucadendron* species showed significant increases in C:N ratio at elevated CO_2, with *L. xanthoconus* increasing by 33% and *L. laureolum* increasing by 134% (Table 3.1, Figure 3.2, $F_{1,29}$ = 13.778, P = 0.001 and $F_{1,29}$ = 5.753, P = 0.023 respectively).

Phosphorus

Phosphorus concentration was significantly higher in *F. aethiopica* relative to the two *Leucadendron* species, which showed similar P concentrations (Table 3.1, Figure 3.2). Elevated CO_2 caused a cumulative decrease in P concentration (Table 3.1). Phosphorus concentration within species was not significantly affected by elevated CO_2 except in *L. laureolum*, which showed a significant decrease of 31% (Table 3.1, Figure 3.2, $F_{1,29}$ = 8.476, P = 0.007).

C:P ratio

The *Leucadendron* species showed a significantly higher C:P ratio relative to *F. aethiopica* (Table 3.1, Figure 3.2). Elevated CO_2 resulted in a general increase in C:P ratio (Table 3.1), but did not affect C:P ratio within species ($P > 0.05$).

Total Polyphenols

Total polyphenol concentrations in the *Leucadendron* species were similar, whereas these species were in turn significantly higher than that of *F. aethiopica* (Table 3.1, Figure 3.2). Elevated CO_2 did not affect total polyphenol concentration within species except in *L. laureolum*, where a significant increase of 35% was observed (Figure 3.2, $F_{1,29} = 8.207$, $P = 0.008$).
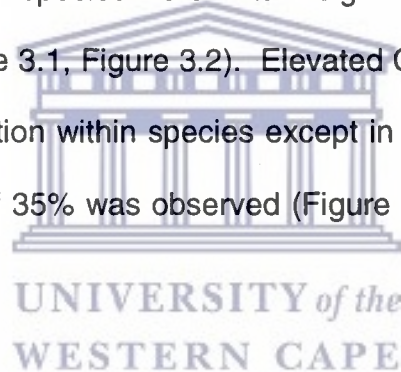
Condensed Tannins

At a species level, *L. xanthoconus* was significantly higher than *L. laureolum*, which in turn was significantly higher than *F. aethiopica* (Table 3.1, Figure 3.2). Condensed tannins were significantly affected by elevated CO_2 only in *L. laureolum* where a 33% increase was detected (Figure 3.2, $F_{1,29} = 5.990$, $P = 0.021$).

Protein-precipitating Tannins

Protein-precipitating tannins were found only in the *Leucadendron* species. These species showed no significant differences between species (Table 3.1, Figure 3.2). Elevated CO_2 induced a general increase in protein-precipitating tannins, however, this increase was significant only in *L. laureolum* (Figure 3.2, 33%, $F_{1,29}$ = 10.456, P = 0.003).

Table 3.1 Multivariate ANOVA testing the effects of CO_2, species and the interaction between these factors on N, TNCs, C:N ratio, P, C:P ratio, total polyphenols and condensed tannins in three fynbos shrubs grown at two different CO_2 treatments. A separate Univariate ANOVA was performed for protein precipitating tannins in the two *Leucadendron* species. Values are *F* values.

	CO_2 effect	Species effect	CO_2 x species
N	17.083***	87.965***	0.978 [NS]
TNCs	6.558*	16.950***	0.134 [NS]
C:N ratio	11.821***	55.459***	1.246 [NS]
P	5.331*	30.116***	0.680 [NS]
C:P ratio	4.728*	29.864***	0.705 [NS]
Total polyphenols	1.653 [NS]	27.446***	1.416 [NS]
Condensed tannins	0.347 [NS]	100.02***	1.593 [NS]
Protein precipitating tannins	8.294**	0.091 [NS]	2.974 [NS]

Significant differences at ***$P < 0.001$; **$P \leq 0.01$; *$P < 0.05$; NS = not significant *F* values
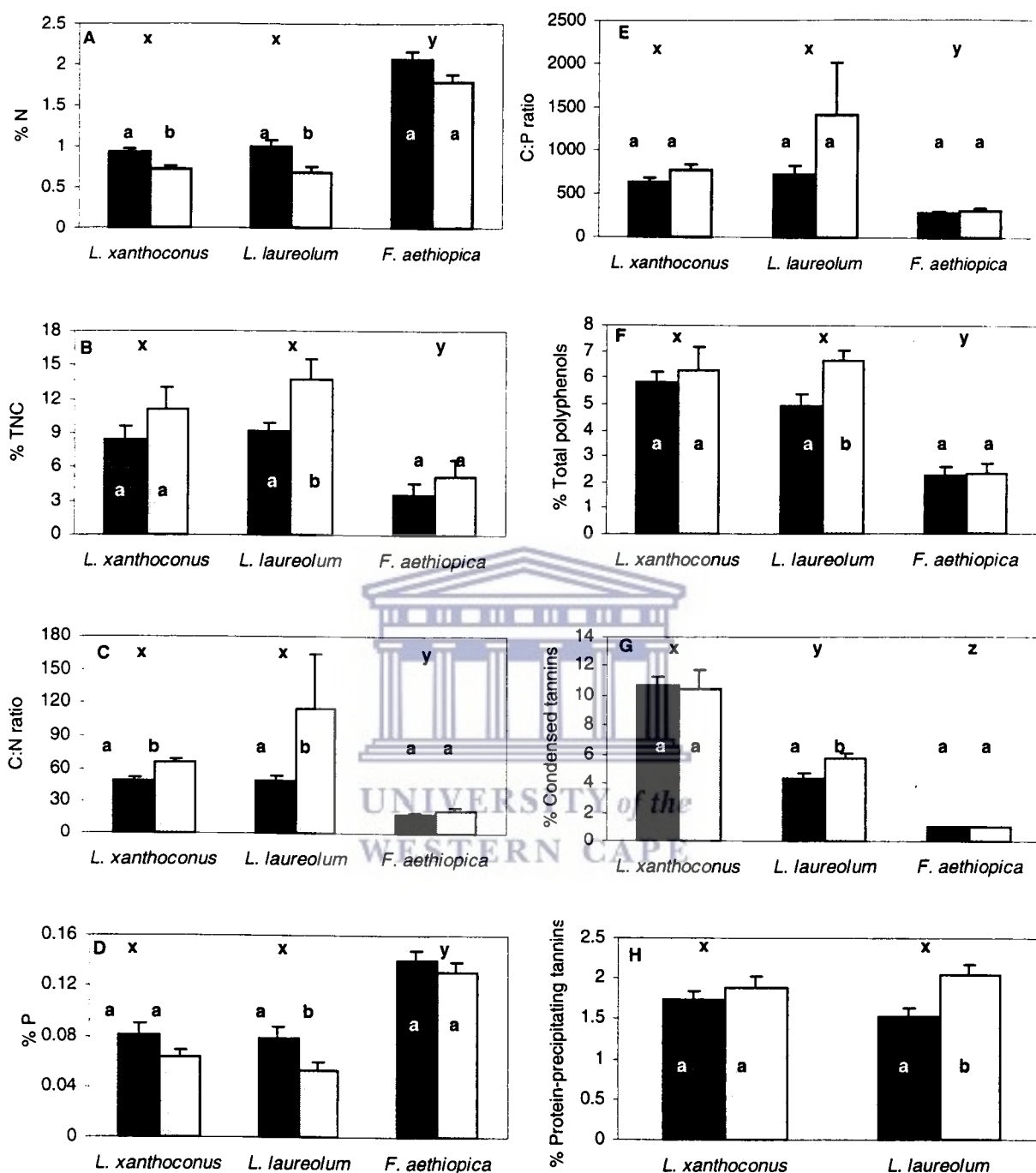
Figure 3.2 The effects of CO_2 level (ambient *solid column* and elevated *dotted column*) on percentage nitrogen (A), percentage total non-structural carbohydrates (B), C:N ratio (C), percentage phosphorus (D), C:P ratio (E), percentage total polyphenols (F), percentage condensed tannins (G) and percentage protein precipitating tannins (H) in *L. xanthoconus* (n=15), *L. laureolum* (n=15) and *F. aethiopica* (n=10) leaves. Values are means ± SE. Different letters (*x, y* and *z*) indicate significant differences between species and (*a* and *b*) indicate significant differences within species ($P < 0.05$).

3.4. Discussion

Consistent with general findings of plant responses to CO_2 fertilization, results showed a general decrease in N and P concentrations and a general increase in TNC concentration and C:N and C:P ratios (Table 3.1). Additionally, protein precipitating tannin concentrations in the *Leucadendron* species showed a general increase.

The Asteraceous *F. aethiopica* was unaffected by elevated CO_2. Nitrogen and P concentrations were higher in this species relative to the *Leucadendron* species. *Felicia aethiopica* is a mycorrhizal species and theoretical principles governing mycorrhizal symbiosis may predispose the allocation of photosynthates to mycorrhizas (Saxe *et al.* 1998), which could facilitate the uptake of growth limiting nutrients in infertile fynbos soils. This postulation is supported by Stock & Allsopp (1992) who noted that mycorrhizal symbiosis retards seedling development by imposing severe nutrient and carbon demands on seedling development in certain fynbos species (especially during the infection phase). Furthermore, mycorrhizal colonization is often higher on roots grown under P limited conditions and elevated CO_2, which may be due to photosynthate allocation to roots, a condition often associated with low root nitrogen concentration (Lewis *et al.* 1994) as experienced by shrubs studied here. However, mycorrhizal root colonization response is not always positive

as Rillig *et al.* (1999) and Rillig *et al.* (1998) showed species-specific responses in grasslands. Mycorrhizal forest trees grown under elevated CO_2 conditions also showed a general increase in root carbohydrate concentration, though evidence that mycorrhizal colonization and abundance is enhanced as a result of CO_2 enrichment is fragmented in forest systems (Saxe *et al.* 1998). The allocation of assimilated sugars to mycorrhizal maintenance (Salisbury & Ross 1992) may then explain the relatively low TNC concentration observed in *F. aethiopica*. Therefore, it may be that mycorrhizal demand for carbon and other nutrients may hinder elevated CO_2 response in this species. However, the effect of P concentration on biomass and mycorrhizal colonization and abundance is varied (Saxe *et al.* 1998), which suggests that P concentration may not be the determining factor in mycorrhizal plant response to elevated CO_2. Therefore, the low TNC concentration, relatively high N and P concentrations and overall non-response of *F. aethiopica* to elevated CO_2 may merely be an artefact of the plants ontogenic growth mechanism.

Many authors attribute the decrease in nitrogen content in elevated CO_2 grown plants to enhanced nitrogen use efficiency in plant leaves (Bowes 1993, Saxe *et al.* 1998, Davey *et al.* 1999, Tognetti & Johnson 1999). This is supported by Midgley *et al.* (1995) who found small increases in CO_2 responsiveness (mass at 700 ppm/mass at 350 ppm) for three (including *L. laureolum* and *L. xanthoconus*) of the four *Leucadendron* species studied at low nutrient supply and proposed

increased NUE as explanation. Taking this study a step further, Midgley *et al.* (1999) clearly showed an increase in photosynthetic nitrogen use efficiency with increase in CO_2 level and low nutrient concentration in both *L. laureolum* and *L. xanthoconus*. However, they did not find any change in N concentration due to elevated CO_2 at low nutrient supply in either species. Contrary to these findings, nitrogen was reduced in both *L. xanthoconus* and *L. laureolum* studied here. Nitrogen also showed a general decrease with CO_2 enrichment. Long *et al.* (1996) reported that plants grown in N limited conditions showed a decrease in Rubisco concentration, which in many species is coupled with a change in RuBP regeneration capacity. Theoretical principles governing photosynthetic carbon fixation suggest that this change in RuBP regeneration capacity is manifested in a reduction. Leaf N content and photosynthesis show a strong positive correlation across many species (Evans 1989, Woodward 1990, Saxe *et al.* 1998). Midgley *et al.* (1999) confirmed this in four *Leucadendron* species at ambient and elevated CO_2 concentrations, further showing that both carboxylation efficiency and RuBP regeneration capacity is positively correlated with leaf nitrogen content. It is therefore proposed that the *Leucadendron* species studied here experience acclimation imposed by soil N limitation.

Response in P concentration under elevated CO_2 is expected to mimic N response (Bowes 1993), i.e. it should show a decrease with increase c_a. As with N, P concentration showed a general decrease with

CO_2 fertilization (Table 3.1), however, *L. laureolum* was the only species that showed a significant decrease at elevated c_a. Midgley *et al.* (1999) showed that *L. xanthoconus* increased its starch but not sugar concentration at low P (a trait of low nutrient fynbos sands) and elevated CO_2. This reaction is consistent with *in* vitro studies, which showed that low levels of cytosolic orthophosphate (P_i) decrease the export of triose-P from the chloroplast to the cytosol, by limiting RuBP regeneration. This results in an increase in starch synthesis in the chloroplast (Terry & Rao 1991). Conroy *et al.* (1990) also showed that leaf starch concentration increased under CO_2 fertilization and low P in two pine species. This supports the suggestion that elevated CO_2 response in *L. xanthoconus* is limited by P availability. Indeed, Davis, Flynn & Midgley (1992) showed that an increase in biomass accumulation in *L. xanthoconus* was achieved by the addition of a balanced nutrient supply, than by N or P alone (Midgley *et al.* 1995).

Grown in the same low P soil medium, it was expected that *L. laureolum* experience a similar decrease in triose-P export from the chloroplast. However, contrary to findings for TNCs in this experiment, Midgley *et al.* (1999) showed a non-significant increase in both starch and sugar concentration in *L. laureolum*. They further showed a marked decrease in Rubisco activity and RuBP regeneration capacity in *L. laureolum*, which signalled down-regulation. This down-regulation involves an increase in sucrose and glucose (as found in TNC

concentration here) that can repress *rbcs*, the nuclear gene that codes for the small subunit of Rubisco (Long *et al.* 1996). Thus low cytosolic P_i, which result from low P availability, may be the indirect cause of down-regulation in this species. Furthermore, P deficiency, as experienced by *L. laureolum*, leads to an increase in unphosphorylated carbon compounds (starch, sucrose and glucose) and may have increased non-structural carbohydrates (Terry & Rao 1991).

Despite being significant only in *L. laureolum*, TNC concentration showed a general increase at elevated CO_2, which is consistent with empirical data (Poorter *et al.* 1997, Poorter & Pérez-Soba 2001). Midgley *et al.* (1999) showed that the maximum light-saturated rate of net CO_2 uptake (A_{max}) at low nutrient supply increased in both *Leucadendron* species under investigation in this experiment. They found that A_{max} was similar at ambient CO_2 concentration in both species, but that the increase in A_{max} for *L. xanthoconus* at double ambient CO_2 was two-fold higher than that of *L. laureolum*. Down-regulation mediated by low soil P and N availability in *L. laureolum* may therefore explain the higher concentration in TNCs found here.

Increase in C:N ratios as a consequence of decreases in N concentration (Tognetti & Johnson 1999) in the *Leucadendron* species is consistent with widely reported observations (Poorter *et al.* 1997, Peñuelas & Estiarte 1998, Saxe *et al.* 1998, Tognetti & Johnson 1999). However, Midgley *et al.* (1999) showed that N concentration was not

affected by increased CO_2 at low nutrient supply, implying that C:N ratio would not be affected in the *Leucadendron* species studied. The limitation imposed on protein synthesis by a low N supply in the presence of carbohydrates in excess of growth requirements (manifested by an increase in TNC concentration), found here in *L. laureolum*, may have availed phenylalanine from the shikimate pathway to polyphenolic synthesis (Lambers 1993, Peñuelas & Estiarte 1998, Jones & Harley 1999). From the TNC data shown by Midgley *et al.* (1999) for *L. laureolum*, it is reasonable to assume that the increase in TNCs found here represents an increase in both starch and sugar concentration. This would present sucrose levels higher than required for protein synthesis. Lambers (1993) proposed two models by which phenolics may be synthesised under these conditions: 1) sucrose in excess of protein requirements may then be diverted to phenolic synthesis, or 2) protein synthesis may not be able to keep pace with the rate of phenylalanine production, availing the precursor for secondary metabolite synthesis. These predictions are consistent with the carbon-nutrient balance hypothesis (Bryant *et al.* 1983). Furthermore, it should be noted that the decrease in phosphate concentration will indirectly affect the rate of protein synthesis (Lambers 1993), and the cumulative effect of the decrease in P and N concentrations shown here for *L. laureolum*, is expected to affect the accumulation of secondary compounds. It is therefore not surprising that polyphenolics and tannins increased in *L.*

laureolum. However, the hypothesis does not hold for *L. xanthoconus* that showed a decrease in N, increase in TNCs and a decrease in C:N ratio, but no increase in polyphenolics and tannins.

Consistent with the carbon-nutrient balance hypothesis (Bryant *et al.* 1983), it has been shown that nutrient paucity, which decreases carbon demand for growth, promotes the accumulation of TNCs (as found in this study) in the leaves of plants (Lambers 1993, Peñuelas & Estiarte 1998), even at ambient CO_2 conditions (Stock & Midgley 1995). This accumulation of carbon in excess of growth requirements may be allocated to the synthesis of carbon-based secondary compounds (Bryant *et al.* 1983, Lambers 1993, Stock & Midgley 1995, Peñuelas & Estiarte 1998, Tognetti & Johnson 1999, Kuokannen *et al.* 2001). Indeed the increase in TNC concentration in *L. laureolum* appears to be related to an increase in polyphenolic compounds in this species. This finding is consistent with general observations for species grown under low availability of N and other nutrients (Lambers 1993, Peñuelas & Estiarte 1998). It also corroborates the source-sink balance hypothesis (Peñuelas & Estiarte 1998) especially since Stock (in proceedings of Medecos 2001) reported that none of the species studied here showed any gain in biomass under increased CO_2 level, which suggest a sink limitation. Midgley *et al.* (1995) also showed that biomass was unaffected in *L. laureolum* and *L. xanthoconus* grown at low nutrient supply and elevated CO_2 levels, but found that the addition of nutrients

had a significantly positive effect on biomass in *L. xanthoconus*. Therefore it is reasonable to assume that low soil N is the primary limiting factor in protein synthesis and thus growth in *L. xanthoconus*. In the case of *L. laureolum*, genetically controlled growth mechanisms may govern biomass production.

Interestingly, Midgley *et al.* (1999) showed an increase in starch concentration but no change in sugar concentration for *L. xanthoconus* grown at elevated CO_2 and low nutrient supply. They also showed that carboxylation and RuBP regeneration capacity was negatively correlated with starch concentration, implying that increase in starch resulted in a decrease in photosynthesis. Indeed, starch storage has been reported to represent excess photosynthates (Salisbury & Ross 1992), and an increase in starch grains in the chloroplast is reported to press thylakoids unusually close together, thereby physically preventing light from reaching the thylakoids and causing photosynthesis. This may be the underlying mechanism in the observed negative correlation between starch concentration and carboxylation and RuBP regeneration capacity found by Midgley *et al.* (1999).

Increases in the concentrations of protein precipitating tannins, condensed tannins and total polyphenolics in *L. laureolum* were similar, suggesting that the biological activity that accounts for the protein precipitation effect appears to be associated with the condensed tannin group (Stock, Le Roux & Van der Heyden 1993) and that condensed

tannins in turn are associated with the total polyphenolic group of compounds.

In conclusion, chemical response to elevated c_a followed generally observed trends, but the magnitude of change (if any) is species-specific. The findings of this experiment suggest that the source-sink balance hypothesis does not explain carbon allocation in all the species studied here. This is underscored by the differential response by the two *Leucadendron* species. The low growth rate and hence low sink demand in the species studied here, preclude its response to CO_2 fertilization (Stock & Midgley 1995). Furthermore, these results suggest that the availability of N and P in soil have a pivotal role in carbon fixation and allocation, and that genetic and biotic traits may dictate elevated CO_2 response in these species. Finally, these results suggest that elevated atmospheric CO_2 concentration could alter ecosystem processes by altering C:N ratio, C:P ratio, and polyphenolic and tannin concentrations in the leaves of the *Leucadendron* species studied here.

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CHAPTER 4 - Synthesis

This study has shown that changes in chemical composition at elevated atmospheric CO_2 concentration in C_3 and C_4 species are species-specific. Changes in chemical composition were not limited to the often reported N and TNC concentrations, but the C_3 grass *A. semialata* and *L. laureolum* showed decreases in P concentration at elevated CO_2. Both these species experienced down-regulation at elevated CO_2 (results for *A. semialata* in Wand (1999) and for *L. laureolum* in Midgley *et al.* (1995)) and the role of P nutrition in photosynthetic down-regulation, particularly its importance in the regeneration of RuBP in the carboxylation step, suggests that not only N but also P nutrition may have a role in controlling photosynthetic response at elevated atmospheric CO_2 concentrations. This result warrants further investigation into the role of P in photosynthetic down-regulation at elevated CO_2.

All C_3 species studied here experienced sink limitation manifested by a lack of enhanced growth at CO_2 concentrations above ambient (results for fynbos shrubs in Stock (proceedings of Medecos 2001) and for *A. semialata* in Wand (1999)). Despite being significant only in *L. laureolum*, this sink limitation was associated with an increase in TNC concentration and a decrease in N and P concentrations in fynbos shrub species. In *A. semialata*, the reported down-regulation was also associated with a non-significant increase in TNC and decrease in N

concentrations, whereas P concentration was significantly decreased at elevated CO_2. These results provide further evidence that N and P nutrition together may limit CO_2 responsiveness, though the effect of N may be more pronounced.

Grass species did not invest assimilated carbon into polyphenolic and tannin production at elevated CO_2. This is consistent with the source-sink balance hypothesis (Peñuelas & Estiarte 1998) as Wand *et al.* (1999) showed that grasses allocated assimilated carbon to biomass production, while Wand (1999) showed that grass species did not show any source-sink imbalance and invested carbon into growth. However, Wand (1999) also showed that positive biomass response was species-specific with *A. semialata* among those that did not show growth stimulation at elevated CO_2. This non-response was attributed to down-regulation.

Despite showing sink limitation in all fynbos species, only *L. laureolum* showed an increase in polyphenolic and tannin concentrations, which is thought to be an indirect effect of nutrient paucity rather than a direct effect of elevated CO_2 (Lambers 1993). The non-response in *L. xanthoconus* and *F. aethiopica* is contrary to the source-sink balance hypothesis. However, the two species did not show an increase in TNC concentration, which is integral to the theoretical basis on which this hypothesis is based. Total nonstructural carbohydrate results suggest these species did not accumulate carbon in excess of growth

requirements. However, Midgley *et al.* (1999) showed that the maximum light-saturated rate of CO_2 uptake in *L. xanthoconus* was twice that of *L. laureolum.* What then is the fate of assimilated carbon in *L. xanthoconus*? An in-depth investigation of carbon allocation at elevated CO_2 in this species may cast light on the fate of carbon assimilates.

Forage quality in the Bongwan grassland will generally not be negatively affected. In fact, Wand (1999) showed a 72 and 118% increase in biomass for *A. appendiculatis* and *T. triandra* respectively, and considering that these species did not show significant changes in chemical composition, the quantity of these grasses will be enhanced without any change in quality at elevated CO_2. However, *S. pyrmidalis* that showed a 50% increase in biomass (Wand 1999) showed a decrease in N and hence C:N ratio. Assuming that the significant increase in overall biomass at the elevated CO_2 site (Stock submitted) is not due to slope position, forage quantity of the Bongwan grassland will be enhanced without any change in forage quality with an increase in atmospheric CO_2 concentration.

Owing to marginal changes in chemical composition in the leaves, litter decomposition in Bongwan should not be affected. The increase in moisture due to CO_2 fertilization (as shown by Stock submitted) may in fact accelerate the rate of litter decomposition. However, as have been shown for chemical responses, theoretical principles formulated from

controlled studies cannot be directly applied in complex natural ecosystems.

The findings in fynbos species suggest that litter decay may be negatively affected in the fynbos biome. These predictions are based on increases in C:N ratios in *Leucadendron* species and an increase in polyphenolic and tannin concentrations in *L. laureolum*. The extent to which decomposition rate in *Leucadendron* species, particularly *L. laureolum*, will affect nutrient cycling within the fynbos biome is relative to the species composition of these species.

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