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THE EFFECTS OF ELEVATED ATMOSPHERIC CO₂
ON BELOW-GROUND PROCESSES IN FYNBOS
AND C₄ ECOSYSTEMS

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This thesis investigated ways in which elevated atmospheric carbon dioxide (CO₂) concentrations influence the plant:soil interface and soil biological processes. The studies focussed on microbial populations associated with the plant rhizosphere, and how these responses could feed into changes in the soil carbon pool. The study comprised three sections: (i) a single Fynbos species, pot-based investigation of *Felicia aethiopica* ssp. *aethiopica* grown in open top chambers in a glasshouse, (ii) a mixed species microcosm investigation of five representative Fynbos plants, including *F. aethiopica*, grown in open top chambers in a glasshouse and (iii) investigation of a natural CO₂ spring situated on a C₄ grassland slope in southern KwaZulu Natal. Long growth periods (between 1.5 and 2.25 years) coupled with natural, low-nutrient soils were used in the glasshouse studies to mimic natural growth conditions. The main questions that were answered through the course of this project were as follows.

- How does the growth, biomass and nutrient allocation in *F. aethiopica* change as a result of a doubling in the atmospheric concentration of CO₂?
- How does soil microbial biomass and community structure react to the indirect effects of the changed atmosphere?
- How are the flows of recently fixed carbon influenced by CO₂?
- Is it possible to distinguish between root and soil microbial respiration in *F. aethiopica* and are there any differences caused by CO₂ treatment?
- What is the long-term carbon storage potential of the two systems studied?

F. aethiopica growth was non-significantly reduced while few CO₂ treatment effects were observed on biomass or nutrient allocation patterns. There was a significant increase in leaf

herbivory among the CO₂ treated plants. Below-ground it was found that root growth remained constant while the elevated CO₂ treatment resulted in a significant 85% increase in soluble carbon in the soil. ¹⁴C labelling revealed the apparently contradictory observation of a reduction in root exudation of labelled products. This was reconciled by the observation that soil microbial biomass was slightly reduced under elevated CO₂ implying that carbon may not be the limiting resource in the Fynbos soil system. The soil microbial community structure was found to remain constant with CO₂ treatment in the single species experiment while there was a clear, and significant change in the structure in the mixed species experiment implying that an increase in the complexity of the study system allowed for a change in the soil microbial community. It was found that the soil microbes were generating approximately 90% of the recently fixed CO₂ being released from the soil however there was no measurable CO₂ treatment effect. This high value shows that a large proportion of the carbon being respired was passing through a soil intermediate phase meaning that small changes in the root-microbial relationship could have large impacts on the carbon flows within the soil. It was concluded that it is unlikely for the Fynbos system to become a significant sink of carbon, due to the plants' small stature and the low-carbon, low-nutrient soils. It is, however, still important to ascertain the response of this system to global change due to the Fynbos's value as a repository of tremendous biological diversity.

There was a significant accumulation of soil moisture in the C₄ grassland, particularly during periods of low rainfall. There was a slight (non-significant) accumulation of soil carbon after a long period (70+ years) of CO₂ treatment while there were no long-term effects on the soil microbial biomass or soluble carbon in the soil. Three years of treatment using a crude fumigation ring resulted in a moisture effect alone. It was concluded that more intensive sampling was required in order to elucidate seasonal and climatic changes in the grassland soil system. It was predicted that there could be moderate accumulation of carbon in the soil but it was not as dramatic as is frequently predicted from short-term, well-resourced model studies.

It was found that many of the responses to CO₂ treatment were subtle and difficult to measure in these natural and semi-natural experiments. This was due to low responsiveness combined with the rather crude analytical methods currently available. These subtle effects are in agreement with current thought proposing that natural systems are frequently less responsive to global change than was predicted from short-term laboratory based studies.

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INTRODUCTION

Introduction

This thesis investigated ways in which elevated atmospheric carbon dioxide concentrations influence the plant:soil interface and soil biological processes. The studies focussed on elevated carbon dioxide (CO₂) effects on soil microbial biomass and microbial populations associated with plant roots, and how these relationships could potentially feed into responses of the soil carbon pool. The study comprised three sections. The first component was a single species, pot based investigation of *Felicia aethiopica* (Burm.fil.) Bol & Wolley Dod ex Adams & Salter ssp. *aethiopica* grown for nearly three years in open top chambers to see how the isolated plant and its associated soil microbial communities respond to CO₂ treatment. The second component was a mixed species microcosm investigation of representative Fynbos plants, also grown in open top chambers, allowing comparison of the single species results with the more complex mixed species system. The third section investigated below-ground processes as part of a collaborative research initiative centred around a natural carbon dioxide spring situated on a C₄ grassland slope in southern KwaZulu Natal.

An increasing atmospheric carbon dioxide concentration

The current state of knowledge regarding human caused global change is extensively reviewed in three *Climate Change 2001* reports compiled by the Intergovernmental Panel on Climate Change (IPCC; Houghton *et al.*, 2001). Since 1750 the atmospheric CO₂ concentration has increased from 280 $\mu\text{mol}\cdot\text{mol}^{-1}$ to 367 $\mu\text{mol}\cdot\text{mol}^{-1}$ in 1999. It is quite certain that the current atmospheric concentration has not been exceeded in the past 420 000 years and it is believed that these CO₂ levels have not been seen during the past 20 million years. Furthermore the current rate of change in CO₂ concentration has not been exceeded in the past 20 000 years (Houghton *et al.*, 2001). Human industrial development and population growth have very high demands for energy and food production which has

resulted in extensive fossil fuel burning and land use changes that have caused the atmospheric changes seen in the past century (Houghton *et al.*, 2001).

The *Amsterdam Declaration on Global Change*, signed in July 2001, provides a clear warning that human activities are having an effect on earth equal to that of the great forces of nature in their extent and impact (Canadell & Noble, 2001). The declaration concludes by highlighting the critical issues that need to be faced in order to prevent the planet from becoming uninhabitable. This is of relevance to this thesis due to the vital need for scientific data on the responses of all of the earth's systems to human induced perturbations, not just highly productive agricultural systems. Sadowsky & Schortemeyer (1997) showed that there are conflicting results in soil microbial responses to global changes and they attribute these differences to either (i) the specificity of the response being dependent on the particular plant species, community or ecosystem being investigated or (ii) the lack of sensitivity in the methodologies used. In both cases the urgent need for more work in the field is emphasised. Scholes & van Breemen (1997) reviewed the effects that global changes may have on tropical ecosystems, i.e. areas where low temperatures are not a primary constraint on ecosystem function and they noted that few studies have been done in these regions. They also highlighted the problems associated with applying results from temperate systems to these, very different, systems. Radiant energy inputs are usually very high while soils are often nutrient deficient and so tropical systems are frequently nutrient- rather than carbon-constrained (Scholes & van Breemen, 1997). The rate limiting step is often decomposition (nutrient release) and so it is vital to gain an understanding of how decomposers respond to atmospheric changes as well as looking at the changes occurring at the root:soil interface (rhizosphere) since this zone might be responsible for the control of many plant processes.

The Cape Floristic Region and atmospheric change

Why is the Cape Floristic Region special?

The Cape Floristic region is a phylogenetically, structurally and functionally distinct flora occurring in the Mediterranean climate zone on the southern and western coasts of South Africa (Linder *et al.*, 1992, Stock & Allsopp, 1992). The dominant vegetation type is Fynbos, a sclerophyllous evergreen shrubland adapted to frequent fires and the leached, low nutrient soils found in the area (Richardson *et al.*, 1995). Plant species diversity is remarkably high with over 8500 species occurring in the Cape Floristic Region, 68% of

which are endemic (Bond & Goldblatt, 1984). In a hierarchical classification of global floristics, the Cape Flora is defined as a floral kingdom, equivalent to vast areas of the earth represented by the other kingdoms such as the Holarctic, Paleotropic and Neotropic regions (Bond & Goldblatt, 1984). The beta and gamma diversity in the landscape is particularly high (Cowling *et al.*, 1992) while the structural diversity in this vegetation is quite low (Campbell, 1985). Tissue turnover is very low and above ground litter is very slow to decompose (Mitchell *et al.*, 1986) meaning that above ground sources of carbon for soil microbes is very limited. The photosynthetic rates of the sclerophyllous vegetation can be quite low (van der Heyden & Lewis, 1989), but a large amount of carbon can be fixed over the lifespan of the leaf due to its longevity. Due to the slow growth rate, much of the fixed carbon is not used in vegetative growth and is thus available for other functions such as the construction of fire resistant seed-pods or it may be allocated to meeting the carbon requirements of mycorrhizas (Allsopp, 1992). If this is the case, then the carbon could also be available to support other soil microbial activities.

Predictions of Mediterranean ecosystem responses to changes

Houghton *et al.* (2001) reviewed many studies that have shown that some systems maintain increased photosynthesis while others down regulate completely under elevated CO₂. This effect persists through photosynthetic, allocation and mycorrhizal responses leading to the conclusion that each of the earth's systems behaves in a different, currently unpredictable, way. This highlights the need for data on the responses of systems other than well-resourced, agricultural models.

Many results of global change studies are from short-term experiments, performed on crop plants grown in optimum conditions using high nutrient or disturbed soils (Hu *et al.*, 1999, Schimel, 1995). Frequently, the experiments are undertaken on single plants grown in pots, which is not a good model to use when wanting to make predictions of how natural ecosystems will change under changed atmospheres (Cannell & Thornley, 1998). The problem, though, is that pot experiments allow for the greatest control of experimental conditions (Pitelka, 1994) along with the ability to economically increase replication which allows for meaningful statistical analysis.

For terrestrial plants following the C₃ photosynthetic pathway, net primary productivity is reduced by photorespiration (Farquhar *et al.*, 1980). It has been shown that an increase in atmospheric CO₂ concentration would relieve some of this limitation which

could result in changes in how plants grow and interact within their ecosystem (Houghton *et al.*, 2001). The effects may be quite subtle since CO₂ concentration has a smaller influence on plant photosynthesis and growth than other resources such as light and water (Bazzaz, 1990). The carbon dioxide issue is however still important because the atmospheric concentration is projected to be twice pre-industrial levels from the middle to the end of this century, depending on the projection models used (Houghton *et al.*, 2001). This is a very dramatic change in the growth environment of plants. As with the tropical systems discussed by Scholes & van Breemen (1997), the limiting factor in the Fynbos is nutrients and not carbon (reviewed by Stock *et al.*, 1992). The authors discuss ways in which excess carbon is allocated to plant secondary compounds, mechanical structures and non-structural carbohydrates. From the above evidence, one can see that there is a good chance of stimulated carbon gain in Fynbos plants under conditions of elevated atmospheric CO₂. Part of this increase could be released from the roots into the soil thus changing the rhizosphere environment.

Houghton *et al.* (2001) reviewed a number of studies that showed that low nitrogen availability does not consistently restrict plant responsiveness to CO₂ and suggested that it is important to investigate nutrient-poor systems to see if there are any generalised trends. Through the use of models, Cannell & Thornley (1998) have shown that the carbon acquisition potential of nutrient-poor systems may be relatively greater than that found in nutrient-rich systems. A key difference between these two systems is that under nutrient limitation a longer time is needed to re-establish equilibrium. Most investigations are completed before the end of this transient unresponsive state, a time when severe nitrogen limitation restricts plant growth. The model showed that in nutrient-poor systems nutrient leaching was lower and so over a long period of time the accumulation of nitrogen in the system would allow the plants to strongly respond to the environmental conditions (Cannell & Thornley, 1998).

Changes in root exudation can have an important influence on long-term responses to elevated atmospheric CO₂ in phosphorous limited ecosystems (Canadell *et al.*, 1996). The Fynbos is such a system, exposed to both soil nitrogen and phosphorus limitations (Richardson *et al.*, 1995). The need for below-ground investigations of responses of Fynbos plants to CO₂ treatment is thus demonstrated.

In terrestrial ecosystems elevated atmospheric CO₂ and climate change are expected to be significant threats to biodiversity along with more direct effects such as land use change and introduction of foreign species (Sala *et al.*, 2000). By using models and scenarios based

on expert opinion, the authors show that Mediterranean and grassland systems are probably most at risk due to their sensitivity to all of the drivers of biodiversity change investigated (land use change, climate change, biotic exchange, nitrogen deposition and atmospheric CO₂ change). These results highlight the urgent need for studies of the effects of these changes on Mediterranean systems. Richardson *et al.* (1995) concludes that much of the species diversity in the Cape region allows for community and landscape stability with the species providing a back up in times of stochastic recruitment failures. It is vital to know if this back up function would continue in a human altered world.

Actual studies of the responses of Mediterranean systems to CO₂

There are few global change studies of Mediterranean ecosystems despite their importance of being repositories of a great deal of biological diversity. For the south-western Cape, Midgley *et al.* (1999) showed that elevated CO₂ had positive, negative and neutral effects on a range of photosynthetic and leaf chemistry measurements of 4 species of *Leucadendron* (Proteaceae). In addition to this, *Leucadendron* spp. native to nutrient-poor, acid sands showed no response to CO₂ treatment at two nutrient supply rates while those species adapted to slightly richer soil environment were able to react to the extra atmospheric CO₂ in both nutrient treatments but the effect was still not statistically significant (Midgley *et al.*, 1995). Neither of the studies by Midgley looked at below-ground response, not even total root mass.

De Angelis *et al.* (2000) showed that the chemical composition of recently senesced litter changed after elevated CO₂ exposure. Initial decomposition of the litter was slower but after a year the remaining mass was not different. This investigation was made in an evergreen Mediterranean forest, but may be of some application to the current project in that it shows that above ground Carbon inputs are likely to be little changed especially considering the small stature and low litter production of the plants investigated.

The response to global changes of the Mediterranean grassland situated at the Jasper Ridge Biological Preserve has been extensively investigated. Lou *et al.* (1996) showed that below-ground respiration (the sum of root and heterotrophic respiration) was both strongly seasonal and influenced by elevated atmospheric CO₂ with the overall carbon budget being balanced. Increased net primary productivity was matched by increased respiration. Investigation of fungal root colonisation revealed that nonmycorrhizal infection was generally lower in both sandstone and serpentine annual grassland communities (Rillig *et al.*, 1999^a). Rillig *et al.* (1999^b) showed that the soil aggregate size and stability was increased in

both the grassland types present at the Jasper Ridge site. This change in the soil structure also occurred at the Sky Oaks CO₂ study in southern California, another Mediterranean climate study site (Rillig *et al.*, 1999^b). The increased soil aggregation is important for preventing erosion, allows carbon storage and can change the physical, chemical and biological properties of the soil.

At a gross level the soil microbial biomass was unchanged with 12 weeks of CO₂ treatment of the southern Californian plant, *Artemisia tridentata* under low nutrient conditions (Klironomos *et al.*, 1996). Upon more detailed investigation, mycorrhizal fungi increased while non-mycorrhizal fungi and bacteria did not respond to the CO₂ treatment. This shows that soil microbial responses are quite unpredictable and need to be investigated at a higher resolution than what is generally done at the moment. The Biolog™ Bacterial Identification System (Biolog Inc., Hayward, California) was used by Rillig *et al.* (1997) to show functional changes in the soil microbial biomass in the soil surrounding *Gutierrezia sarothrae* roots while the number of viable bacteria did not change. Dhillon *et al.* (1996) used the Biolog™ system and other techniques to investigate soil microbial responses in natural Mediterranean soil monoliths planted with *Bromus madritensis*. It was found that there were significant and substantial increases in most of the variables assessed (for example: microbial biomass, microbial activity, extra cellular enzyme activity and mycorrhizal infection). There was a non-significant increase in the total number of substrates utilised on the Biolog™ plates while evaluation of the chemical groups represented on the plates showed that significantly more amines and amides were metabolised by the microbes extracted from the treated monoliths.

Nijs *et al.* (2000) investigated the carbon fluxes through an old field microcosm planted with early successional Mediterranean plants. After three years of study, the monoliths tended towards being net CO₂ sinks although the carbon dynamics were largely influenced by the water relations of the monoliths, particularly early season soil saturation. Investigations by Hungate *et al.* (1996) at the Jasper Ridge research site revealed that after 4 seasons of growth at elevated atmospheric CO₂ there was no change in the soil stored carbon. They showed that this was largely due to a lack of statistical power in revealing small changes within a very large, heterogeneous soil carbon pool. Small, largely undetectable, changes in the carbon input into the soil can have significant large scale and long-term effects on the biosphere (Hungate *et al.*, 1996).

Natural CO₂ springs in Italy have also provided a useful resource to investigate how native herbaceous plants and evergreen trees will respond to global changes when grown

under Mediterranean conditions (Körner & Miglietta, 1994, Miglietta *et al.*, 1993). It was found that there were no gross effects of long-term CO₂ treatment on the plant size, growth rate or flowering time. There were, however, large differences in the tissue quality of the 40 species that were investigated, particularly a great increase in non-structural carbohydrate content of the leaves (Körner & Miglietta, 1994). This indicates that the photosynthetic systems of the plants have not completely down regulated and that there is more carbon being taken up by the system. The study did not extend beyond the leaf level and so predictions of below-ground responses are not possible.

Below-ground issues

Many authors highlight the lack of understanding of the fate of photosynthetically fixed carbon once it is deposited below the soil level (Rogers *et al.*, 1992, Sadowsky & Schortemeyer, 1997, Tinker & Mooney, 1996). This is due to the difficulty in measuring undisturbed root systems along with the complexity of the processes that need to be investigated (Tinker & Mooney, 1996). Generalisations of responses are difficult because it has been found that different systems and component species behave in quite different ways when exposed to elevated atmospheric CO₂ levels. (Tinker & Mooney, 1996, Scholes & van Breemen, 1997).

Despite the difficulties discussed above, Killham & Yeomans (2001) still stress that it is currently a very exciting time to be studying the rhizosphere environment of plants due to the number of investigative techniques which are currently being developed.

Root Growth

Elevated atmospheric CO₂ concentrations can have profound effects on root architecture, micromorphology and physiology (Rogers *et al.*, 1992). Furthermore, roots are the interface between the biosphere and lithosphere and therefore the processes occurring in this region must be understood in order to be able to predict plant, and ultimately biosphere, reactions to global changes (Rogers *et al.*, 1992). Canadell (1996) showed that there is a growing body of information indicating that elevated atmospheric CO₂ concentrations increases the amount of carbon allocated below-ground. This allocation can be in the form of increased root growth or root exudation, which can increase the ability of the plant to acquire nutrients or compete with its neighbours.

The rhizosphere is the region in the soil around a root that is influenced by the root (Bowen & Rovira, 1991). It is a dynamic region that has different dimensions depending on the prevailing environmental conditions. The rhizosphere's extent also varies with the nature of the compound being investigated. For example, secreted high molecular weight compounds diffuse a much shorter distance into the soil when compared to plant released gases such as ethylene. Understanding rhizosphere processes is vitally important because of their intimate linkage with nutrient cycling and organic matter decomposition which feed back to plant growth, soil carbon storage and ecosystem level changes (Cheng, 1999, Sadowsky & Schortemeyer, 1997).

Ten to twenty percent of the carbon fixed by a plant is released from the roots into the soil environment (Bowen & Rovira, 1991). This allocation to processes external to the root is especially large in plants native to semi-arid environments (Whipps, 1990). By using mathematical simulation models based on kinetic equations, Darrah (1996) showed the importance of non-sterile soil conditions with considerably more carbon released from the roots when grown in the presence of rhizosphere microorganisms. Under conditions of increasing atmospheric CO₂, the response often found among herbaceous plants is an increase in total carbon input into the rhizosphere of the plants (Lekkerkerk *et al.*, 1990, Cheng, 1999). Soil microorganisms appear to preferentially metabolise these easily decomposable root products and changes in exudates could cause a change in rhizosphere bacterial population size and structure (Lekkerkerk *et al.*, 1990). This idea is supported by Bowen & Rovira (1991) who found that organic material in the rhizosphere largely determines the types and numbers of microorganisms present. Whether these responses occur in other growth forms such as sclerophyllous shrubs needs further investigation.

Microbial reactions

O'Niell *et al.* (1987) cited a number of studies that have shown that rhizosphere populations respond to light, temperature, and foliar fertilisation while soil microbial functional diversity is influenced by the addition of simple organic substrates (Degens, 1998). From the observed changes in root exudation, one can infer that atmospheric CO₂ changes may well have an influence on soil microbial population structure.

The different methods used in below-ground investigation measure different aspects of the soil microbial population structure or its dynamics. One thus needs to be acutely aware of the limitations of the methods being used so that comparisons are not made between

incompatible data (Darrah, 1996). This is demonstrated by Sadowsky & Schortemeyer (1997) who distinguish among microbial biomass, microbial numbers and microbial activity in their review of soil microbial responses to increased CO₂ concentrations in the atmosphere.

Scientists now appreciate that below-ground processes and carbon flows will make a significant impact on the way in which ecosystems will respond to a changing atmosphere (See papers in the special issues of *Plant and Soil* volumes 165(1), 1994 and 187(2), 1996.). Progress has been hampered by the slow development of techniques that allow for the detailed analyses required to monitor the effects of changes in root exudation on the below-ground biology and biogeochemical cycling. A wide range of response of soil microbial populations has been observed and this can be attributed to the diversity of ecosystems investigated and experimental conditions used (Hu *et al.*, 1999). The authors assert that there is also strong time dependence in the microbial response and that most experiments are not run for long enough to allow the microbes to establish a new equilibrium.

Most global change studies have not determined the atmospheric CO₂ effects on the soil microbial biomass (Cheng, 1999). The few investigations that have measured this property have shown a general increase in soil microbial biomass under the influence of an elevated atmospheric CO₂. Sadowsky & Schortemeyer (1997) reviewed changes in microbial numbers and showed that there can also be temporal differences in the response depending on the season of sampling. This is probably due to the changes in the size of the active pool of organisms. Global change can affect soil processes either by changing the functioning of resident organisms or by fundamentally restructuring the community of organisms present (Schimel & Gullledge, 1998)

Authors rarely distinguish between root respiration and microbial respiration although there are important differences between these two processes (Canadell *et al.*, 1996). Microbial respiration relies on carbon substrate being released from the root and passing through a soil intermediate phase. In contrast, root respiration is a direct process occurring within the root. Lambers *et al.* (1996) predicted that when plants are grown under optimal conditions of water and nutrient supply elevated CO₂ does not change carbon partitioning nor root respiration per unit root mass although the overall respiration may be increased due to a larger root system. Canadell *et al.* (1996) suggested that under natural systems with a restricted nutrient supply, root respiration would remain constant or increase according to the relative effect on biomass partitioning between root and shoot and the total root biomass production.

The effects of changed atmospheres on the responses of soil microbial communities were recently reviewed by Hu *et al.* (1999). It is clear from this review that there are few studies that have explicitly looked at soil microbial community structure. The limited results available indicate that there are increases in mycorrhizal colonisation and rhizosphere bacterial numbers along with changes in the composition of the fungi and bacterial nutritional groups. Most of these early studies used fast growing plants in optimum growing conditions. The authors also highlighted the importance of distinguishing between active and inactive microbial pools since most soil microbes are in an inactive state (Hu *et al.*, 1999). The different investigative tools that are available often yield conflicting results due to the different techniques measuring different activity levels in the soil microbial population.

There is also a great need to link soil microbial community structure with the microbial functioning so that changes in microbial communities can act as a predictor of how other processes (such as soil carbon cycling) may be affected (Schimel & Gullledge, 1998)

Carbon flows into long-term storage

Merckx *et al.* (1987) found that less organic rhizodeposition was incorporated into microbial biomass in soils with low nutrient availability as compared to higher nutrient soils. It was suggested that nutrient limitation limited the microbial degradation of the organic products. If the microbes are unable to metabolise the predicted excess rhizodeposition under elevated CO₂ conditions then a significant accumulation of carbon in the soil could occur.

The balance between plant production and microbial decomposition is thought to be what controls the carbon storage of a system (Hu, *et al.*, 1999). Canadell *et al.* (1996) highlighted the significance of soils in carbon sequestration. It is not only the size of this organic pool but it is also the carbon fractions contained in the soil that have the longest residence times in the biological environment. Changes in these fractions would therefore have the longest lasting effects on the mitigation of increases in atmospheric carbon dioxide. In the IPCC report, Houghton *et al.* (2001) showed that terrestrial systems are currently a net sink for CO₂ in the atmosphere. The challenge now is to try to predict how long this sink will continue and whether it will become stronger as atmospheric concentrations of CO₂ increase. It is important to investigate soil microbial population changes because there are indications that changes in soil functioning can feed back to influence trace gas dynamics at ecosystem and larger scales (Schimel & Gullledge, 1998).

Most studies on responses to elevated atmospheric CO₂ concentrations have focussed on plant responses to the changes; but it is now apparent that the soil will provide important clues as to the likelihood of the terrestrial biosphere sequestering substantial amounts of carbon (Canadell *et al.*, 1996). The effect of microbial processes on the carbon balance of the soil is poorly understood due to the very complex nature of below-ground interactions among plant roots, microbes and soils within the soil food web (Hu *et al.*, 1999). What is known, is that elevated CO₂ has a range of effects on soil organic matter decomposition, from stimulating breakdown to retarding it and so Cheng (1999) concluded that CO₂ effects on soil organic matter are dependent on the plant-soil system and are not unidirectional. This highlights the need to investigate the responses in as many of the major systems in the world as possible. The rapid response of soil microbial biomass to perturbations also has the potential to indicate long-term trends in total soil carbon dynamics that have proved difficult to measure in laboratory based studies (Cheng, 1999).

One of the big issues in global change research is the question of how the changes can be mitigated in order to prevent the reduction in the habitability of the planet. One can either use direct technological solutions to the problem, for example Herzog *et al.*, 2000 who reviewed methods being developed to inject CO₂ into storage areas such as the deep oceans or depleted oil and gas reservoirs, or one can look at the biosphere for help. Bruce *et al.* (1998) released a report outlining the state of the knowledge regarding carbon sequestration in soils. Some of their findings were that through the restoration of degraded soils and more widespread adoption of soil conservation practices there is tremendous potential for increasing carbon uptake. I feel that this would, in the most successful scenario, only re-absorb the carbon that was released by the soils in the first place, before the perturbations started. This issue is to some extent addressed by Bruce *et al.* (1998) where they state that soil sinks for carbon cannot replace the need for greenhouse gas emission reductions, but they can contribute significantly to achieving the Kyoto protocol targets.

Study objectives and key questions

This project was undertaken to gain a better understanding of how plants from South African ecosystems, grown in natural soil, respond to elevated atmospheric CO₂ concentrations and how native soil microbial populations respond to these changes.

The goal was to use low nutrient soils collected from the field along with long growth periods to try to mimic natural growing conditions. The one difference was the constant

water supply and so the plants were not exposed to the potentially confounding effect of water stress in addition to the expected CO₂ effects. *Felicia aethiopica* ssp. *aethiopica* was used as the predominant study species due to its ease of growth from seed. It is a widespread species that is tolerant to a wide range of environments (Bond & Goldblatt, 1984) and so one would expect it to respond to CO₂ treatment and give insight into how other, widespread and variable species would react.

The main questions that were answered through the course of this project were as follows.

- How does the growth of a native Fynbos species (*Felicia aethiopica* ssp. *aethiopica*), from a low nutrient environment, change as a result of a doubling in the atmospheric concentration of CO₂?
- How does the biomass allocation of the plant along with ratios such as root to shoot ratio change as a result of an increase in CO₂ concentrations?
- Are nutrient allocation patterns through the plant affected?
- How does soil microbial biomass react to the indirect effects of the changed atmosphere in both the Fynbos shrubland and C₄ subtropical grassland?
- In what ways does the soil microbial community structure change in response to the applied CO₂ treatment?
- How are the flows of recently fixed carbon influenced by the *Felicia aethiopica* ssp. *aethiopica* plants being grown in an atmosphere containing high levels of CO₂?
- From the investigation of the carbon flows is it possible to distinguish between root and soil microbial respiration and are there any differences caused by CO₂ treatment?
- What is the long-term carbon storage potential of the systems studied?

The results of the investigations are presented here in a sequence from individual plant responses to CO₂ (Chapter 2), through gross measurements of soil microbial response (Chapter 3), detailed investigation of the microbial community responses (Chapter 4), investigation of the allocation of recently fixed carbon along with the separation of root respiration and rhizomicrobial respiration (Chapter 5) and ending with an overview of how plant and microbial responses could interact to influence the carbon flows through the system from the atmosphere into stable soil storage pools (Chapter 6). It can be seen that the bulk of the investigation was at the level of soil microbial interaction with the plant roots. Data was collected at the individual whole-plant level to provide a framework for the interpretation of

the microbial data and results from these two levels were combined to try to gain insights into larger spatial scale (community and ecosystem) responses to the CO₂ treatment.

Answering the questions posed above allows one to direct future research efforts and formulate urgent questions that need be answered within the limited research resources available.

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FELICIA AETHIOPICA SSP. *AETHIOPICA* PLANT PERFORMANCE AND GENERAL GROWTH RESPONSES TO ELEVATED ATMOSPHERIC CO₂ CONCENTRATIONS.

Introduction

The study of the effects of elevated atmospheric carbon dioxide concentrations on the growth of plants is now a large and well established field of research (Bowes, 1993). Recently, interest has been focussed on how plants from natural communities will respond to the expected changes because workers have come to realise that plant responses to CO₂ can become unpredictable when the system being studied has other limitations on it, such as light, water or nutrients (Insam *et al.*, 1999). Very little is known about the potential responses of natural systems to these changes due to their tremendous complexity (Rogers *et al.*, 1994) and lack of significance in terms of agricultural productivity.

Two key areas where there is insufficient research are the responses of roots and surrounding rhizosphere soil to global change (Murray, 1995) along with very few investigations done on the evergreen, sclerophyllous vegetation growing in very low nutrient, Mediterranean type ecosystems such as that found in the Cape Floristic region (Midgley *et al.*, 1995 and Midgley *et al.*, 1999). This vegetation type is referred to as *Fynbos*. Stock and Allsopp (1992) show that plants from the Fynbos region show unpredictable responses to nitrogen and phosphorus additions. Therefore, if CO₂ is considered to behave in a similar way to other nutrients, unexpected results could occur in this system.

This work serves as a preliminary investigation into the gross responses of *Felicia aethiopica* ssp. *aethiopica* to elevated atmospheric CO₂ and acts as the foundation for the other, more detailed, experiments that were performed in this study. Three themes emerged from this investigation. These were the general growth of the plant in response to the addition of CO₂, the nutrient relations of the plant and soil system and the unexpected effect of insect herbivory that occurred during the experiment. The plants were grown for almost

two and a quarter years in open top chambers situated in a glasshouse. During this time two harvests occurred, the first after about 19 months with the second at the end of the experiment (27 months).

Materials and Methods

Plant material and establishment

Felicia aethiopica (Burm.fil.) Bol & Wolley Dod ex Adams & Salter is a member of the Asteraceae and has a widespread distribution from the Cedarberg in the west, along the coast up to the eastern Cape and Kwa Zulu Natal (Bond & Goldblatt, 1984), occurring from sea level to an altitude of 2000 feet (610 m). The genus *Felicia* consists of 83 species distributed in tropical and southern Africa (Bond & Goldblatt, 1984). The seed used in this study was collected in the Cape Infanta and Stilbaai areas of the South Western Cape (34°20'S, 21°00'E) by the staff at Kirstenbosch National Botanical Gardens in October 1995 (Accession number 775/88).

Felicia aethiopica ssp. *aethiopica* plants were germinated from seed in approximately 850 g (dry weight) Fynbos soil in PVC planting tubes (7.0 cm i/d, 20 cm long) in a phytotron chamber with a 12 hour, 20°C/8°C day/night cycle. The daytime light intensity was 139 $\mu\text{mol.m}^{-2}.\text{s}^{-1}$. The pH, nitrogen (Kjeldahl digestion followed by colorimetry) and phosphorus (Tri - acid digestion followed by Murphy and Riley colorimetric determination) contents of the soil was 3.48, 0.76 mg.g^{-1} and 35.08 $\mu\text{g.g}^{-1}$ respectively. The soil was saturated with deionised water, three seeds were planted in each tube, then watered with 2 ml of *Kirstenbosch Instant Smoke Plus Seed Primer* (National Botanical Institute, Claremont, South Africa) and covered with a petri dish to maintain humidity. This cover was removed once the cotyledons on the seedling were established. After 40 days of growth (11 March 1996) the pots of seedlings were randomly assigned to open-top chambers situated in the University of Cape Town Botany Department's new glasshouse. One month later, planting tubes containing dead plants were replaced with tubes containing plants that had been grown in the phytotron chamber for this time. A second batch of seed was germinated in seed trays filled with Fynbos soil in the phytotron under the same conditions as the experimental plants and 42 of these plants were harvested after 40 days to serve as $t = 0$ controls.

Growth chambers and experimental conditions

The open-top chambers consisted of a plastic rubbish bin (50 cm in diameter) with an 80 cm high, removable polyethylene cylinder on top. A fan drew air from outside the glasshouse through a 16.0 cm diameter manifold before being divided into six 7.0 cm i/d PVC tubes that led into the centre of the chamber. The air was deflected horizontally by a 10 cm diameter PVC baffle 3 cm above the opening of the tube. Air flows were high enough to ensure at least 3 replacements of the chamber air every minute. Pure CO₂ was bled into the individual 16 cm diameter pipes that led off the manifold before the diameter was reduced to the 7.0 cm pipe. Six chambers were used, 3 ambient and 3 elevated (ambient + 342 $\mu\text{mol}\cdot\text{mol}^{-1}$ CO₂), and 17 pots were placed in each chamber giving a total of 50 plants plus one soil control per treatment. The CO₂ concentration in the chambers was monitored for periods of 24 hours at different times throughout the experiment using an ADC LCA2 infrared gas analyser (The Analytical Development Co. Ltd., Hoddesdon, Herts, England) attached to a monitoring computer. The plants were regularly watered to saturation with deionised water and were never allowed to become water stressed. The plants were not fertilised during the experimental period (Norby *et al.*, 1987). By 107 days after planting, the seedlings were established enough to thin the pots down to one seedling per pot. The plant to remain was identified as either being the one closest to the centre of the pot or one was randomly selected if there was more than one in the centre of the tube. The plants were removed by cutting them at the soil level and leaving the roots in the soil to ensure that the remaining seedling was not disturbed. This harvested material was dried and weighed in order to get an estimate of plant growth rates. The plants were shuffled three times within each chamber to eliminate position effects on their growth. It was noticed that some of the plants were being damaged by insects. To control this the plants were treated once at 122 days after planting with *MetasystoxR* (250 g.l⁻¹ Oxydemeton-methyl (organophosphate), Bayer Chemical Co.) made up to the recommended dosage of 1ml.l⁻¹. From this time until the end of the experiment the foliage of the plants was sprayed with deionised water every time the plants were watered.

Two distinct seedling variants were evident in the experiment from the same seed source. To eliminate seedling effects, only the tall variant was used in the analyses although within this group the plants were randomly selected. At two stages through the experiment (after 545 days and 758 days) the plants were assessed for herbivory damage to the apical meristem regions of the plants. The total number of meristems was counted and the number of actively growing tips was also noted. The degree of damage was assessed and two groups

were established: 0 no damage and 1 some degree of herbivory ranging from light, with meristems still alive, to severe damage with a dead meristem.

Two harvests were performed. The first harvest period started 568 days after the start of the experiment (22 August 1997) and finished 24 days later. Four plants were taken from each chamber giving a total of 12 plants per treatment. The second harvest began 810 days after the start of the experiment and occurred on three distinct days; 21 April 1998, 5 May 1998 and 18 May 1998. Due to the seedling variation and the previous harvest there was a shortage of suitable plant material and so 15 plants were taken from each treatment regardless of the chamber that they had been growing in.

Harvesting and analyses

The plants were cut at soil level and the top parts were separated into green leaves, dead leaves and stems. The soil was tipped out into a tray and the roots were gently separated from the soil. Soil adhering to the roots was shaken off and was defined as being rhizosphere soil. The remaining bulk soil was passed through a 2 mm analytical sieve and visible root material was picked out of the soil. A subsample of the bulk soil was collected for analysis of moisture content by means of oven drying at 107 °C for 24 hours. The rest of the plant and soil material was dried at 80 °C. This harvesting procedure resulted in 6 distinct compartments or pools of material that could then be analysed further. The pools were: green leaves, dead leaves, stems, roots, rhizosphere soil and bulk soil. Once dry, all of the samples were weighed on an analytical balance. The leaf and root material were then ground in a mortar and pestle using liquid nitrogen while the stem material was ground in a Wiley mill (20 mesh). All of the samples were analysed for total nitrogen content (Kjeldahl digestion, with colorimetry) and total phosphorus content (Tri-acid digestion followed by Murphy and Riley colorimetric determination). The pH of the soil was measured on an Orion pH meter in a soil solution of 20g of air dry soil shaken with 50ml 0.01M CaCl₂.

Statistics

Statistical analysis was performed using *STATISTICA for Windows*, release 5.1 (StatSoft, Inc, 1984 - 1996). All of the data were tested for normality and homoscedascity. Extreme values in the nutrient data were deleted assuming that they were a result of experimental error in the analysis. These values were identified using a routine from *STATISTICA* meeting either of the following criteria: datum > mean + 7se or datum < mean - 7se (with se being the

standard error). The most values disregarded in any data set was six in the rhizosphere phosphorus group. This high number was due to the combination of small samples sizes which prevented the analysis from being performed and extreme values which caused further data to be discarded. The percentage carbon dioxide effect was expressed as $((E-A)/A)*100$ (Drake *et al.*,1996, where E= the elevated value and A= the ambient value). Due to the structure of the experiments performed at the different harvest times, different statistical designs were used to analyse the data. For the first harvest (568 days) the data were subjected to a nested ANOVA with the chamber being nested within the treatment. In the second harvest (810 days) the ambient values were compared to the elevated data using t-tests. The relevant non-parametric test was used (Chi Squared test and Mann Whitney U test), when median and percentage data were presented. Soil data from the two harvests were compared using a t-tests.

Results

Chamber CO₂ concentration.

The concentrations of CO₂ in the chambers can be seen in Figure 2-1. From this graph one can see that the CO₂ concentration remained reasonably constant throughout the 810 day experiment. There was slight variation among the three elevated CO₂ open top chambers with average CO₂ enrichment above ambient of 322 $\mu\text{mol}\cdot\text{mol}^{-1}$ for chamber S1, 357 $\mu\text{mol}\cdot\text{mol}^{-1}$ for chamber S3 and 354 $\mu\text{mol}\cdot\text{mol}^{-1}$ for chamber S4.

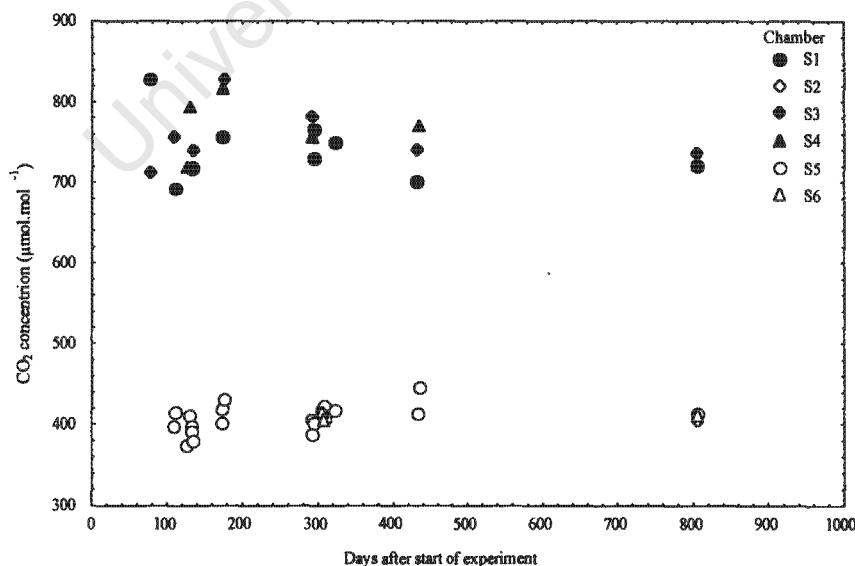


Figure 2-1: Carbon dioxide concentrations (in $\mu\text{mol}\cdot\text{mol}^{-1}$) within the open top chambers (S1-S6) used in this experiment. The measurements were made 10 cm above the PVC baffle on top of the air inlet tube. Each point is the mean of 24 hours of sampling at a rate of one sample every 111 seconds.

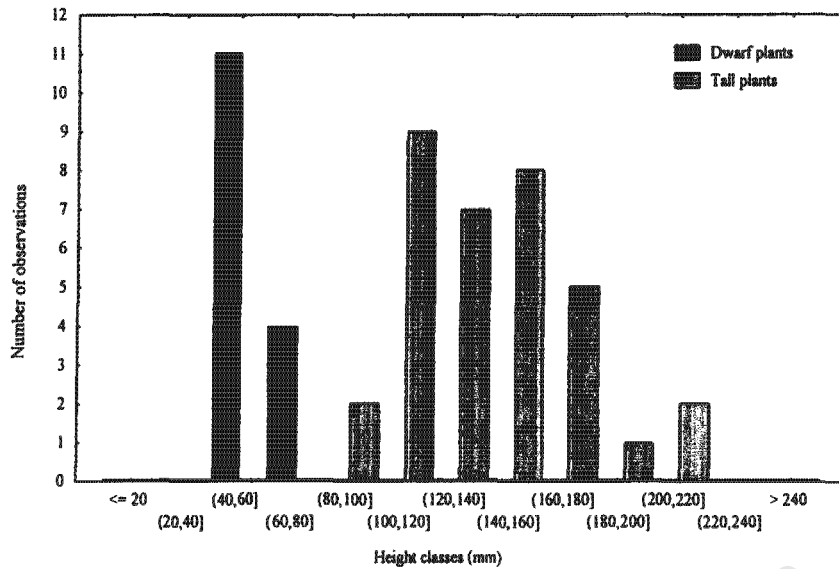


Figure 2-2: *Felicia aethiopica* ssp. *aethiopica* height variation under ambient CO₂ treatment. The plants were measured 568 days after the start of the experiment.

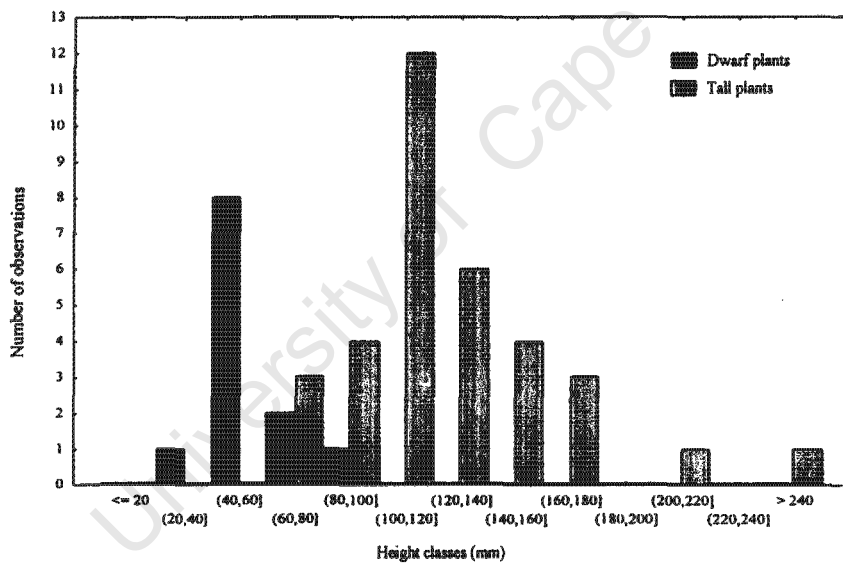


Figure 2-3: *Felicia aethiopica* ssp. *aethiopica* height variation under elevated CO₂ treatment. The plants were measured 568 days after the start of the experiment.

Seedling variation.

Figures 2-2 and 2-3 show the seedling variation observed in the *Felicia aethiopica* ssp. *aethiopica* plants in terms of the height of the plants. In these figures the two groups are those identified from observation of the plants growing in the pots. These figures confirm the distinction between the two groups. The dwarf variety was not only shorter but it also had smaller leaves, more compact growth and much denser branching patterns. For all further experiments only the tall variant was used.

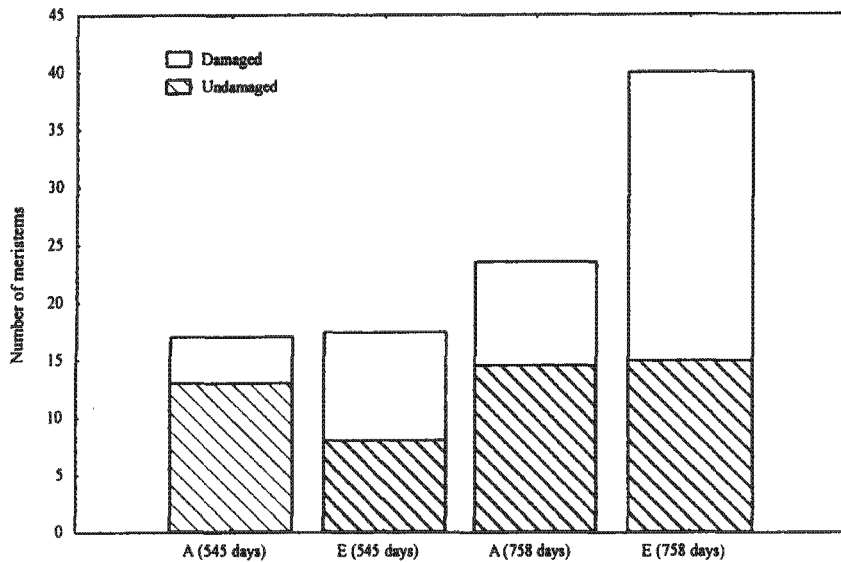


Figure 2-4: Median meristem damage to *Felicia aethiopica ssp. aethiopica* plants at 545 days and 758 days after the start of the experiment. A = Ambient treatment, E = Elevated treatment (n = 34 for ambient and elevated treatments at 545 days and for 758 days n = 22 for the ambient treatment while n = 19 in the elevated chambers)

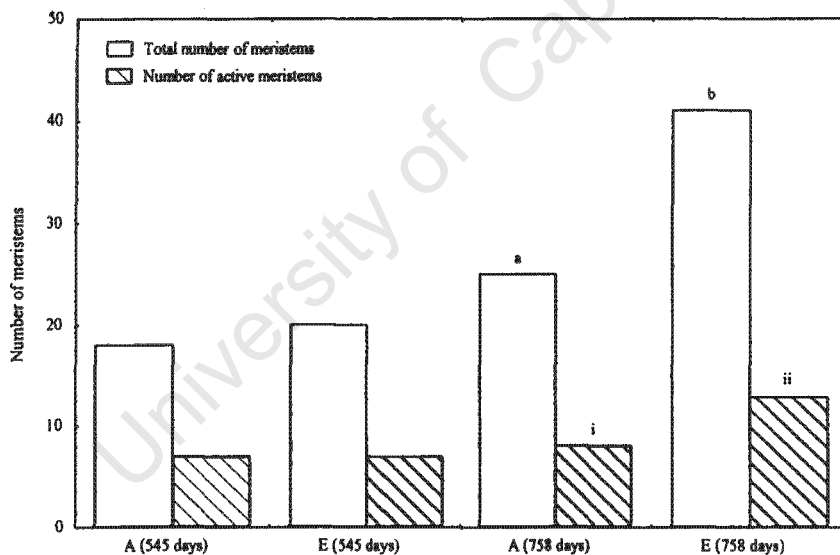


Figure 2-5: Median number of meristems in the *Felicia aethiopica ssp. aethiopica* plants along with the number of these tips that are active. Lower case letters within each category indicate statistical difference, Mann Whitney U Test. A = Ambient treatment, E = Elevated treatment (n = 34 for ambient and elevated treatments at 545 days and for 758 days n = 22 for the ambient treatment while n = 19 in the elevated chambers)

Herbivory damage.

Figure 2-4 shows both the median total number of meristems (height of the bar) along with the degree of damage in the different categories. A 2x2 Chi Squared test of the median values for both times gave results at the $p \leq 0.10$ significance level ($p=0.0532$ at 545 days and

Table 2-1: Percentage of the total number of meristems that are active. (n = 34 for the ambient readings at both 545 days and 758 days, n = 22 for the elevated readings at 545 days and n = 19 at 758 days) *p<0.05; ns Not Significant.

	Ambient	Elevated	Significance ^a
545 days	39%	35%	*
758 days	32%	32%	ns

a: Mann Whitney U test.

p=0.0524 at 758 days). There is significantly more herbivory occurring on the plants being exposed to elevated levels of atmospheric CO₂.

The number of currently active meristems were also counted (Figure 2-5). These were defined as shoot tips that were newly developed with fresh, green growth. There is no difference between the ambient and elevated condition for the active meristems at the first assessment (545 days, Mann Whitney U test with p > 0.10) while at 758 days there is a significant difference (Mann Whitney U test with p ≤ 0.05). This trend is repeated for the total number of meristems yielding p values of 0.121 and 0.0125 for the 545 and 758 day observations respectively.

To assess the overall vigour of the plants, the number of currently active meristems were expressed as a percentage of the total number of meristems (Table 2-1). At the first assessment (545 days) the plants exposed to ambient conditions showed more active growth when compared to the CO₂ treated plants (Mann Whitney U test with p ≤ 0.05). This pattern was lost by the second harvest (758 days, p > 0.10) showing that the differences in Figure 2-5 are due to a uniform proportional increase in both meristem number and activity.

Plant growth

There was a slight, non-significant (p > 0.10), reduction in overall plant growth in elevated atmospheric CO₂ at both the 568 day and 810 day harvests (Figure 2-6). The ambient and elevated values for the 568 day harvest are 3.29g ± 0.270g and 2.90g ± 0.297g respectively while after 810 days of growth the values had increased to 3.82 ± 0.245g and 3.60 ± 0.178g. The weight of the elevated plants was similar to that of the ambient ones in spite of the significant damage that occurred to the plants. The root to shoot ratio of the plants was not significantly influenced by CO₂, Ambient = 1.65 and Elevated = 1.69 at 568 days and A= 1.07 E= 1.04 at 810 days. Table 2-2 shows the statistical analysis of the dry weight data. The proportion allocation to each plant part has also been considered by means of expressing the weights as a percentage of the total plant weight (Table 2-3).

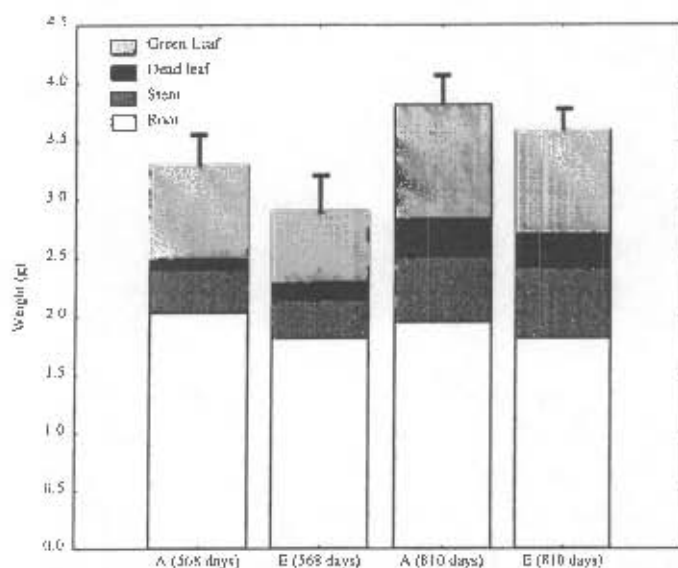


Figure 2-6: Mean dry weights (in g) of *Felicia aethiopica* ssp. *aethiopica* plant parts under different treatments, at different times. A = Ambient treatment, E = Elevated treatment (n = 12 at 568 days and n = 15 at 810 days) The error bars are whole plant mean + 1 se.

Table 2-2: The carbon dioxide effect (expressed as a percentage, $\frac{[E-A]}{A} \times 100$, (Drake *et al.*, 1996)) on the dry weight of *Felicia aethiopica* ssp. *aethiopica*. (n = 4 for 568 days and n = 15 for 810 days). + p < 0.10; ns Not Significant.

	568 days	810 days
Green leaves	-22.78 + ^a	-11.11 ns ^b
Dead leaves	+36.36 ns	-9.09 ns
Stems	-10.81 ns	+7.14 ns
Roots	-10.84 ns	-6.19 ns

a: ANOVA with 3 chambers nested within 2 treatments

b: t-test

Too few measurements of root and shoot growth were made to compile a proper growth analysis as described by Poorter (1989) but the series of measurements that were made (40 days, 107 days, 568 days, 810 days) can be seen in Figure 2-7. Here again, the non-significant effect of CO₂ on growth can be seen. One can also see the way in which the root and shoot growth curves are different with the roots initially growing quickly and then ceasing growth while the shoots continued growing until the end of the experiment. This graph explains the change in the root to shoot ratio seen above.

Table 2-3: Percentage allocation of dry matter in *Felicia aethiopica* ssp. *aethiopica* plants exposed to elevated atmospheric CO₂ treatment. (n = 4 for 568 days, n = 15 for 810 days and values are means ± standard error). + p ≤ 0.10; ns Not Significant.

	568 days			810 days		
	Ambient	Elevated	CO ₂ effect ^a	Ambient	Elevated	CO ₂ effect
Green leaves	24.07 ± 1.30	21.34 ± 1.01	-11.34 ns ^b	26.48 ± 1.35	24.69 ± 4.54	-6.58 ns ^c
Dead leaves	3.30 ± 0.75	5.21 ± 0.65	+57.88 +	8.67 ± 1.08	8.74 ± 3.19	+0.81 ns
Stem	11.24 ± 0.56	10.96 ± 1.12	-2.49 ns	14.66 ± 0.83	16.86 ± 2.88	+15.01 +
Roots	61.39 ± 1.75	62.49 ± 1.04	+1.79 ns	50.24 ± 2.50	49.71 ± 7.52	-1.05 ns

a: ((E-A)/A)*100 (Drake *et al.*, 1996)

b: ANOVA with 3 chambers nested within 2 treatments

c: t-test

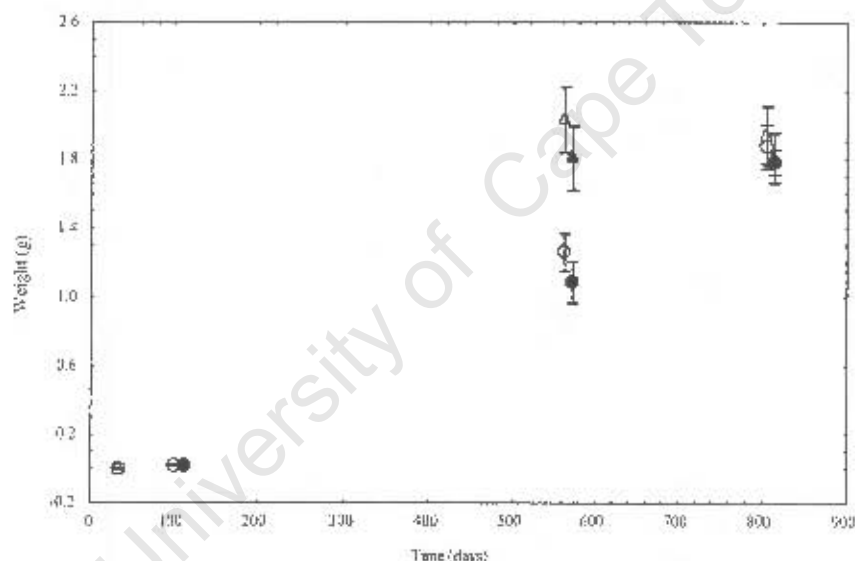


Figure 2-7: *Felicia aethiopica* ssp. *aethiopica* root and shoot development (in g of dry weight) over the course of the experiment. (n = 42 at 40 days, at 107 days n = 31 in the ambient treatment and n = 39 in the elevated chambers, n = 12 at 568 days and n = 15 at 810 days) The points are means ± 1 se. Symbol types are as follows: open circle = ambient shoot, open triangle = ambient root, closed circle = elevated shoot and closed triangle = elevated root. In the interests of clarity, the ambient symbols are offset 5 days earlier and the elevated symbols are offset 5 days later than the actual measurement.

Plant nitrogen content

The nitrogen content of the different compartments can be seen in Table 2-4. This table also shows the percent CO₂ effect. Although there were some large effects (for example for the stems at 568 days) few of them were statistically significant. Another way of expressing these data is in the form of total pool size (taking the weight of the compartment into

Table 2-4: Nitrogen content of *Felicia aethiopica* ssp. *aethiopica* plant parts in mgN.g⁻¹ dry material. (n = 4 for 568 days, n = 15 for 810 days and values are means ± standard error). + p≤0.10; ns Not Significant.

	568 days			810 days		
	Ambient	Elevated	CO ₂ effect ^a	Ambient	Elevated	CO ₂ effect
Green leaves	12.67 ± 0.615	12.19 ± 0.358	-3.79 ns ^b	13.91 ± 0.766	13.41 ± 0.535	-3.59 ns ^c
Dead leaves				10.72 ± 0.584	12.29 ± 0.535	+14.65 +
Stem	4.40 ± 0.337	5.50 ± 0.613	+25.08 ns	5.70 ± 0.339	6.46 ± 0.469	+13.33 ns
Roots	6.16 ± 0.387	6.58 ± 0.232	+6.82 ns	6.27 ± 0.465	6.25 ± 0.307	-0.32 ns

a: $((E-A)/A) * 100$ (Drake *et al.*, 1996)

b: ANOVA with 3 chambers nested within 2 treatments

c: t-test

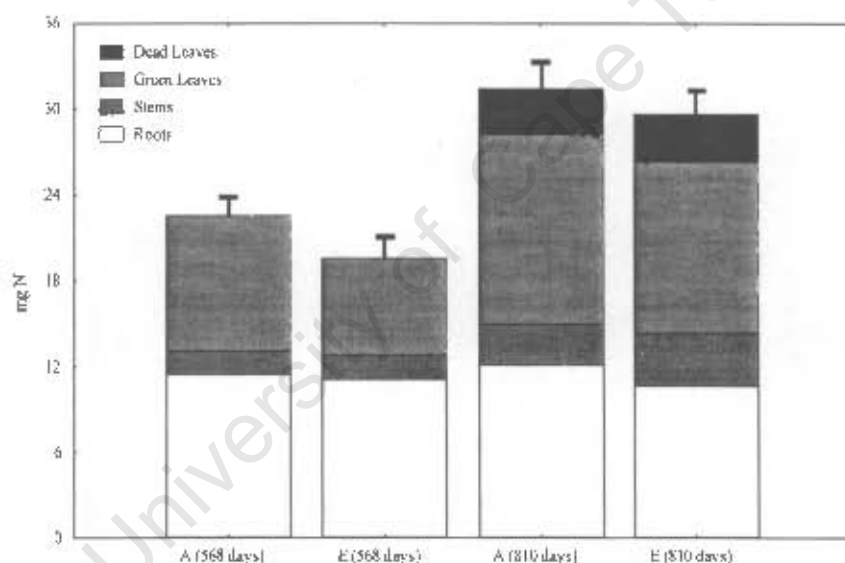


Figure 2-8: The mean total nitrogen (in mg per plant) of the *Felicia aethiopica* ssp. *aethiopica* plant parts under different treatments at different times. A = Ambient treatment, E = Elevated treatment (11 ≤ n ≤ 12 at 568 days and 11 ≤ n ≤ 14 at 810 days) The error bars are whole plant mean + 1 se.

account). Figure 2-8 shows the mean total nitrogen content of the plants in mg (height of the bar) along with the contribution made by each pool investigated.

At the 568 day harvest there was a highly significant ($p \leq 0.01$) 28.72% decrease in the total nitrogen content of the leaves in the elevated CO₂ group. This decrease is related to the observed decrease in leaf weight, Table 2-2. This decrease in leaf nitrogen content was seen again at 810 days but it was a smaller value, 9.82% and not significant (Figure 2-8).

The Dead Leaf data are missing in the 568 day harvest because of the very small amount of material (a mean 0.11g for the ambient plants and 0.15g for the elevated plants). There was a significant ($p \leq 0.10$) 14.65% increase in nitrogen concentration in the dead leaves in the elevated CO₂ group at the 810 day harvest (Table 2-4). This increase in concentration however did not result in significant increase in the total pool size due to the slight decrease in the dead leaf weight for the CO₂ treated plants (Table 2-2).

The stems showed a general increase in nitrogen for both harvests (Table 2-4 and Figure 2-8). This effect, however, was only statistically significant for 810 day harvest for the total nitrogen pool size (+27.24%, $p \leq 0.05$).

Looking at the roots, there is little agreement between the two sets of data gathered. For the 568 day harvest there was a slight increase (+6.82%) in nitrogen concentration in the elevated CO₂ plants with very little increase (+3.65%) in the total N pool size while at the 810 day harvest these trends have changed to no CO₂ effect on nitrogen concentration and a -11.44% decrease in the pool size.

Plant phosphorus content

Table 2-5 shows the concentration of phosphorus in the tissue in units of $\mu\text{g P.g}^{-1}$ dry material while Figure 2-9 shows the various pool sizes in $\mu\text{g P}$.

Investigating the green leaves revealed that at 568 days there was no CO₂ effect (+1.56%) on the phosphorus concentration but the total pool size significantly ($p \leq 0.05$) decreased by 18.47 percent. In contrast to these results, at the 810 day harvest there was a significant ($p \leq 0.05$) increase in P concentration (+12.98%) while the total pool size increased by 8.97%.

In the Dead Leaf pool at the 810 day harvest, the CO₂ treatment resulted in a general increase in P concentration and P total pool size with values of +27.82% and +19.22% respectively. Neither of these values are supported at the $p \leq 0.1$ level.

Stem P values showed general increase in both of the harvests. At 568 days there were no statistically significant results. The P concentration increased by +15.77% but the total pool size was little changed at +4.88%. The reason for this lack of change in the total pool is that the stem weight decreased (Table 2-2). At 810 days the weight and percent weight both increased in the stems (Tables 2-2 and 2-3) which translated in a significant ($p \leq 0.10$) increase in the total phosphorus pool size (+16.13%).

Table 2-5: Phosphorous content of *Felicia aethiopica* ssp. *aethiopica* plant parts in $\mu\text{gP}\cdot\text{g}^{-1}$ dry material. (n = 4 for 568 days, n = 15 for 810 days and the values are means \pm standard error). * $p \leq 0.05$; ** $p < 0.01$; ns Not Significant.

	568 days			810 days		
	Ambient	Elevated	CO ₂ effect ^a	Ambient	Elevated	CO ₂ effect
Green leaves	739.42 \pm 38.317	750.92 \pm 46.452	+1.56 ns ^b	652.98 \pm 29.595	737.72 \pm 27.720	+12.98 * ^c
Dead leaves				432.73 \pm 52.484	553.10 \pm 51.152	+27.82 ns
Stem	427.94 \pm 24.619	495.43 \pm 45.999	+15.77 ns	506.76 \pm 32.242	528.98 \pm 23.938	+4.38 ns
Roots	515.91 \pm 28.482	529.09 \pm 29.923	+2.55 ns	426.67 \pm 10.311	373.62 \pm 13.866	-12.43 **

a: $((I-A)/A) \times 100$ (Drake *et al.*, 1996)

b: ANOVA with 3 chambers nested within 2 treatments

c: t-test

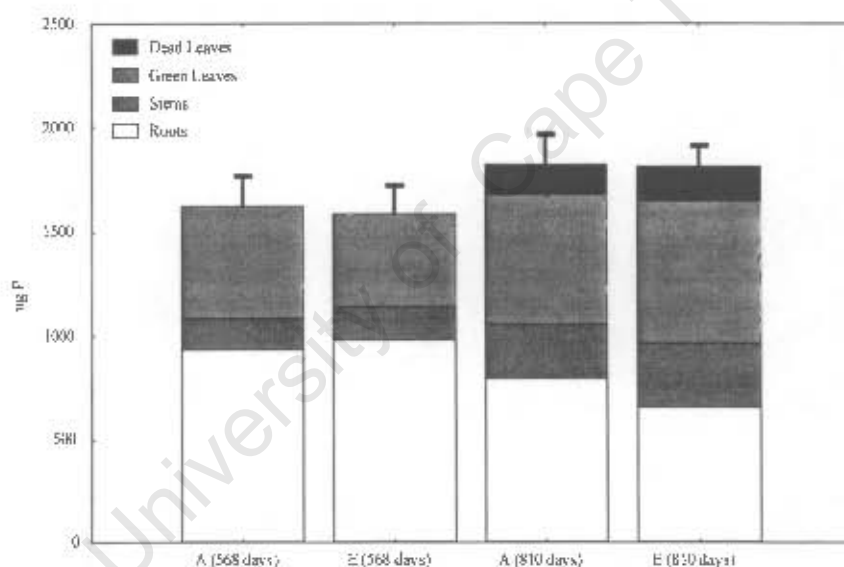


Figure 2-9: The mean total phosphorus (in μg per plant) of the *Felicia aethiopica* ssp. *aethiopica* plant parts under different treatments at different times. A = Ambient treatment, E = Elevated treatment ($9 \leq n \leq 11$ at 568 days and $11 \leq n \leq 14$ at 810 days) The error bars are whole plant mean + 1 se.

There were not very substantial effects of CO₂ on the root phosphorus at the 568 day harvest while there were a number of significant differences at 810 days. At the first harvest, elevated CO₂ had no effect on either phosphorus concentration or pool size in the roots ($p > 0.10$). By contrast, at the final harvest, elevated CO₂ reduced root P concentration by 12.43% ($p \leq 0.01$) and the total pool size by 17.19% ($p \leq 0.05$).

Table 2-6: Analysis of rhizosphere and bulk soils from the *Felicia aethiopica* ssp. *aethiopica* plants. (n=4 for 568 days, n=15 for 810 days and the values are means \pm standard error) ns Not Significant.

	568 days			810 days		
	Ambient	Elevated	CO ₂ effect ^a	Ambient	Elevated	CO ₂ effect
RHIZOSPHERE						
Dry weight (g)	3.26 \pm 0.565	1.59 \pm 0.282	-51.23 ns ^b	5.53 \pm 0.735	5.03 \pm 0.686	-9.04 ns ^c
mgN.g ⁻¹ dry material	1.24 \pm 0.117	1.20 \pm 0.142	-3.21 ns	0.87 \pm 0.066	0.80 \pm 0.066	-8.05 ns
μ gP.g ⁻¹ dry material	57.18 \pm 4.552	62.02 \pm 6.164	-8.46 ns	36.73 \pm 2.123	33.05 \pm 1.88	-10.02 ns
BULK						
Dry weight (g)	820.18 \pm 8.627	835.59 \pm 6.591	+1.88 ns	823.87 \pm 10.400	828.70 \pm 11.899	+0.59 ns
pH	3.68 \pm 0.021	3.66 \pm 0.017	-0.54 ns	3.79 \pm 0.031	3.81 \pm 0.028	+0.53 ns
mgN.g ⁻¹ dry material	0.89 \pm 0.056	0.97 \pm 0.048	+8.99 ns	0.73 \pm 0.022	0.74 \pm 0.024	+1.37 ns
μ gP.g ⁻¹ dry material	31.11 \pm 1.298	33.23 \pm 1.409	+6.81 ns	25.15 \pm 0.805	24.10 \pm 0.363	+4.17 ns

a: $((E-A)/A) \times 100$ (Drake *et al.*, 1996)

b: ANOVA with 3 chambers nested within 2 treatments

c: t-test

Soil investigations

Table 2-6 shows the results of the analyses that were performed on the rhizosphere and bulk soil fractions. There was no significant CO₂ effect in any of these measurements although there are large, and highly significant, changes in some of the values between the two harvests. Most notable among these are the decrease in N and P concentration in the rhizosphere soils ($p \leq 0.001$ for N and P) and the substantial increase in rhizosphere weight ($p \leq 0.001$). In the bulk soil there are statistically significant reductions in N and P concentration ($p \leq 0.001$ for N and P) along with a change in the pH ($p \leq 0.001$) while the total soil dry mass remains constant ($p > 0.10$).

Discussion

The simple design of the open top chambers that were used in this experiment were effective at providing a constant enrichment of CO₂ to the plants (Figure 2-1). The measurement and adjustment of the CO₂ concentration in the chambers was made on a comparative basis

between an ambient and elevated chamber and so the concentrations quoted in the results are the real enrichment values above the ambient CO₂ concentrations in the experimental system.

The experimental design of three replicate chambers was good, and reasonable replication of pots within the chambers was also achieved. There are however a few confounding effects that need to be considered. Open top chambers can introduce some artifacts such as increase in temperature, a constant draft from the air supply and attenuation of the incoming light (Norby *et al.*, 1999). The use of pots can cause pot effects by the restriction of root foraging, depletion of nutrients within the pot, presence of wider soil temperature fluctuations and more rapid soil drying which results in earlier onset of drought conditions (Townend & Dickinson, 1995). McConnaughay *et al.* (1993) question suggestions that results from pot experiments should be discarded due to their observation that CO₂ responsiveness is more sensitive to nutrient concentrations rather than pot effects. Pots and growth chambers are also useful because they allow for the detailed manipulation of plants needed in some experiments. Furthermore, in this *Felicia* study there is no difference in growth between the ambient and elevated plants at the 108 day harvest (Figure 2-7, $p > 0.10$) Thus there is no indication that these plants were responding to CO₂ at this young age and then being restricted by the pots during subsequent growth.

The most significant effect of CO₂ in this experiment was a dramatic increase in the herbivory on the plants in the high CO₂ environment. The surface of the leaves had some evidence of mite damage which is a common problem in glasshouse plant culture (J. Hofmann *pers comm.*). The major tip damage was caused by a Lepidopteran larva from an unidentified group (J. Hofmann *pers comm.*). These insects hollowed out the leaves and evidence of their presence was given by the presence of frass in the meristem regions of the damaged plants; empty pupae cases were found and one plant had a mature larva on it. All of the chambers had a common air supply and there was a flow of air out of each chamber at a rate of at least three air changes per minute and so it would be reasonable to assume that all of the chambers were exposed to an equal chance of introduction of the insect. Other reasons for the increase in the damage could be the increase in carbohydrate in the leaves as was shown by Zhang & Nobel (1996), in the form of starch and sucrose accumulation in *Encelia farinosa*, another member of the Asteraceae. In this *Felicia* experiment the time of day was not noted during the harvest and so an assessment of carbohydrate in the tissue would yield inconclusive results due to the daily fluctuations in carbohydrate content that are observed in photosynthesising plants (Zhang & Nobel, 1996, Midgley *et al.*, 1999). The food quality in terms of nitrogen and phosphorus content are little changed in the leaves

(Tables 2-4 and 2-5). Stock & Allsopp (1992) discuss nutrient content of vegetation in relation to the degree of herbivory and the authors show that there is a reduced degree of herbivory among Fynbos plants that is probably due to the high fibre and low nutrient content of the material. In contrast to this, thicket and Renoster shrubland have much higher foliar nutrient concentration and more herbivory occurs (Stock & Allsopp, 1992). In this case, the foliar nutrient concentrations are relatively high (see below) and so an insect herbivore can clearly gain enough resources from these leaves. These observations support the hypothesis that elevated atmospheric CO₂ concentrations will increase herbivory although the traditional reason, reduced forage quality (Fajer *et al.*, 1989, Whittaker, 2001), is not supported. Reasons for this increase could be a larger insect population (this was not assessed), or the insects themselves could have been larger and better fed.

Increases in herbivory could have very dramatic effects on plant community structure because epidemics of pests in natural systems would not be controlled in the same way as in managed systems (Rogers *et al.*, 1994). Stock *et al.* (1992) discuss the dramatic effect that herbivory has on resource limited systems and highlight some of the measures taken by plants in the Fynbos to defend themselves from this attack. If this observed increase in insect attack were to be widespread under a changed global environment, one can foresee significant changes in the community structure of the Fynbos occurring.

The dramatic increase in the total number of meristems was caused by the killing of the shoot tips which resulted in back budding of the plants. This indirect effect of insect damage significantly changes the architecture of the plants and this could have a effect on the way in which the plant interacts with competing plants in its environment.

Contrary to general observations of responses to CO₂ (Norby *et al.*, 1999), there was a slight, non-significant, reduction in biomass. This lack of response can be expected in a wild plant growing in unfertilised, low nutrient Fynbos soil that would have other constraints limiting the plant's growth (Murray, 1995). This would indicate that the plant is not carbon limited (Stock *et al.*, 1992). Investigation of 4 *Leucadendron* species that grow in the Fynbos region also resulted in very little growth response to CO₂ treatment even though they were treated with up to 20% Long Ashton nutrient solution (Midgley *et al.*, 1995). Baxter *et al.* (1994) showed a significant reduction of growth in *Festuca vivipara*, a perennial montane grass, under CO₂ treatment and also discuss other experiments where no effect of CO₂ on growth was observed. Cannell and Thornley (1998) warn that there may be large responses in nutrient-poor ecosystems but they will take a long time to manifest themselves (over 100

years) and so short-term laboratory studies may be misleading when used to predict how whole ecosystems will respond to changes in atmospheric CO₂ concentrations.

The lack of CO₂ response could also be due to the removal of biomass by the insects that were eating the plants. Although not alone in its influence, a contributing factor could also be sink strength limitation where the plant is unable to use the extra photosynthate fixed from the enriched CO₂ atmosphere (Reekie *et al.*, 1998, Baxter *et al.*, 1994). Removal of meristem tissue would reduce the sink strength even further.

It has been shown that many of the very large responses to CO₂ can be attributed to a compound interest effect, especially when plants are in the exponential phase of their growth cycle (Norby *et al.*, 1999). Under natural conditions this exponential phase is limited by other constraining resources that would slow the growth of the plants down and so it is important in experimental studies that this effect is taken into account (Norby *et al.*, 1999, Winter & Lovelock, 1999). In this experiment it can be seen that at the time of the two harvests, the root development had passed through the exponential growth phase while the shoots showed no clear growth trends. It is difficult to ascertain whether the growth was linear or whether it was at the start of the exponential phase (Figure 2-7). This curve also shows the very slow accumulation of biomass by these plants (1.8 g for the shoots at 810 days with the roots showing a similar weight gain) and so any small, initial changes that may have occurred have not been emphasised by the compound interest effect. Norby *et al.* (1999) cite a study that showed that CO₂ induced photosynthetic enhancement of one year old *Pinus radiata* needles was lower than that of the current year's growth. In this *Felicia* study, as would happen with many of the slow growing sclerophyllous species of the Fynbos, there is slow replacement of leaf material and so the overall CO₂ effect on the plants may be reduced.

The observed cessation of root development could be due to the roots filling the container, although it was observed that the shoot growth did continue indicating that there was no limitation on overall development. It would be surprising for the roots to have been restricted considering the fact that there was less than 2 g of root in pots containing 850 g of soil in a volume of 770 cm³. Another explanation would be Fajer *et al.* (1991) who showed that *Plantago lanceolata* compensated for loss of above ground biomass by reducing root production under conditions of simulated herbivory. In this case, however, there was no difference in root development between treatment and control groups while the herbivory levels were significantly different. The unchanged root to shoot ratio seen in this experiment is in line with the results reviewed by Rogers *et al.* (1994) who showed that

changes in the root to shoot ratio as a result of CO₂ is variable with some cases showing an increase, some decrease, while many others remain unchanged.

Many studies show a dramatic enhancement of the photosynthetic parameters of the plants but these changes are not carried through to ecologically important, integrated CO₂ responses in the overall growth of the plants (Norby *et al.*, 1999). Some of this discrepancy could be due to fine root turnover that can be responsible for significant carbon inputs into the soil but is not sampled during destructive harvesting (Pregitzer *et al.*, 1995). Stock *et al.* (1992) have shown that Fynbos plants generally have low photosynthetic capacities which would be an additional explanation for the lack of a CO₂ response in this vegetation. Low leaf nitrogen has been attributed to this low photosynthetic capacity (Mooney *et al.*, 1983) although in this case the *Felicia* plants do appear to have a relatively high leaf nitrogen content (see below).

It is important to remember that this experiment is not a seedling study. Although the plants were grown in pots, they were grown for a period of one and a half years to the first harvest and almost two and a quarter years for the second one. This is a long exposure period by most experimental standards. Norby *et al.* (1999) reviewed the growth responses of trees to CO₂ after an average of 2.7 growing seasons. The minimum time period included in the study was one growing season. In this *Felicia* case a herbaceous perennial was being investigated and so more rapid responses than trees would be expected.

At the 568 day harvest there was a significant decrease in the green leaf biomass while the dead leaf weight increased, although this increase was not significant. When expressed as a percentage of the total plant biomass, this difference was significant. This earlier senescence of leaves is supported by observations made by McConnaughay *et al.* (1996) along with other studies cited by the authors. The increase in the amount of dead leaf material may be as a result of greater leaf turnover occurring in the elevated plants, or it may be that the plants do not need as many leaves to maintain a positive C balance.

The only significant change in the nitrogen content of the plants occurred in the dead leaves with a 14% increase. The green leaves showed slight reduction in N content but they were not as dramatic as the reductions observed for many other plants (references in Murray, 1995). This would indicate that the plant is not down-regulating its photosynthetic machinery which results in dramatic decreases in leaf nitrogen content. Murray (1995) alludes to the remobilisation of nitrogen away from senescent plant parts. In this investigation the N content of dead leaf material was only slightly lower than that of the green leaves but the difference was smaller in the elevated CO₂ treatment. The question that

needs to be asked is whether the plants are less efficient at withdrawing the nitrogen from their senescent leaves when exposed to elevated atmospheric CO₂ conditions? A similar situation appeared to occur with phosphorus. This change in litter nutrient content could have very significant impacts on N and P cycling in this system.

In the green leaves and stems of the plants there is a general increase in the phosphorus concentration which is consistent with the prediction of a greater P demand under elevated CO₂ conditions (Midgley *et al.* 1995).

When compared to other nutrient values an interesting pattern emerges. Specht & Moll (1983) present typical nutrient contents of soils and vegetation found in Mediterranean type ecosystems from around the world. The soils used in this experiment would be described as strongly leached with values of N and P falling within the range presented for mountain Fynbos soil (0.4 to 1.0 mgN.g⁻¹ and 15 to 27 µgP.g⁻¹). Coastal Fynbos soils have values of 0.05 mgN.g⁻¹ and 600 µgP.g⁻¹ which shows that the balance of nutrients is very different. The pH values also vary markedly, with mountain Fynbos soils having values in the range of 3.5 to 5 while at the coast it is 5 to 8. The seeds of the *Felicia aethiopica* ssp. *aethiopica* plants were collected from near the coast and they were planted in mountain Fynbos soils. The plant's distribution includes both lowlands and mountain slopes (see above, Bond and Goldblatt, 1984 and Adamson and Salter, 1950) and so this flexibility probably allowed it to grow in the soils chosen. It is interesting to note that even though the *Felicia* plants were grown in low-nutrient soils, the plant was still able to achieve leaf nutrient concentrations far in excess of those found in mountain Fynbos species that have the ranges: 3.9 to 7.2 mgN.g⁻¹ and 100 to 470 µgP.g⁻¹ (Specht & Moll, 1983). Stock & Allsopp (1992) present further values of leaf litter found in the Pella region, a Cape coastal foreland. These values are 4.3 to 5.7 mgN.g⁻¹ and 100 to 300 µgP.g⁻¹ and so once again the values for this experiment are very much higher. Unfortunately there are no typical values for Asteraceae from either of these two sources.

On investigating the changes that occurred in the soils between the 568 day and 810 day harvests, many statistically significant differences were noted, however, it is important to assess whether these changes are biologically significant. In the ambient plants there was a 0.11 unit increase in the soil pH while for the CO₂ treated plants the change was 0.15 units. These values are small leading, to the conclusion that they are biologically unimportant. The higher nutrient values in the rhizosphere region of the roots compared to the bulk soil demonstrates the physiological difference between these two components of the soil. A reduction in nutrient concentration was observed in the two soil fractions. Calculating the

system's nutrient budget indicates that this reduction in soil nutrient is not offset by an increase in the plant nutrient content. From this one can conclude that leaching out of the pots was occurring, although calculations of the amounts would be difficult due to the low soil nutrient values that would result in small errors translating into big overall differences.

The data in this experiment were very variable which clouds the assessment of statistical differences between the treatments. Some of this variability was from experimental error but much of it is probably due to the inherent variability of the plants. The study plants were seed grown from an open pollinated, wild population and so considerable genetic variability would be expected. Clear indications of this variability were seen by the presence of two identifiable seedling variants. In their investigation of the responses of an early successional Mediterranean ecosystem, Nijs *et al.* (2000) found that the composites were the species exhibiting the greatest growth variability among the groups studied. Poorter (1989) mentions an unpublished experiment that showed a coefficient of variation of 30% in plant dry weight of a genetically homogenous, inbred line of *Plantago major* ssp. *major* demonstrating that natural variability is widespread and expected. Two further examples are mentioned by Midgley *et al.* (1999) referring to photosynthetic CO₂ responsiveness between ecotypes and genotypes. The variability demonstrated in this experiment is important as it shows the robustness that natural plants have; allowing them to tolerate environmental changes. It is also important to realise that small changes observed in short-term experiments, such as the change in the nutrient content of the litter, could lead to large changes on bigger spatial and time scales although one should be very cautious over the conclusions (Cannell & Thornley, 1998,). An example of this caution can be seen in Winter & Lovelock (1999) where the authors showed large CO₂ effects occurring in tropical tree seedlings over very short periods of time (35 to 84 days) under no nutrient, light or water restrictions while more natural field experiments have not yielded these dramatic effects.

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ELEVATED ATMOSPHERIC CARBON DIOXIDE EFFECTS ON THE SOIL MICROBIAL BIOMASS IN TWO ECOSYSTEMS: C₄ GRASSLAND AND AN OLIGOTROPHIC MEDITERRANEAN SHRUBLAND, THE FYNBOS.

Introduction

It is widely accepted that an understanding of the dynamics of the soil carbon pools under global change scenarios is critical for the development of models of ecosystem responses to these anthropogenic influences at the planetary scale (e.g. Sadowsky & Schortemeyer, 1997). It is, however, surprising to find how few studies have focussed on these below-ground processes. This paucity of data is due to the difficulty in separating intimately linked processes in a tremendously heterogeneous matrix such as that found in the soil environment. These problems with soil investigations were first encountered by O'Neill *et al.* (1987) and the difficulties have not yet been satisfactorily resolved.

Studies of the effects of elevated CO₂ on below-ground processes have investigated a wide range of plant ecological systems. Mediterranean grassland (Hu *et al.*, 2001), alpine grassland (Niklaus *et al.*, 2001, Niklaus & Körner, 1996), maturing pine forest (Allen *et al.*, 2000) and mixed C₃ and C₄ tallgrass prairie (Williams *et al.*, 2000) are all natural, undisturbed systems that have been studied. Other workers have used microcosm-based investigations to look at a tropical system (Insam *et al.*, 1999) and a temperate system (Kandeler *et al.*, 1998). Earlier studies made prior to these are reviewed by Tate & Ross (1997) and Sadowsky & Schortemeyer, (1997).

Soil microbial biomass is a small component of the total soil carbon (usually less than 5%) but it is a key part of many important processes occurring in the soil (Dalal, 1998). The microbes are a labile source of nutrients (C, N, P and S) and an immediate sink of these nutrients when they become available in the soil. They transform nutrients within the soil, form symbiotic relationships with plant roots and can be pathogenic or act as protection against attack by other organisms. The speed of nutrient cycling and flux through the soil microbial population is at least an order of magnitude faster than that of other soil pools and

so it is likely that this pool will show changes brought about by experimental treatments sooner than what would be seen in the other, more conservative carbon pools (Dalal, 1998, Anderson & Domsch, 1989). The microbial quotient (the biomass C to organic C ratio expressed as a percentage) has been shown by Dalal (1998) to be sensitive to the effects of heavy metals on the soil microbes. The microbial quotient decreased much faster than the microbial biomass with increasing metal pollution. There is thus evidence that this measure could be useful in identifying effects of elevated atmospheric CO₂ treatment on soil processes.

The methods used to investigate carbon in the soil vary in sensitivity. Two methods are being used here, the rather crude loss on ignition method to measure total organic carbon content of the soils and the more sensitive chloroform fumigation extraction (CFE) method that measures the microbial biomass carbon in the soil.

The efficacy of the loss on ignition method to determine soil organic C was investigated for sandy, acidic soils from South Western France (Jolivet *et al.*, 1998). This research showed that combustion was a suitable way to measure soil carbon in these soils and so it is therefore probably a suitable method to use for acid soils from the South Western Cape region of South Africa.

Since its development in 1976 by Jekinson and Powlsen, the chloroform fumigation of soils has become a very widespread tool for the assessment of soil microbial biomass. Chloroform fumigation followed by extraction with 0.5 M K₂SO₄ has become the dominant method for assessing soil microbial biomass C due to its perceived ease and quick analysis time (Haney *et al.*, 1999). The CFE method works by the chloroform attacking the membranes of the soil organisms causing them to die and release their cellular contents, particularly cytoplasm, into the soils. These are then degraded by enzymatic autolysis transforming them into extractable components which can be measured (Joergensen, 1996). Voroney *et al.* (1991) assert that the fumigation extraction procedure is a precise measurement method with only three to four replications needed to discriminate a 5 to 10% difference in biomass at the 5% significance level. Concern has subsequently been raised that different classes of microbes may react differently to the fumigation process resulting in different proportions of their carbon being released thus influencing the biomass values (Hu & Van Bruggen, 1998). The authors showed that bacteria isolated on carbon rich and carbon poor media were equally sensitive to chloroform fumigation indicating that oligotrophic bacteria are also measured by CFE. This shows that this method would be suitable for use in the low nutrient environment found in Western Cape soils. CFE is less restricted by soil

conditions compared to other microbial measures making it a popularly used method. One needs to remember that this method, along with fumigation incubation and substrate induced respiration is far from perfect (Beck *et al.*, 1997). The suitability of using K_2SO_4 as an extractant has been questioned by Haney *et al.* (1999), as they have shown that its use has developed for historical reasons rather than strictly practical ones. K^+ ions flocculate soil and can reduce the solubility of carbon especially in acid soils (Haney *et al.*, 1999). Their results did show, however, that the use of water as an extractant resulted in soil microbial biomass being negative at low soil pH values. Furthermore the use of K_2SO_4 is widespread and so needs to be used at this stage to allow comparison of results.

Many authors assert that measurement of soil microbial biomass is important but what the numbers mean, how they can be compared and what their predictive power is, are still unresolved factors (Dalal, 1998). It has been found that microbial biomass assessments can vary more between different laboratories than between different types of land use (Joergensen, 1996) making it difficult to compare soil microbial biomass results from different studies (Dalal, 1998). Beck *et al.* (1997) showed that ten different analysis methods used to assess the microbial C content of soil gave difference absolute values but the rank order of the 20 soils investigated was virtually unchanged. To solve this problem of variability, the relative effect of treatments can be compared without paying too much attention to the actual values presented.

To make predictions of how natural systems will respond to global changes, Niklaus & Körner (1996) advise that one strive to investigate as undisturbed systems as possible. Fertilisation and soil disturbance can confound results obtained from short-term, small-scale pot to microcosm investigations (Niklaus & Körner, 1996).

The aim of this study was to use CFE to see whether CO_2 treatment caused changes in the microbial biomass and total carbon of the soils in a subtropical C_4 grassland and an oligotrophic Mediterranean shrubland (the Fynbos). Answering these questions will help to reveal potential patterns occurring under a changed atmospheric scenario as was highlighted by Tate & Ross (1997). The authors reviewed the potential sequestration of carbon by some grasslands that could mitigate or delay the current rise in atmospheric CO_2 concentration while other results showed no enhanced carbon storage. At this stage there were few experimental observations to support either of the theories.

The subtropical C_4 grassland study site is situated around a natural CO_2 spring and has the great advantage of having been exposed to high levels of CO_2 for a considerable period of time. In other regions of the world such as Iceland, Italy, New Zealand, Venezuela and

the United States of America, studies of natural CO₂ sources have proven to be useful for studying the effects that global changes will have on vegetation (Fernández *et al.*, 1998, Newton *et al.*, 1996, Koch, 1994, Miglietta *et al.*, 1993). An inherent problem with these natural experiments is the difficulty in creating a well controlled experimental design using the resources that are available (Tate & Ross, 1997, Newton *et al.*, 1996, Körner & Miglietta, 1994). The two biggest problems are a lack of replication and the common way in which the vents constitute a point source of CO₂ which diffuses into the environment creating a steep gradient (Stock *et al.*, in prep, Fernández *et al.*, 1998) whose shape depends on the direction of the wind. Even with these problems, natural experiments are very valuable in revealing the long-term effects of CO₂, which are not currently possible to measure with artificially created experiments.

Materials and methods

Site description and experimental set up.

The C₄ grassland was situated at a natural carbon dioxide source in southern KwaZulu Natal, South Africa (Harris *et al.*, 1997). The release of CO₂ was first noted by geologists in the 1920s (Forman, 1927). Local traditional healers use the water from a nearby river for medicinal purposes indicating that this area is part of the indigenous folklore showing that these exhalations have been active for a long time. The study area was situated on the farm *Pleasant View* (30°40'S 30°01'E; altitude 660m) about 30 km west of Paddock and 15 km south east of Harding (Stock *et al.*, in prep). The site is an east facing, grass covered hillside with an average slope of 9 degrees. Dry CO₂ of 98.39 % purity (with 1.38% CO and 0.20% CH₄, data provided by landowner G Clegg from analyses performed by ED Booysen of SASOL) is released from a steel pipe that has been sunk into the gas fault underlying the area. Mr. Clegg reported that soil patches around the main pipe bubble when they are saturated by rainfall thus indicating additional gas is released from the surrounding soil. In 1996 a simple free air CO₂ enrichment ring was erected 19 meters away from the steel pipe. The CO₂ was supplied by attaching a cap to the top of the pipe and running the gas into a 5 cm diameter irrigation pipe that surrounded the treated plot. This tube was buried to protect it from fires while fireproof copper risers were inserted into the tube. A more complete description of the treatment can be found in Stock *et al.* (in prep). A CO₂ concentration of approximately 600 µmol.mol⁻¹ was achieved using this simple fumigation system (Stock *et*

al., in prep). At a distance of 39 m and 72 m up slope from the CO₂ source pipe control plots were established.

The Fynbos study was conducted at the University of Cape Town's new glasshouse and consisted of two parts; a single species, pot study of *Felicia aethiopica* ssp. *aethiopica* plants (ss experiment) and a mixed species, microcosm study that contained four representative Fynbos species along with the *Felicia* plants (ms experiment, Stock *et al.*, 1997). Open-top chambers were used to treat the plants with carbon dioxide. The chambers consisted of a plastic rubbish bin (50 cm in diameter) with an 80 cm high, removable polyethylene cylinder on top. A fan drew air from outside the glasshouse through a 16.0 cm diameter manifold before being divided into six 7.0 cm i/d PVC tubes that led into the centre of the chamber. The air was deflected horizontally by a 10 cm diameter PVC baffle 3 cm above the opening of the tube. Air flows were high enough to ensure at least 3 replacements of the chamber air every minute. Pure CO₂ was bled into the individual 16 cm diameter pipes that led off the manifold before the diameter was reduced to the 7.0 cm pipes. The CO₂ concentration in the chambers was monitored for periods of 24 hours at different times throughout the experiment using an ADC LCA2 infrared gas analyser (The Analytical Development Co. Ltd., Hoddesdon, Herts, England) attached to a monitoring computer. An average CO₂ enrichment of ambient plus 340 $\mu\text{mol}\cdot\text{mol}^{-1}$ CO₂ was achieved. The soil used in the ss experiment was collected from Orange Kloof Reserve on Table Mountain. The pH, nitrogen (Kjeldahl digestion followed by colorimetry) and phosphorus (Tri-acid digestion followed by Murphy and Riley colorimetric determination) contents of the soil was 3.48, 0.76 $\text{mg}\cdot\text{g}^{-1}$ and 35.08 $\mu\text{g}\cdot\text{g}^{-1}$ respectively. The soil used in the ms study came from the same source but was amended with approximately 50% v/v *Fynbos potting mix* (Kirstenbosch Botanical Gardens).

Plant material and establishment

In the ss experiment, *Felicia aethiopica* ssp. *aethiopica* (Kirstenbosch Botanical Gardens accession number: 775/88) plants were germinated from seed in approximately 850 g (dry weight) Fynbos soil in PVC planting tubes (7.0 cm i/d, 20 cm long) in a phytotron chamber with a 12 hour, 20°C/8°C day/night cycle. The daytime light intensity was 139 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. Three seeds were planted in each tube, then watered with 2 ml of *Kirstenbosch Instant Smoke Plus Seed Primer* (National Botanical Institute, Claremont, South Africa) and covered with a petri dish to maintain humidity. This cover was removed once the cotyledons on the

seedling were established. After 40 days of growth (11 March 1996) the pots of seedlings were randomly assigned to the open-top chambers. One month later, planting tubes containing dead plants were replaced with tubes containing plants that had been grown in the phytotron chamber for this time. After 107 days of growth the pots were thinned down to one plant per pot. The plants were removed by cutting them at soil level and leaving the roots in the soil to ensure minimum disturbance to the remaining seedling. The plants were regularly watered with deionised water and never allowed to become water stressed. The plants were not fertilised throughout the experimental period and they were shuffled three times within each chamber to eliminated position effects on their growth. Two distinct seedling variants developed; one tall and one short with a dense arrangement of small leaves. To avoid the confounding effect of this seedling variation only the tall plants were used in the analysis although within this group the plants were randomly selected.

Felicia plants germinated from the same batch of seeds, referred to above, were used in the ms experiment. The other four species used in the ms experiment were *Leucadendron laureolum*, *L. xanthoconus*, *Salvia africana-lutea* and *Podalyria sericea*. The chamber was filled with soil and five *L. laureolum* plants were equidistantly arranged around it. A ring of five individuals of the five study species were planted around each *L. laureolum* plant.

Soil sampling

In the C₄ grassland, soil samples were collected three times at one-year intervals. In 1996 (the year that the fumigation treatment system was established, sampled on 14 May) a base line sampling strategy was implemented working on the assumption of a steep CO₂ gradient moving away from the point source of the gas caused by the steel pipe. Samples were collected at distances of 5, 10, 15, 80 and 85 m from the pipe. For the bottom three distances, a sector of 90 degrees to the NW of the pipe was chosen and three 10 to 15cm deep soil samples were collected from each of the rings. At 80 and 85 meters from the pipe three samples were collected from each ring, near the top of the hillside close to the position of the top permanent plot. To allow for comparisons with the data collected in subsequent years, only three sampling sites are presented, the 5 m, 15 m and the combined 80 and 85 m presented as 82 m.

In 1997 (one year after starting CO₂ treatment, sampled on 24 April) 20 cm deep soil cores were collected from each of the three permanent plots that were established the year before. Five samples from each site were collected. In addition to this, 5 samples were

collected 2 m away to the NW of the CO₂ pipe and 5 samples were collected from the North-facing hillside, opposite the treatment area. This site was assumed not to have had any elevated atmospheric CO₂ exposure.

In 1998 (two years after the start of CO₂ fumigation, sampled on 9 July) five 20cm deep cores were again collected from each of the permanent plots along with 5 samples collected in the NW sector 3 m from the CO₂ pipe. In addition to this, an array of smaller (10cm deep) cores were assessed for soil moisture content. Eight to 10 samples were collected at 2 m intervals on various contours moving up the slope.

In the single species Fynbos study, the soils were sampled 568 days after the start of the experiment (22 August 2001). Four pots were sampled from each of the 3 ambient and elevated chambers giving a total of 12 soil samples per treatment. The soil was tipped into a sterile tray and the roots were gently separated from the soil.

In the mixed species experiment, two soil cores were taken (#17 corer, approximately 100 mm deep) from near the ring of *Felicia aethiopica* ssp. *aethiopica* surrounding the central *Leucadendron lauroolum* plant. The litter and top 10 mm of soil were removed before placing the sample in a polyethylene bag. Nine ambient and nine elevated chambers were analysed.

In all cases the bulk soil was passed through a 2 mm analytical sieve and visible root material was picked out of the soil (Mueller *et al.*, 1992). A subsample of the bulk soil was analysed for moisture content by means of oven drying at 107°C for 24 hours and the organic matter content was assessed by weight loss on combustion at 450°C for 24 hours. The soils were stored in polyethylene bags at 4°C until they were analysed for soil microbial carbon content. Due to practical constraints, the soil samples were stored at for an average period of 55 days with a range from 10 to 127 days prior to analysis. Many authors have investigated the effects of storage on microbial biomass measurements and most of them show that although changes do occur on storage the significance of the effect is disputed (Lovell & Jarvis, 1998, Stenberg *et al.*, 1998, Ross, 1991). With this in mind, it was felt that the CFE method used in the current studies was sufficiently robust to be effectively used to gather data on soil microbial biomass changes.

Fumigation, extraction and carbon analysis

Microbial biomass carbon was assessed using the fumigation extraction technique (Jenkinson & Powlson, 1976 and Vance *et al.*, 1987). Briefly, duplicate sieved soil samples (an

equivalent of 20g dry weight, calculated from earlier moisture content determination) were fumigated with alcohol free CHCl_3 for 24 hours. Prior to fumigation, the soils were adjusted to 15% moisture content (dry mass basis) by the addition of distilled water (Ross, 1989). After the removal of the CHCl_3 by repeated evacuation of the fumigation chamber, soluble carbon was extracted from fumigated and duplicate unfumigated samples using 100 ml 0.5 M K_2SO_4 for 30 minutes in an end over end shaker. The extracts were filtered using vacuum filtration through a Whatman GF/B filter. Organic carbon was determined using acidic dichromate consumption (Vance *et al.*, 1987). Microbial biomass was calculated using the equation:

$$\text{Biomass C} = 2.64 E_C$$

where $E_C = (\text{organic C from fumigated soil}) - (\text{organic C from unfumigated soil})$. It should be noted that the reciprocal of 2.64 is a k_{EC} value of 0.38 that is the value recommended by Joergensen (1996). The calculations used to obtain values of the carbon contents of the extracts can be found in Rowell (1994). Total carbon in the soil was calculated from the percentage mass loss on combustion of dry soil multiplied by a factor of 5.8 (Rowell, 1994) to give values in mg carbon per gram of dry soil, while the extractable carbon content of the soils was assessed using the values obtained from the unfumigated soil samples used in the fumigation extraction analysis. The microbial quotient of the soil was calculated by dividing the microbial biomass carbon (in mg.g^{-1} dry soil) by the organic carbon content and expressing this result as a percentage.

Statistical analysis

Statistical analyses were performed using *STATISTICA for Windows*, release 5.1 (StatSoft, Inc, 1984 - 1996). The data were tested for normality and analysed using Analysis of Variance, nested ANOVA, Student's t-test or non-parametric equivalents, where appropriate. The specific tests used have been elaborated in the results.

Results

The Natal C_4 Grassland

The natural carbon dioxide spring is situated on a gently sloping site and it was found that there was a significant gradient of increasing moisture moving down the slope (Figure 3-1).

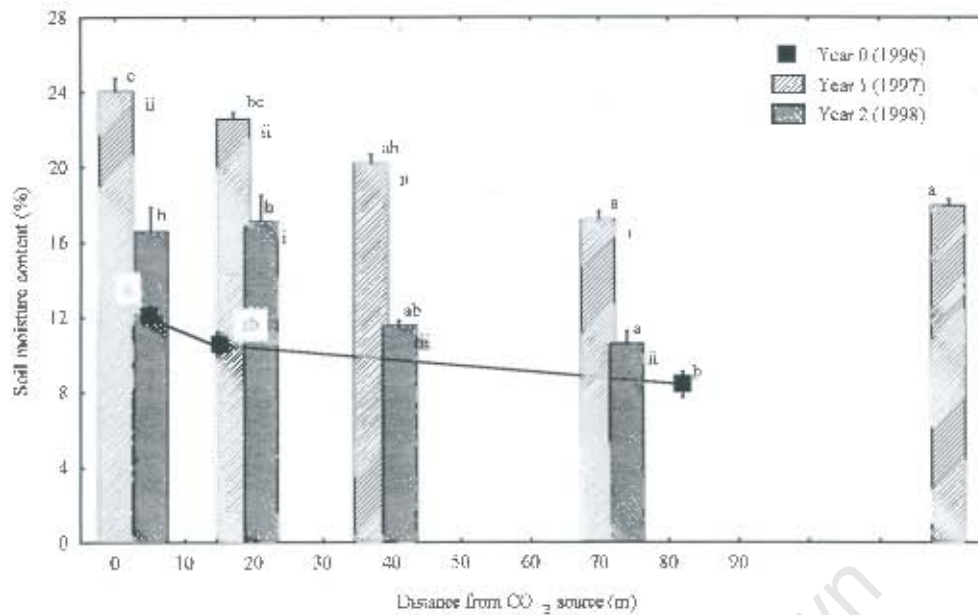


Figure 3-1 : Moisture content of soils collected from Pleasant View Farm. The points and bars are means \pm se. Closed square = 1996 baseline sampling of vegetation naturally exposed to a point source of CO₂ (n = 3), hatched bar = 1997 sampling, one year after establishing the fumigation ring 19 m from the CO₂ source (n = 5, bar offset by -2 m), grey bar = 1998 sampling, two years after establishing the fumigation ring (n = 5, bar offset by +2 m). The 1997 bar on the far right of the figure is a sample taken from the N facing hillside opposite the study area. The letters and symbols indicate statistically significant (ANOVA, $p \leq 0.05$) differences within each year of sampling.

This gradient could be attributed to either a geographical slope effect or it could be moisture accumulation as a result of improved water use efficiency under the elevated CO₂ conditions. Figure 3-2 shows the results of the array of moisture samples taken in July 1998. There is a slope effect on the soil moisture content but in addition to this there is a significant ($p \leq 0.01$, Kruskal Wallis ANOVA & Median test) CO₂ effect within the bounds of the treated plot. In contrast to this, there is no difference between inside and outside the top control plot ($p > 0.05$, Kruskal Wallis ANOVA & Median test). This effect can be seen in Figure 3-1 with the inflexion of the line at the 19m sample while the effect is not as apparent at the other two sampling times.

Investigation of rainfall records (Table 3-1) show that prior to the 1998 sampling there had been very little precipitation while at the other times more rain had fallen. From this one can conclude that the system is not often water stressed but at times of stress such as occurred in 1998 there is an improvement of water use efficiency by the plants and thus the soil remains moist for a longer period of time.

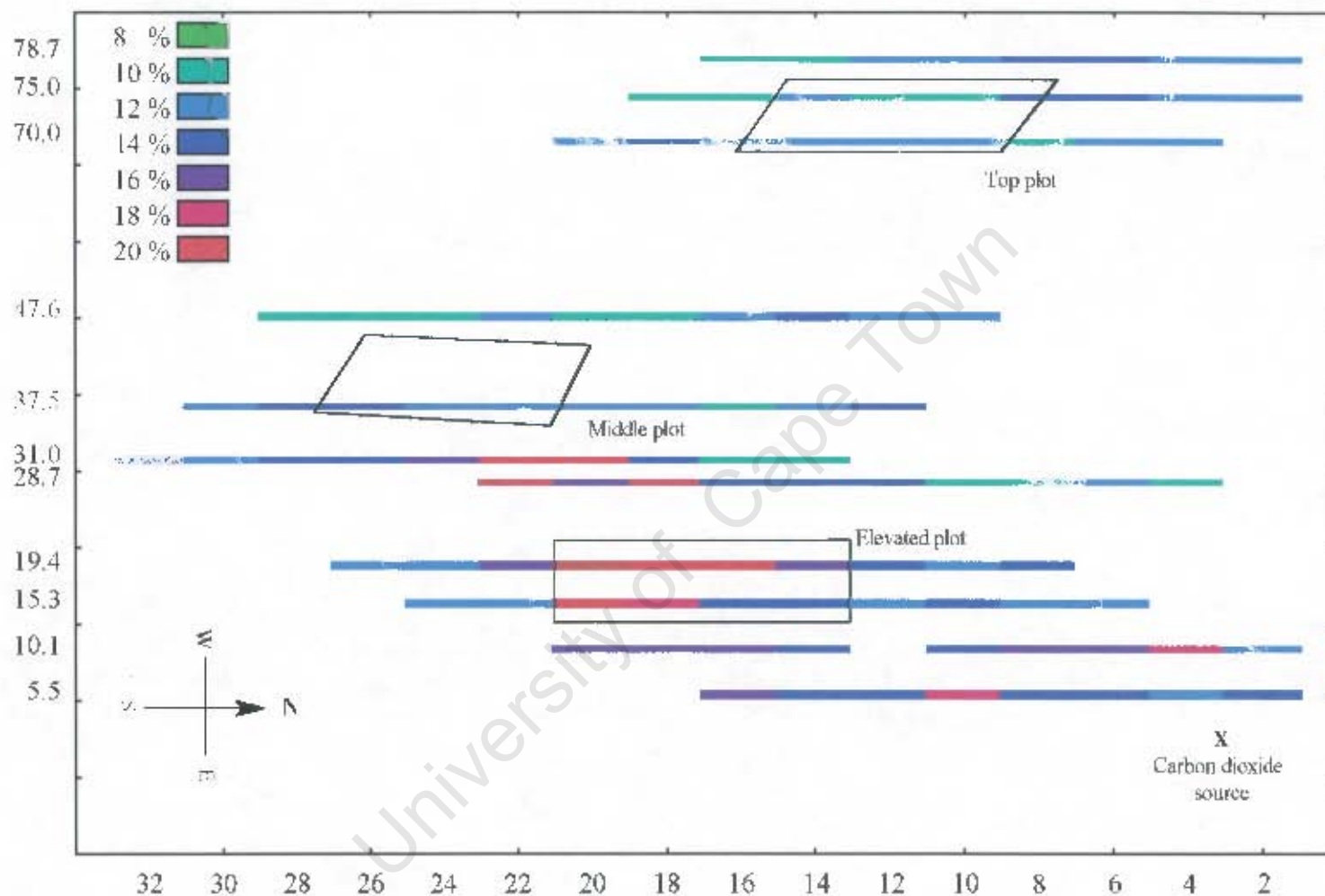


Figure 3-2 : Array of soil moisture measurements taken over the whole study area at Pleasant View Farm in 1998. The natural carbon dioxide source is indicated along with the positions of the permanent plots that were established in 1996. The values on the x axis are distances, in meters, away from the fence running down the north side of the site while the values on the y axis are distances, in meters, away from the CO₂ source westwards up the slope.

Table 3-1: Rainfall data for the Eureka weather station (30°43'S 30°01'E : altitude 671m) for the periods prior to the sampling dates at the natural CO₂ spring on *Pleasant View* farm . The average data is a mean for the period 1936 to 1999. Data supplied by the South African Weather Bureau.

Year	Sampling Date	Rainfall data		
		Month	Rainfall	Average
1996	14 May 1996	March	94.0	97.1
		April	25.0	53.7
		May	5.0	34.0
1997	24 April 1997	March	198.0	97.1
		April	42.0	53.7
1998	9 July 1998	May	54.5	34.0
		June	0.0	18.4
		July	0.0	28.9

Although it was well below average, more rain did fall in 1996 before sampling but it was found that the soil was drier. This is probably due to the higher temperatures of autumn along with a larger green leaf area causing more depletion of soil water. In this year there was no CO₂ treatment present at 19 m, while five meters away from the pipe there was a CO₂ concentration of between 480 and 596 $\mu\text{mol mol}^{-1}$ (Stock *et al.*, in prep) and this has resulted in a small peak in the soil moisture content at this point ($p \leq 0.05$, ANOVA). From Table 3-1, 1997 was a wet year prior to soil sampling and this is borne out by only the slope effect being apparent in Figure 3-1.

Moving to the investigation of the below-ground carbon. Figure 3-3 shows organic carbon content of the soils. Apart from the curiously high value for the 39 m samples collected in 1997, the results over the three years of sampling maintained a very similar pattern with a higher soil organic matter content close to the CO₂ source with the value decreasing as one moves away from the source, up the slope. Only the data from 1997 has statistical support for the differences between the values (Figure 3-3). There is no evidence of carbon accumulation after two years of treatment within the simple fumigation ring placed 19m away from the CO₂ source. Although not statistically supported, there is some evidence to suggest that long-term treatment (more than 70 years) is causing changes in the carbon storage.

Figure 3-4 shows the soil microbial carbon content of the C₄ grassland over a period of three years under different CO₂ treatments. The variability in the data was large and so there are not statistically supported differences among the samples within each year. The matched samples collected in 1997 and 1998 are remarkably similar including the peak seen at 39 m.

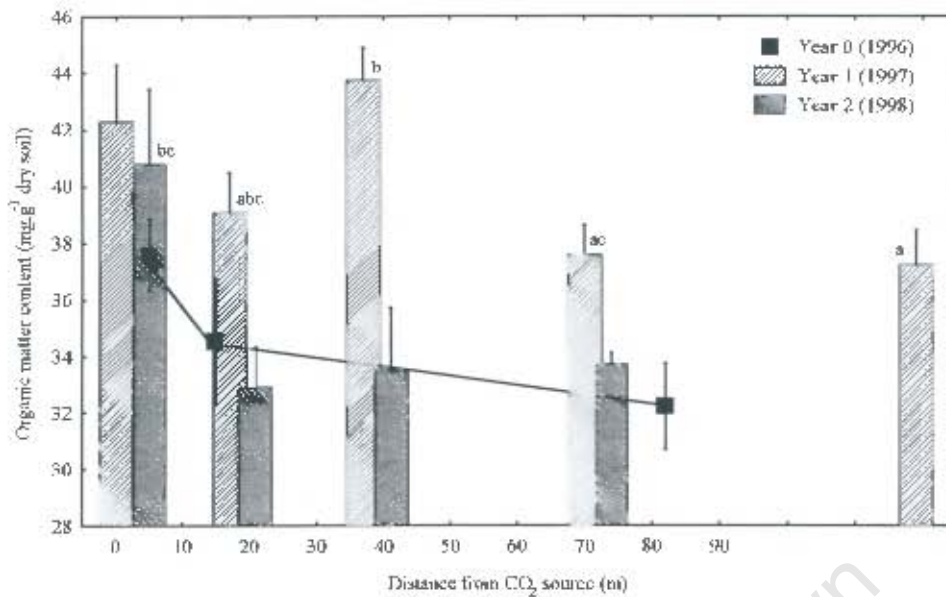


Figure 3-3 : Total organic matter content of soils collected from Pleasant View Farm. The points and bars are means \pm se. Closed square = 1996 baseline sampling of vegetation naturally exposed to a point source of CO₂ (n = 3), hatched bar = 1997 sampling, one year after establishing the fumigation ring 19 m from the CO₂ source (n = 5, bar offset by -2 m), grey bar = 1998 sampling, two years after establishing the fumigation ring (n = 5, bar offset by +2 m). The 1997 symbol on the far right of the figure is a sample taken from the N facing hillside opposite the study area. The letters indicate statistically significant (ANOVA, $p < 0.05$) differences within each year of sampling.

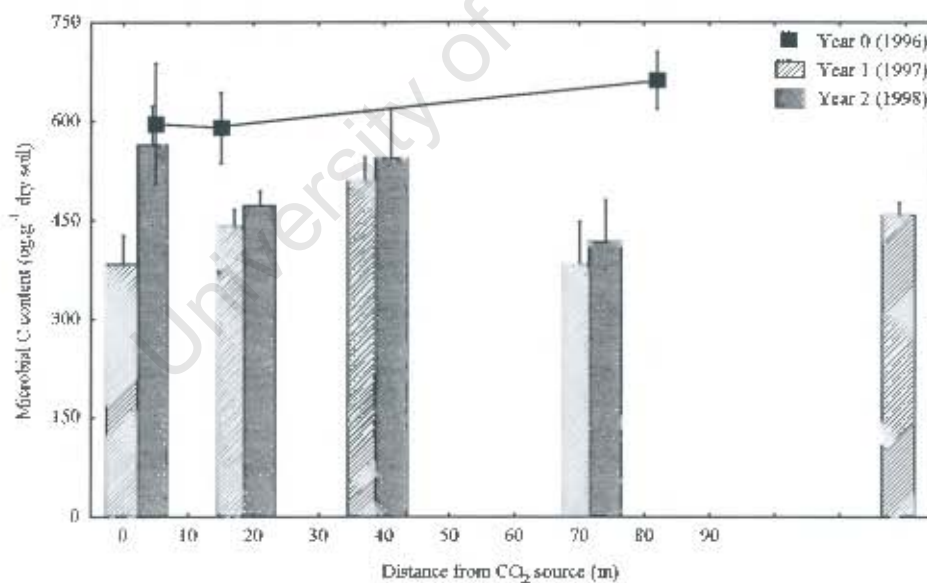


Figure 3-4 : Microbial carbon content (measured using chloroform fumigation extraction) of soils collected from Pleasant View Farm. The points and bars are means \pm se. Closed square = 1996 baseline sampling of vegetation naturally exposed to a point source of CO₂ (n = 3), hatched bar = 1997 sampling, one year after establishing the fumigation ring 19 m from the CO₂ source (n = 5, bar offset by -2 m), grey bar = 1998 sampling, two years after establishing the fumigation ring (n = 5, bar offset by +2 m). The 1997 bar on the far right of the figure is a sample taken from the N facing hillside opposite the study area. There are no statistically (ANOVA, $p > 0.1$) significant differences within each year of sampling.

The only difference is seen in the sample collected at the CO₂ pipe with the one taken in 1998 having a much higher value than that of 1997.

The extractable carbon content of the soil can be equated to the soluble carbon in the soil. The changes over time and with different CO₂ treatments can be seen in Figure 3-5. The only statistically significant differences were found in 1997 with the value at 72 m being different from the 2 m value (Figure 3-5).

The microbial quotient values can be seen in Table 3-2. Within each year there was no difference in the values ($p > 0.10$). Further, there were no clear trends showing changes of this integrated measure over the three year study period thus indicating that the CO₂ treatment was not affecting the functioning of the soil microbes nor the allocation of carbon between the two pools (total carbon and microbial carbon).

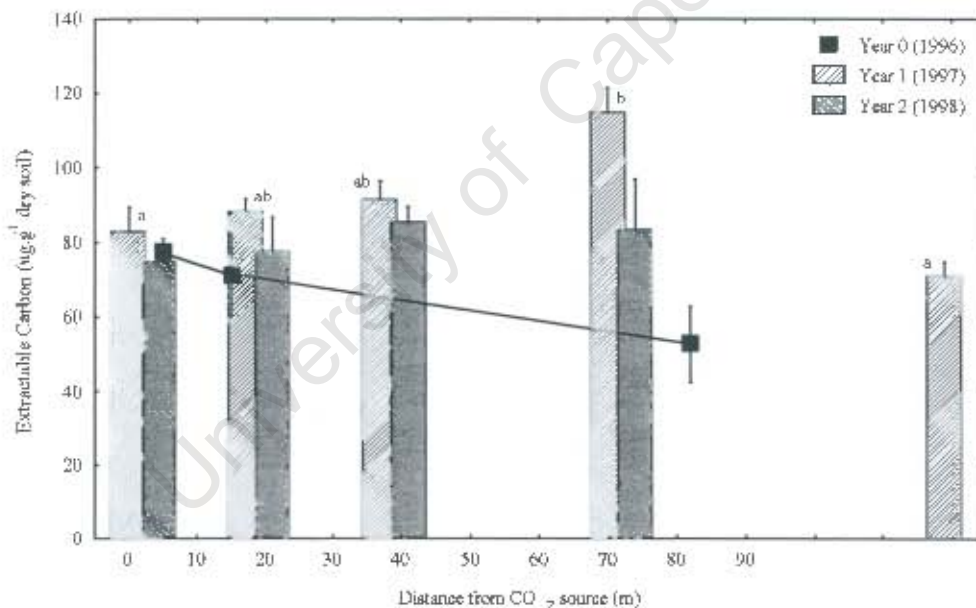


Figure 3-5 : Extractable carbon from soils collected from Pleasant View Farm. The points and bars are means \pm se. Closed square = 1996 baseline sampling of vegetation naturally exposed to a point source of CO₂ ($n = 3$), hatched bar = 1997 sampling, one year after establishing the fumigation ring 19 m from the CO₂ source ($n = 5$, bar offset by -2 m), grey bar = 1998 sampling, two years after establishing the fumigation ring ($n = 5$, bar offset by $+2$ m). The 1997 bar on the far right of the figure is a sample taken from the N facing hillside opposite the study area. The letters indicate statistically significant (ANOVA, $p \leq 0.05$) differences within each year of sampling.

Table 3-2 : Microbial quotient values (microbial biomass C / organic C expressed as a percentage) for the Natal grassland study.

Year	Distance from CO ₂ source (m)	Site	n	Mean microbial quotient	se
1996	5		4	1.59	0.22
	15		2	1.81	0.05
	82		6	2.07	0.17
1997	2	CO ₂ pipe	5	0.94	0.07
	19	CO ₂ plot	5	1.19	0.11
	39	lower control	5	1.22	0.13
	72	top control	5	1.06	0.26
		N hillside control	5	1.26	0.03
1998	3	CO ₂ pipe	5	1.39	0.13
	19	CO ₂ plot	5	1.47	0.14
	39	lower control	5	1.70	0.37
	72	top control	5	1.25	0.28

The oligotrophic Fynbos

Carbon dioxide treatment did not have much effect on the soils in the Fynbos systems investigated (Table 3-3). As was found in earlier investigations, Chapter 2, some large CO₂ effects were seen but these were not statistically significant due to the variability in the data.

In the single species experiment the total soil carbon was unchanged in 568 days of treatment while there were quite large percentage changes in the soil moisture content and microbial carbon content although these differences were not statistically supported. The data in this experiment were analysed using a nested ANOVA design which separates between chamber effects and treatment effects. Generally there were no significant chamber effects. The one exception was the significant chamber effect obtained for the total soil carbon. In this case the treatment (CO₂) effect was the smallest of all the parameters measured.

There was a significant ($p \leq 0.05$) increase in 0.5 M K₂SO₄ extractable carbon content after treatment with CO₂. In percentage terms, there was nearly twice the amount of soluble carbon present in the soils that had been treated with CO₂.

In the mixed species experiment, there were no significant treatment effects and when expressed in terms of CO₂ effect one can see that the effects were much smaller in this system, ranging from +10 to - 8% (Table 3-3).

There was no CO₂ effect on the microbial quotient of the soils in either the ss or ms experiments ($p > 0.1$, Table 3-4).

Table 3-3 : Moisture and carbon pool assessments of the soils used in the single species and mixed species studies of the effects of elevated atmospheric CO₂ on the Fynbos plant-soil system. Values are means ± standard error (n = 4 samples per chamber for the ss experiment and n = 16 in the ms study.) ns Not significant, * p ≤ 0.05

	Single species experiment			Mixed species experiment		
	Ambient	Elevated	CO ₂ effect ^a	Ambient	Elevated	CO ₂ effect
Soil Moisture (%)	5.94 ± 0.704	4.68 ± 0.878	-21.21% ns ^b	10.08 ± .460	9.94 ± 0.415	-8.31% ns ^c
Total soil carbon (mg.g ⁻¹ dry soil)	17.6 ± 0.96	17.9 ± 1.13	+1.71% ns	51.0 ± 1.87	48.6 ± 1.28	-4.72% ns
Microbial carbon (µg.g ⁻¹ dry soil)	244.1 ± 24.3	201.9 ± 18.4	-17.26% ns	858.5 ± 48.4	822.2 ± 68.1	-2.13% ns
Extractable carbon (µg.g ⁻¹ dry soil)	13.9 ± 3.4	25.8 ± 4.1	+85.14% *	96.1 ± 8.5	105.4 ± 8.4	+9.68% ns

a: ((E-A)/A)*100 (Drake *et al.*, 1996)

b: ANOVA with 3 chambers nested within 2 treatments

c: t-test

The soil moisture, total organic carbon, soil microbial carbon and extractable carbon was different between the two experiments (p ≤ 0.01) largely as a result of the addition of organic material to the soil of the ms study.

The differences between the two experiments was less marked for the microbial quotient (Table 3-4); even though they were different (p ≤ 0.01). The ss elevated value was statistically different to the two ms values while all of the other numbers were statistically equal (ss ambient and the two ms groups).

Table 3-4: Microbial quotient values (microbial biomass C / organic C expressed as a percentage) for the Fynbos study.

Study type	Treatment	n	Mean microbial quotient	se
Single species	Ambient CO ₂	10	1.34	0.12
	Elevated CO ₂	12	1.13	0.07
Mixed species	Ambient CO ₂	16	1.70	0.10
	Elevated CO ₂	16	1.74	0.17

Discussion

In broad terms, three classes of experiments have been used to investigate the CO₂ effect on carbon pools within the soil: a long-term field trial with short-term components (the Natal grassland), a medium-term microcosm study (the Fynbos system) and a medium-term pot investigation (a Fynbos species).

In the Natal C₄ grassland system, four CO₂ treatments were investigated. They were a long-term (more than 70 years) treatment (next to the pipe in 1996 to 1998), a two-year

treatment (1998 sampling), a one-year treatment (1997 sampling) and no CO₂ exposure. There was a clear accumulation of soil moisture as a result of CO₂ treatment. No trends were apparent in the soil microbial biomass and soil extractable carbon measurements while there seemed to be a slight accumulation of total soil carbon after a long-term treatment with CO₂.

It was only possible to collect samples once a year and this was at slightly different times each year. The results indicate that season and rainfall affect the carbon economy of the soils. The challenge would be to separate these climatic effects from the CO₂ effects. It would have been prudent to take measurements down slope of the CO₂ source but this was not possible due to a long band of excavation running along the contour immediately below the pipe which would severely affect the hydrology of the slope. More intensive sampling through the year would help to reveal the effect of the interaction between CO₂ and moisture on the microbial population and soil carbon. Wardle (1998) highlights the fact that soil microbial biomass is not a static entity and that temporal dynamics in the biomass are likely to be extremely important in understanding nutrient cycles. Hu *et al.* (2001) demonstrated a strong temporal effect in their elevated atmospheric CO₂ investigations while Dalal (1998) concludes that better understanding of seasonal trends in soil microbial dynamics is needed, while showing that the soil microbial biomass appears to peak during maximal root activity. Insam (1990) found that mean annual temperature significantly influenced soil microbial biomass with high temperatures resulting in lower microbial biomass values. In his review of 58 studies that investigated the temporal dynamics of the soil microbial biomass, Wardle (1998) found that, on qualitative assessment, soils from warm temperate and tropical regions (those that do not freeze during winter) show no consistent temporal trends with any kind of seasonality. Correlation studies of the coefficients of variation revealed that latitude, soil organic carbon and pH were all important determinants of biomass C values but a substantial amount of the variation is accounted for at an individual site level (Wardle, 1998). There was a temperate ecosystem bias in this analysis due to the fact that most of the studies included were performed in temperate systems. Further investigation of the tropical soils studied in this project would help to confirm or refute Wardle's (1998) qualitative observations.

Morris & Boerner (1999) made a detailed investigation of spatial variability of microbes in an Ohio hardwood forest developing a model of the microbial patterns that was independent of scale. They found that bacterial biomass and soil carbon were significantly affected by soil chemistry and soil moisture at the time of sampling while fungal biomass was

constrained by long-term moisture patterns and soil texture. When one uses CFE (as opposed to differential fluorescence staining as was used by Morris & Boerner, 1999) no distinction is made between the bacterial and fungal components of the soil microbial biomass. Applying the above findings to the Natal study one can assume that soil texture and chemistry was consistent over the sampling area. The soil microbial biomass was thus being influenced by both the current soil conditions along with long-term moisture conditions highlighting the value of repeated measures in order to separate the influence of these two factors. Considering the variability seen in the microbial biomass C values, it can be concluded that either the soil fungi are not being influenced by any long-term soil moisture effects of the CO₂ treatment or the fungal component in the soil is small when compared to the bacterial component which is more variable. The need for an investigation at this level of detail at this site is thus emphasised.

In a six year elevated CO₂ investigation of a moderately fertile grassland sited at the Jasper Ridge Biological Preserve, Hu *et al.* (2001) found that there was a moderate (ns) increase in soil carbon and a significant increase in soil microbial biomass at certain times. Nitrogen contents were unchanged causing a higher C:N ratio in the microbial biomass possibly caused by changes in the bacterial to fungal ratios. The authors also highlight the assertion that future ecosystem carbon dynamics need to be considered in terms other than merely flows of carbon and rates of decomposition. In their case they highlight nitrogen changes while in the current study it can be seen that moisture was having an influence (Hu *et al.*, 2001). Allen *et al.* (2000) found no effect on the soil microbial biomass carbon in a maturing loblolly pine forest exposed to elevated atmospheric CO₂. In contrast to these two reports, Williams *et al.* (2000) found that in tallgrass prairie there was a significant increase in total soil carbon as a result of eight years of CO₂ treatment. Along with this, soil moisture contents were increased while soil microbial biomass showed some increases but the results were not as consistently different as was found for other measurements. The authors concluded that there is the potential for other drought prone grasslands from around the world to act as carbon sinks.

Campbell *et al.* (1997) showed in a small microcosm study how plants in an artificial sward responded in different, non linear ways throughout a range of applied CO₂ treatments (350, 525 and 700 ppm) while Gill *et al.* (2002) have confirmed this non-linearity in grassland systems with regards to nitrogen availability. It is possible that the Natal C₄ system was showing a variable response depending on surrounding carbon dioxide concentration or even period of CO₂ exposure. It would only be possible to differentiate

between seasonal effects and non-linear effects after a long period of regular sampling so that generalised patterns of response could be elucidated.

The slight accumulation of carbon around the CO₂ spring did not develop in the fumigated plot due to the short period of the experiment coupled with the conservative nature of soil organic matter. Tate and Ross (1997) show that one would be unlikely to detect changes in soil carbon storage over short-term experiments because of the gradual way in which carbon pools can change along with the great spatial variability present in soils that would obscure any small changes that are occurring. Even at the very early stages of investigating soil microbial responses to elevated atmospheric CO₂, O'Neill *et al.* (1987) found that there was little treatment effect on natural forest soils and that the variability within samples was too large to allow distinction of the treatments. Hungate (1996) has also shown that the power of statistical analyses is too low to identify small, consistent changes in carbon addition that could have a dramatic overall effect on ecosystem carbon dynamics.

Campbell *et al.* (1997) highlighted the scarcity of data on the direct effects of elevated CO₂ on water supply at the landscape scale in grassland ecosystems. The current study showed that there was a clear CO₂ effect on the soil moisture content that was particularly apparent during dry periods. This could lead to a late season effect on plant growth and soil carbon dynamics; but from these results and others (Stock *et al.*, in prep), this extra moisture did not encourage more overall plant biomass production nor changes in microbial carbon content.

Turning attention to the Fynbos investigations. In order to draw more generalised conclusions on the responses of the below-ground processes to elevated atmospheric CO₂ one needs to identify similarities and differences between the two experiments. Both the single species and the mixed species experiments were run for a long period of time (about two seasons) and kept under similar growth conditions in the same glass house allowing sufficient time for stable, mature microbial communities to develop.

Plants are the intermediaries between the atmosphere and the soil and so plant responses to atmospheric change will influence the pattern of response of the soil microbes, especially those present in the rhizosphere. The number of plants and number of species would affect the microbial responses with the ss study investigating interactions between the microbes and *Felicia* roots alone. The ms study was much more complicated with the presence of many more plants along with four other species. The samples were collected from near the *Felicia* plants in order to achieve a level of consistency between the two experiments. Pots were used in the ss experiment which would have resulted in wider

fluctuations in temperature and moisture than that encountered in the ms chambers that contained a large soil volume. These abiotic factors have the potential to affect soil microbial populations as well. Furthermore, additional nutrients and organic matter were added to the ms chambers. This increased the microbial growth (Table 3-3) and has the potential to change the population dynamics due to the availability of nutrients allowing for the decomposition of carbon additions to the soil. Soil moisture, organic matter and extractable carbon was also enhanced by the *Fynbos mix* additions to the soils of the ms chambers.

The results show that these factors along with the applied CO₂ treatment had very little effect on the soil carbon dynamics (Table 3-3). This indicates that the soil microbes are not carbon limited; other factors are responsible for controlling the flow of carbon through the soil. Fynbos is a typical Mediterranean-type ecosystem that is characterised by having very low nutrient soils (Specht & Moll, 1983). It is possible that this nutrient limitation is having more of an effect on the soil microbial growth than the presence of additional carbon being added to the soils in the form of root exudates. This is supported by the increase in soil microbial biomass in the ms study compared to the ss experiment whose soils had not been augmented with nutrients. The significant increase in extractable carbon resulting from CO₂ observed in the ss experiment provides further evidence of the microbes being unable to utilise the additional carbon supply. There was an increase (non-significant) in extractable carbon in the ms experiment but it would be difficult to measure this effect in the larger soil volumes where the roots are more dispersed and the soluble carbon can leach a greater distance away from the rooting zone. The observation that plants from nutrient-poor systems such as the Fynbos are not carbon limited has been discussed (Stock *et al.*, 1992). It appears that the limits on the plant growth and development are constrained by the availability of soil nutrients. These results are interesting because it can be seen from the current experiment that a similar constraint also appears to be operating on the soil microbial biomass. Although there is no statistical support, the soil microbial biomass in the ss study was reduced by 17% after treatment with CO₂ while in the ms study it only dropped by 2%. This smaller difference in biomass could be the reason why the ms experiment did not show as great an accumulation of soluble carbon. Similar microbial biomass is being supported on the higher nutrient availability soils in the ms chambers allowing any additional soluble carbon to be consumed (Niklaus & Körner, 1996). Similar to the ss study, a drop in total count of soil bacteria has been observed in other experiments, e.g. Hodge *et al.* (1998) and non-significantly in Rillig *et al.* (1997) although the explanation of it is unclear. One

possibility could be the increase of the C:N ratio of the exudate which would disfavour microbial growth especially in nutrient limited soils. Hodge *et al.* (1998) used a short-term study (21 days) on ryegrass (*Lolium perenne*). A longer investigation (115 days) of the same species gave an increase in soil microbial ¹⁴C when assessed using a fumigation based method with the increase being in proportion to the increase in the root biomass (van Ginkel & Gorissen, 1998).

The total organic carbon in the soil was little changed and this is not surprising considering the fact that this soil pool is about 85 times larger than the more labile pools investigated. This pool is also predominantly made up of “historical carbon”, carbon that was present in the soil prior to the start of the experiment, and so it can take considerable time for the treatment effects to be observable in the total soil carbon pool.

Kandeler *et al.* (1998) concluded their investigation of an artificial temperate system by saying that under conditions of low soil nutrient availability, the effects of climate change on the soil microbial community and processes are likely to be minimal and largely unpredictable. Many authors have shown that in natural and semi natural systems soil microbial biomass is largely unresponsive to increased atmospheric CO₂ concentrations (Oren *et al.*, 2001, Allen *et al.*, 2000, Insam *et al.*, 1999 Niklaus & Körner, 1996, as examples).

Many studies have shown little to no change in soil microbial activity or biomass in response to CO₂ alone but frequently a response is shown with the addition of nutrients to the system, particularly nitrogen (Niklaus & Körner, 1996, Klironomos *et al.*, 1996). In the two systems studied here, C₄ grassland and Fynbos, there is little chance of generalised enhancement of the soil's nutrient status through pollutant deposition and so these issues have been ignored in the interests of economy.

In the Natal system, the soil extractable carbon did not change indicating that the plants' roots are not releasing more carbon and so the soil microbes are effectively unresponsive to the atmospheric changes due to the effect not penetrating to this level in the ecosystem. In the Fynbos there are indications of an increase in exudation but no additional microbial growth. There appears to be a slight decrease in microbial biomass. It can be seen that these two systems are behaving in quite different ways that may lead to different outcomes under global change scenarios. Following the predictions outlined by Niklaus & Körner (1996), (i) in the absence of fertiliser addition, microorganisms cannot respond to addition carbon inputs caused by elevated carbon dioxide on the plants, (ii) Under ambient CO₂ conditions, fertilising causes an increase in above ground plant growth with no

additional carbon release below-ground and so soil microbes become carbon limited even though their mineral needs are satiated and (iii) when CO₂ and nitrogen are supplied together, both limitations on the microbes are relieved and so this pool is able to respond to the changes. From the above evidence one can conclude that the Fynbos was behaving according to prediction (i) with further support for the conclusion coming from the observations made in the more nutrient-rich mixed species study. Another study comparable to the species and environment investigated for the Fynbos study has also shown the lack of response due to CO₂ alone while responding strongly to the combination of CO₂ and nutrients (Klironomos *et al.* 1996). In the C₄ system, the limitation on the microbial response was coming from the plants themselves as opposed to nutrient factors.

It is suggested by Wardle and Ghani (1995) that microbial C to organic C ratios are more sensitive to ecological changes than measures of microbial biomass alone. Other authors such as Barajas Aceves *et al.* (1999) and Dalal (1998) state that linked variables such as these may prove useful as indicators of soil quality once a large enough database of values are available. The reason why these ratio measurements are useful is because the active soil organic matter (microbial measurements) react quickly to change but may be masked by fluctuations in the total carbon content of the soil. Integrating the two measures highlights the relative changes that are occurring. Lundquist *et al.* (1999) warn that the microbial biomass can change extremely rapidly and so may not be a stable indicator of active soil organic matter. By looking at these ratios in the context of elevated atmospheric CO₂ research one is able to see if there is a net accumulation of soil carbon occurring or whether the soil microbial population is increasing with increasing organic matter and thus the net effect is unchanged (Anderson & Domsch, 1989). Insam (1990) presents an equation that can be used to ascertain the expected equilibrium C_{mic} to C_{org} ratio using the ratio of mean annual precipitation and mean annual evaporation. One can then see if the actual C_{mic} to C_{org} ratio is above or below this expected equilibrium line, indicating whether the soils are accumulating or losing carbon. In these experiments the evaporation values were not known and so one is unable to draw conclusions on the possible changes in carbon storage occurring under the CO₂ treatment.

The soil microbial population is a small but key component in the soil sequestration of carbon (Dalal, 1998). The capacity of the soil to store additional organic compounds is also highlighted by the author. If the soil has absorbed all of the organic material that it can, or if the resources available are fully utilised by the maximum density of soil microbes, then if more of a non limiting resource (carbon in this case) is added to the system there would be no

effect on sequestration in the soil (Gill *et al.*, 2002). Wardle and Ghani (1995) state that small differences in the microbial biomass in the soil are likely to be ecologically insignificant and so the limitations of the CFE method in only identifying large changes is all that is necessary if one wants to make predictions on the long-term effects of global change. Conclusions do however need to be made with care considering the assertion by Hungate *et al.* (1996) that changes may be occurring but we are not sensing them due to the crudeness of our methods and weakness of statistics.

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SOIL MICROBIAL COMMUNITY RESPONSES TO ELEVATED ATMOSPHERIC CO₂ TREATMENT IN NUTRIENT-POOR FYNBOS SOIL.

Introduction

The carbon dioxide released by human activities is currently being absorbed by the oceans, accumulating in the atmosphere and being taken up by the terrestrial biosphere (GCTE COST, 2000). The net carbon storage within a terrestrial ecosystem depends on the balance between carbon fixation by the plants and carbon release from microbial decomposition (Hu *et al.*, 1999). Changes to the decomposition rates through disturbance or restoration efforts could have a marked effect on the release or uptake of carbon from the soils. It has now become clear that an understanding of the response of soil microbial populations to global changes is critical for the understanding and prediction of how ecosystems will respond to these changes (Sadowsky & Schortemeyer, 1997 and Hu *et al.*, 1999).

In the past, investigators studied gross aspects of carbon sequestration, soil microbial biomass and change in nutrient flows under conditions of global change in a wide range of habitats and environmental conditions. Results were frequently unclear which prompted the research teams to try to elucidate more subtle changes in the responses of soil microbes to imposed perturbations. It is likely that changes will occur much sooner in microbial communities due to their short generation time, rapid turnover rate and brisk evolution. Bending *et al.* (2000) showed how microbial substrate utilisation patterns were very sensitive to soil management practice while traditional measures of soil quality such as soil organic matter did not respond to the changes being studied.

Researchers are also now considering potentially less responsive systems such as natural ecosystems and areas that are very low in nutrients. Insam *et al.* (1999) state that their investigation of an artificial tropical ecosystem is among the first to report on a microcosm scale investigation of CO₂ effects on a low nutrient system. The authors show few effects on a suite of factors investigated and conclude by highlighting the difference between their results of a low nutrient system versus the established results and predictions of

soil microbial response that were based on fertile experimental systems with a large background pool of “old” carbon.

It is important to appreciate the indirect effect of CO₂ enrichment on soil microbes through changes in root growth, fine root turnover and exudation. The direct carbon dioxide effect is minimal due to natural enrichment from respiration within the soil (Sadowsky & Schortemeyer, 1997). Because soil microbes are primarily fed from root derived substances one would expect that any environmental factor that is changing the growth of a plant should change the behavior of the soil microbial community surrounding the plant (Klironomos *et al.*, 1996). The authors presented a detailed study of the responses of rhizosphere microbes around *Artemesia tridentata* and suggest that below-ground processes need to be studied in greater detail than has been done before in order to show many of the subtle effects that are occurring within below-ground food webs.

There are a variety of methods that can be used to analyse microbial populations. These include metabolic tests (Biolog, Crystal, Minitek and API), Genetic analyses (DNA, rRNA) and measurement of cellular components (PLFA, phospholipid fatty acid analysis and FAME, Fatty acid methyl esters) (Truu *et al.*, 1999). The Biolog bacterial identification system was developed to taxonomically identify pure cultures of bacterial strains (Bochner, 1989, Bochner & Savageau, 1977). The technique of using these identification plates to investigate whole environmental microbial samples was developed by Garland & Mills (1991) and expanded upon by Zak *et al.* (1994). This system has been used in many areas of soil biology including investigations of pollution effects (Kelly *et al.*, 1999), farming practice (Bending *et al.*, 2000) and characterisation of tropical and temperate *Rhizobium* strains (McInroy *et al.*, 1999).

Biolog has been criticised because it does not capture the complete bacterial diversity. Only aerobic, fast growing eutrophs that are accessible within the soil matrix (not bound to particles or buried in soil pores) are being measured with Biolog (Mayr *et al.*, 1999^a). Furthermore, Verschuere *et al.* (1997) found that the Biolog pattern was most strongly influenced by the fastest growing microbes, especially when the inoculation density was low; as would be found in environmental samples. These limitations need to be kept in mind when interpreting the results but there are advantages of this method over other metabolic methods including the fine resolution obtained with the 95 carbon substrates that are tested (Truu *et al.*, 1999). Another key advantage is that Biolog is measuring components of the active microbial pool which makes it potentially more sensitive to changes than the other

methods that monitor whole community parameters which includes a large microbial component in an inactive state (Hu *et al.*, 1999).

Waldrop *et al.* (2000) attempted to link PLFA biomarker data from several soil microbial communities with functional measures of soil microbial populations such as microbial biomass, enzyme activities and Biolog analysis. No correlation was found between the PLFA and Biolog population measures. As has been mentioned above, Hu *et al.* (1999) highlights that these methods are investigating components of the population in different activity states. This was acknowledged by Waldrop *et al.* (2000). Furthermore, although Waldrop *et al.* (2000) used a widely accepted analysis method for the Biolog data, the Garland and Mills scaling method has raised concerns of distortion of the data. Earlier, Buyer and Drinkwater (1997) investigated the use of Biolog in parallel with the better defined and studied method of PLFA emphasising the value of Biolog due to its technical simplicity. Analysis involved normalising the Biolog data by dividing the well absorbances by the average well colour development (AWCD) followed by a complicated two step data reduction and subsequent analysis procedure. This procedure uses first Principal Component Analysis, then Analysis of variance and Canonical Discriminant Analysis. I feel that the analysis could have been improved by using canonical correspondence analysis (CCA, ter Braak, 1986) which allows direct investigation of the environmental effects on the species data rather than hoping sensible groups will be resolved from the latent structure in the data (Kent and Coker, 1992). Sampling date was found to be the most significant factor in Buyer & Drinkwater's (1997) analysis and this factor could have been used as a co-variable in a CCA analysis to allow for analysis of the management history and residue quality on the microbial populations. This influence of time on the results affirms the findings of Hu *et al.* (1999). Buyer & Drinkwater (1997) found that both analysis methods demonstrated the difference between management history of the soils, although the difference between assessing community structure versus potential community function was highlighted with the conclusion that these two methods are complementary rather than being equal.

One of the problems with Biolog investigations is the low level of replication compared to the number of response variables (carbon substrates)(Lowit *et al.*, 2000, Insam & Hitzl, 1999, Hitzl *et al.*, 1997 and Howard, 1997). Lowit *et al.* (2000) point out that these problems have been discussed for a long time. The authors cite investigations that used Monte Carlo simulation tests to show that significant eigenvalues for the factors can be obtained reliably when the percentage of variance explained by the factors exceeds a set

value. Furthermore, it has been shown (Legendre & Legendre, 1998) that the first few eigenvectors are little affected by a low sample to variable ratio. A correct interpretation of the ordination in reduced space is thus still possible.

Lowit *et al.* (2000) investigated the statistical power of the Biolog system in natural water samples and found that it was sufficient to use two Gram negative (GN) plates at each sampling site to separate the sites. The authors go on to say that in most cases environmental variability is greater than analytical variability and so it is better to increase the number of environmental samples rather than the number of replicate plates per sample.

The Biolog system is therefore an attractive analytical tool due to the availability of the microtitre plates along with the technical simplicity and minimal equipment requirements (Verschuere *et al.*, 1997). This analysis generates a tremendous amount of data and with them it is possible to derive three components from the response: overall rate of development, diversity of substrate use and pattern of substrate utilisation (Garland 1997).

This chapter reports on the use of the Biolog Bacterial Identification System to investigate the community level physiological profiles (CLPP) of soils from two experiments using *Felicia aethiopica* ssp. *aethiopica* plants grown at two different atmospheric CO₂ concentrations using an open top chamber system in a glasshouse..

The objectives of the study were:

- (1) to see if there were measurable CO₂ induced changes in the CLPP of microbial communities from the root and surrounding bulk soil of the fynbos plant *Felicia aethiopica* ssp. *aethiopica* grown in nutrient-poor soil.
- (2) to compare the results from the single species, pot study to the more natural mixed species microcosm study to see if any predictions could be made on how wild systems would respond to the expected global changes that are occurring.

Some of the broader scale implications of the results along with comments on the analytical procedure are also discussed.

Materials and methods

Plant material and establishment

Felicia aethiopica ssp. *aethiopica* (Kirstenbosch Botanical Gardens accession number: 775/88) plants were germinated from seed in approximately 850 g (dry weight) fynbos soil in PVC planting tubes (7.0 cm i/d, 20 cm long) in a phytotron chamber with a 12 hour, 20°C/8°C day/night cycle. The daytime light intensity was 139 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. The pH,

nitrogen (Kjeldahl digestion followed by colorimetry) and phosphorus (Tri-acid digestion followed by Murphy and Riley colorimetric determination) contents of the soil was 3.48, 0.76 mg.g⁻¹ and 35.08 µg.g⁻¹ respectively. Three seeds were planted in each tube, then watered with 2 ml of *Kirstenbosch Instant Smoke Plus Seed Primer* (National Botanical Institute, Claremont, South Africa) and covered with a petri dish to maintain humidity. This cover was removed once the cotyledons on the seedling were established. After 40 days of growth (11 March 1996) the pots of seedlings were randomly assigned to open-top chambers situated in the University of Cape Town Botany Department's new glasshouse. One month later, planting tubes containing dead plants were replaced with tubes containing plants that had been grown in the phytotron chamber for this time. After 107 days of growth the pots were thinned down to one plant per pot. The plants were removed by cutting them at the soil level and leaving the roots in the soil to ensure minimum disturbance to the remaining seedling. The plants were regularly watered with deionised water and never allowed to become water stressed. The plants were not fertilised throughout the experimental period and they were shuffled three times within each chamber to eliminate position effects on their growth. The plants were harvested after 568 days of growth (22 August 1997). Three plants were taken from each chamber giving a total of 9 plants per treatment (3 ambient and 3 elevated chambers). Two separate soil extracts and duplicate samples from the one root extract were analysed using the Biolog bacterial identification system (Biolog, Inc., Hayward California, USA).

Felicia plants germinated from the same batch of seeds, referred to above, were used in another study investigating the effects of CO₂ on fynbos competition (Stock *et al.*, 1997). Four other species were used in the experiment (*Leucadendron laurosum*, *L. xanthoconus*, *Salvia africana-lutea* and *Podalyria sericea*). The mixed plant communities were grown for 700 days in large volumes of soil within the open-top chambers. Five *L. laurosum* plants were equidistantly arranged around the chamber with a ring of five individuals of the five study species planted around each *L. laurosum* plant. The soil in these chambers was from the same source as that used in the pot experiment (Orange Kloof Reserve on Table Mountain) except that it had been amended with 50% v/v *Fynbos potting mix* (Kirstenbosch Botanical Gardens). The *Felicia aethiopica* ssp. *aethiopica* roots were sampled along with bulk soil from around the *Felicia* plants and analysed using the Biolog identification system. Three root and 3 soil samples were analysed from each of the 16 microcosm chambers (8 ambient, 8 elevated).

Growth chambers and experimental conditions

The open-top chambers consisted of a plastic rubbish bin (50 cm in diameter) with an 80 cm high, removable polyethylene cylinder on top. A fan drew air from outside the glasshouse through a 16.0 cm diameter manifold before being divided into six 7.0 cm i/d PVC tubes that led into the centre of the chamber. The air was deflected horizontally by a 10 cm diameter PVC baffle 3 cm above the opening of the tube. Air flows were high enough to ensure at least 3 replacements of the chamber air every minute. Pure CO₂ was bled into the individual 16 cm diameter pipes that led off the manifold before the diameter was reduced to the 7.0 cm pipe. The CO₂ concentration in the chambers was monitored for periods of 24 hours at different times throughout the experiment using an ADC LCA2 infrared gas analyser (The Analytical Development Co. Ltd., Hoddesdon, Herts, England) attached to a monitoring computer. An average CO₂ enrichment of ambient plus 340 ppm CO₂ was achieved.

Harvesting and analyses

In the single species pot experiment (ss), the plants were cut at soil level with the above ground parts being analysed separately. The soil was tipped out into a sterile tray and the roots were gently separated from the soil. Soil adhering to the roots was shaken off. The bulk soil was passed through a 2 mm analytical sieve and visible root material was picked out of the soil. A subsample of the bulk soil was analysed for moisture content by means of oven drying at 107 °C for 24 hours. The pH of the soil was measured by adding 20g of the air dry soil to 50ml 0.01M CaCl₂ and the organic matter content was assessed by weight loss on combustion at 450°C for 24 hours.

In the mixed species microcosm study (ms), two cores (#17 corer, approximately 100 mm deep) were taken from three equilateral points around the *Felicia aethiopica* ssp. *aethiopica*. Litter and the top 10 mm of soil were removed before placing the sample in a plastic bag. Root samples were gathered as soon as the microcosm was cut open. Three *Felicia* roots were identified, removed from the soil and bagged separately.

In all of these operations with both the soil and root extracts, the equipment was sterilised with ethanol.

BIOLOG Analysis

The community level physiological profile (CLPP) of soil and root samples were assessed using the Gram negative (GN) microplates from the Biolog™ Bacterial Identification System

(Biolog Inc., Hayward California, lot numbers 1106031 and 1206091) using methods adapted from Garland and Mills (1991). Each 96 well microtitre plate contains a complex, low concentration nutrient medium, redox dye chemistry and one of 95 sole carbon sources or a water control (Bochner, 1989). Utilisation of the carbon is monitored by the irreversible reduction of the colourless tetrazolium violet to the coloured, insoluble formazan (Bochner & Savageau, 1977). The peak absorbance of this dye is 595 nm but it is possible to use other wavelengths (Zak *et al.*, 1994, Garland, 1996). In the current case the plate reader used (Anthos Reader 2001, Anthos Labtech Instruments, Austria) was set to read at 620 nm as this filter was the closest to 595 nm available on this machine. The increase in absorbance in each well is a synergistic combination of increasing well colour from the production of formazan along with increasing turbidity from bacterial growth. This was reported by Lindstrom *et al.* (1997) and was very apparent in this experiment.

Samples of 0.5 g (fresh weight) of unwashed root material or 1.0 g (wet weight) soil were suspended in 50 ml phosphate buffer (0.025M KH_2PO_4 and 0.025M K_2HPO_4 , pH 6.9, M Rillig *Pers comm.*) and agitated for 5 minutes and 20 minutes respectively. The root extract was diluted to 10^{-2} while the soil extract was diluted by 10^{-1} . After a maximum of 16 hours after sampling, 140 μl of the diluted extracts were inoculated into each well of the GN microplate. The soils used in these experiments were low in organic matter and high in sand content and so it was not necessary to filter or centrifuge the extract before inoculating the Biolog plates. The plates were incubated at 25 °C in a humid atmosphere created by laying wet paper towel on the floor of the incubator cabinet. The absorbance values of each well on each plate was measured at 48, 72, 96 and 120 hours after inoculation.

Any plate that showed an absorbance of greater than 0.5 in the water control (well A1) at any stage in the incubation was removed entirely from subsequent analyses. The net absorbance was calculated by subtracting the absorbance of the water control from the response wells (Howard, 1997 and Garland & Mills, 1999^a). Each substrate value was calculated by taking the average of the replicate absorbance values and any negative mean values were replaced with zero (Bossio & Scow, 1995). The average well colour development of the Biolog plates was calculated by adding all of the absorbance values and then dividing by 95 (Garland & Mills, 1991).

In this study, a positive well response was defined as one that had a net absorbance of greater than 0.2 (values discussed by Garland, 1997). Using this criterion an estimate of the richness of substrate use is possible.

No scaling or transformation was applied to the absorbance data prior to the ordination analysis. To confirm gross similarity of the samples, several factors (moisture content, organic matter content, pH, wet weight of root material used, and dry weight of the soil samples used) were compared. There were no statistical differences between the samples analysed and so were considered to be sufficiently similar to allow ordination analysis with no scaling of the data. Further confirmation is provided by the average well colour development of the plates over time (see results).

Statistical analysis

Statistical analyses were performed using *STATISTICA for Windows*, release 5.1 (StatSoft, Inc, 1984 - 1996) and *Canoco for Windows* (version 4.02, Microcomputer Power, Ithaca, NY, USA). Details of the tests used are explained with the results.

The absorbance data were analysed in a similar way to that of Bossio and Scow (1995) using Canonical Correspondence Analysis (CCA, ter Braak, 1986). CCA is a direct gradient analysis method that produces an integrated ordination of species together with the associated environmental data (ter Braak, 1987). The carbon substrates on the Biolog plate were treated as the species while the samples were either the averaged soil and root samples from each of the ss or ms experiments. For the ss experiment, actual time of plate reading, CO₂ treatment, soil organic matter content, soil pH and soil moisture were included in the analysis as environmental variables. Similar environmental variables were used in the ms microcosm analysis except for soil pH and actual plate reading time. Time of plate reading (48, 72, 96 or 120 hours) and whether the sample was a root or soil sample were defined as co-variables and these were used to define blocks in the ordination analysis.

The statistical significance of each environmental variable in explaining the observed changes in substrate utilisation patterns was assessed using the automatic forward selection subroutine in *Canoco* along with Monte Carlo permutation tests.

The results of a CCA analysis can be displayed in the form of biplots where the relationships among environmental variables and either the samples from the growth chambers or the weighted averages of the Biolog substrates are shown. The environmental variables are represented by means of arrows that show the direction of the environmental gradient and their length is proportional to the importance of the variable in describing the pattern seen on the diagram.

Carbon substrates that reacted significantly positively or negatively to particular environmental factors were identified using Van Dobben-circles drawn on t-value biplots

generated from the analysis (ter Braak and Looman, 1994). This region of significance is identified by circumscribing a circle with a diameter of the line segment joining the origin and the environmental point. Any species that have their t-value coordinates within this region react positively to the environmental variable. The region of negative influence is found by drawing a similar circle between the origin and the mirror image of the environmental point (ter Braak and Looman, 1994).

Results

Similarity of the soil samples

Carbon dioxide treatment in both the ss experiment and in the ms study yielded no gross effects on the soils used (Table 4-1). This table also shows the similarity in the amount of material that was collected for the Biolog analyses. From this one can see that the results from the Biolog plates can be compared without having to rescale the data. It is possible that there were different concentrations of microbes in the soils of the two CO₂ treatments, however Table 4-1 shows that the analyses were performed on equal amounts of soil and root and so these differences would be due to the CO₂ treatment.

Table 4-1: Investigation of the soil samples in the ambient and elevated CO₂ treated chambers. (Values are means ± standard error. n = 9 for all the single species samples, n = 8 for the elevated mixed species samples and n = 7 for the ambient ms samples) ns Not significant.

	Single species experiment			Mixed species experiment		
	Ambient	Elevated		Ambient	Elevated	
Moisture (%)	6.23 ± 0.761	4.91 ± 0.974	ns ^a	10.25 ± 0.574	10.05 ± 0.591	ns ^b
Organic matter (%)	3.11 ± 0.189	3.16 ± 0.259	ns	8.80 ± 0.425	8.41 ± 0.310	ns
pH	3.68 ± 0.03	3.66 ± 0.02	ns			
Dry weight of Biolog soil samples (g)	0.966 ± 0.005	0.964 ± 0.007	ns	0.911 ± 0.005	0.912 ± 0.005	ns
Wet weight of Biolog root samples (g)	0.503 ± 0.002	0.503 ± 0.002	ns	0.501 ± 0.004	0.499 ± 0.003	ns

a: t-test and ANOVA with 3 chambers nested within 2 treatments

b: t-test

This table also shows the effect adding *Fynbos potting mix* had to the soil in the microcosm study. The unamended soil (ss experiment) shows an organic matter content of about 3 percent while the addition of the potting mix in the ms experiment raised the content to 8.5%. The moisture content has also been increased by the addition of the organic matter although soil volumes and time since the last watering would also play a large part in any differences observed between the soils.

Average Well Colour Development

Garland and Mills (1991) recommend that average well colour development (AWCD) be assessed when investigating Biolog data because this gives one an idea of any large scale differences in the microbial development on the plates.

AWCD of the plates over time in the ss experiment is shown in Figure 4-1. There were no differences in the AWCD at any of the time points (ANOVA with 3 chambers nested within 2 treatments). Colour development was very slow in the first 48 hours (lag phase) followed by a much more rapid development after the first reading. Only 4 readings were made on each of the plates and so it is not possible to see whether the plates had passed through the exponential growth phase. After 120 hours of incubation the plates often had very dense microbial growth on the bottom of the wells and occasionally thick hyphal growth extending above the liquid in the wells. In addition to this, the corner wells contained less liquid than the central wells despite the chamber being lined with saturated paper towel. It is for these reasons that it was not feasible to continue reading the plates after this period of time.

The development of well colour in the samples taken from the mixed species experiment are seen in Figure 4-2. These results are very similar to the single species experiment. There are no statistical differences among the samples at each of the times (ANOVA, $p > 0.1$) along with a clear lag phase up to 48 hours of incubation followed by what appears to be a linear growth phase. The AWCD values in this experiment are higher than those from the ss experiment. This is probably due to the richer carbon environment resulting from the addition of organic material to the soil. More organic carbon in the soil would have two effects, one is a direct effect on allowing a denser microbial community to develop while there would be more soluble carbon in the soil extract and this may allow the microbes to grow better on the Biolog plates.

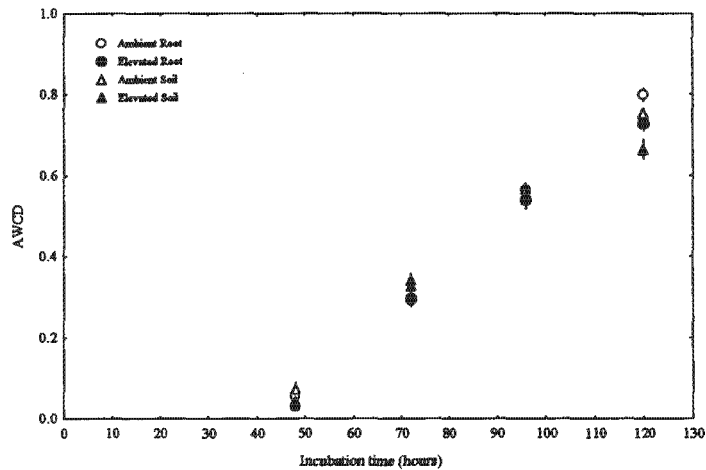


Figure 4-1: The Average Well Colour Development (AWCD) of the Biolog plates over time in the *Felicia aethiopica* ssp. *aethiopica* single species experiment. The points are means \pm se ($n = 9$) with none of the points at each time step significantly different from one another.

This second problem was overcome by discarding any plates that showed a water control (well A1) colour development of more than 0.5. Fourteen ms *Felicia aethiopica* ssp. *aethiopica* root samples, four ms soil samples and no ss experiment samples were excluded from the analyses. This clearly shows the increased presence of soluble carbon contamination in the ms soil analyses.

The undifferentiated AWCD results provide further support to running the ordination analysis on the unscaled plate data (Garland & Mills, 1994). The main reason to normalise the data using methods such as Garland and Mills Scaling (well value/AWCD, Garland and Mills, 1991 and Garland 1997) is when the overall colour development on the plates is so different that any grouping analysis would be excessively influenced by the degree of development of the wells rather than the pattern of well colour formation (Garland, 1996).

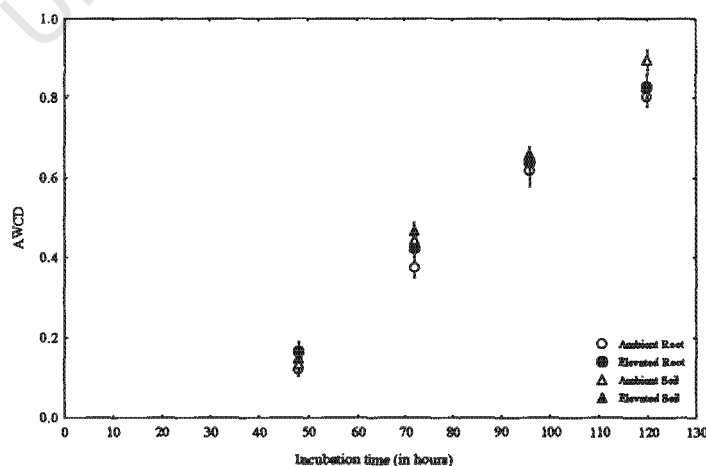


Figure 4-2: The Average Well Colour Development (AWCD) of the Biolog plates over time for the mixed species experiment. The points are means \pm se ($n = 7$ for root extracts and $n = 8$ for the soil extracts) with none of the points at each time step significantly different from one another.

Table 4-2: Numbers of wells showing a positive colour response (net colour development of > 0.2 optical units) on the Biolog plates in the ambient and elevated CO₂ treated chambers. (Values are medians with interquartile range in parenthesis. n = 9 for all the single species samples, n = 8 for the elevated soil mixed species samples and n = 7 for the other three ms samples)

			Time			
			48 hours	72 hours	96 hours	120 hours
Single species experiment	Ambient	Root	1 (6)	46 (10)	65 (6)	76 (4)
		Soil	4 (9)	54 (6)	70 (4)	76 (10)
	Elevated	Root	1 (4)	49 (7)	61 (7)	74 (7)
		Soil	1 (6)	49 (22)	65 (5)	74 (4)
Mixed species experiment	Ambient	Root	18 (30)	55 (10)	74 (9)	82 (3)
		Soil	16 (13)	56 (13)	78 (9)	85 (6)
	Elevated	Root	31 (50)	65 (32)	77 (23)	81 (13)
		Soil	27.5 (19)	65.5 (10)	79 (4)	87.5 (8)

Richness and Substrate Utilisation

An indication of the richness of the microbial communities is obtained by assessing the number of positive wells on the Biolog plates (Table 4-2).

In the ss experiment a Kruskal-Wallis ANOVA by Ranks revealed that at 96 hours there was a significant ($p \leq 0.05$) difference among the 4 groups. At the other times there were no statistically significant differences. Multiple Mann Whitney U tests on the 96 hour data revealed that there was a difference between ambient root and ambient soil samples ($p \leq 0.05$) and between ambient soil and elevated root samples ($p \leq 0.01$). This latter difference has no biological meaning because dissimilar samples were being compared as opposed to the other tests that compared samples within a CO₂ treatment group or sample type (root or soil) group.

Another way of investigating differences among Biolog plate responses is to assess changes in the utilisation of different substrate groups that occur on the plate. It is possible that the physiological profile of a microbial community may react strongly to a particular substrate class but this effect is hidden by the other substrates that have not responded or even responded in the opposite direction. This assessment is made by dividing the 95 absorbance values into 11 substrate groups as outlined by Garland and Mills (1991). An average colour development is then calculated for the wells in each of these groups. Analysis of variance of followed by post-hoc comparisons (Tukey honest significant difference) of all the 11 groups at all 4 times yielded few significant differences in the data (Figure 4-3). The utilisation of amides between ambient root and elevated soil samples after 120 hours of incubation were shown to be different (ANOVA $p \leq 0.05$) although, as has been pointed out earlier, this is a meaningless comparison.

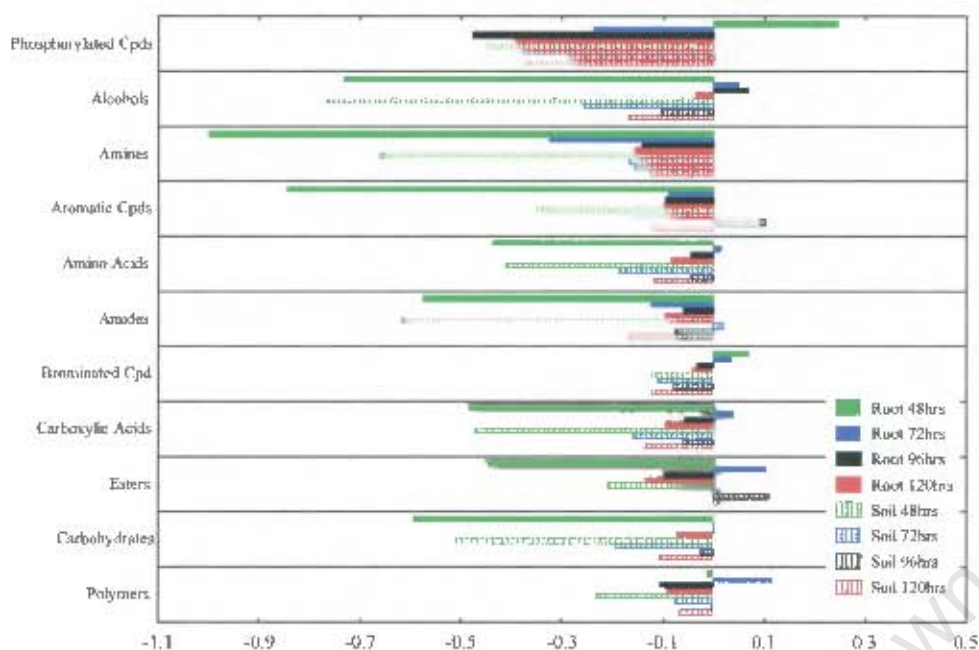


Figure 4-3: The proportional change ((Elevated colour development - Ambient colour development)/Ambient colour development) in microbial utilisation of substrate groups present on the Biolog GN plate inoculated with soil extracts from the single-species experiment. There was a significant difference ($p \leq 0.05$ for 48 hours and $p \leq 0.01$ for the other three times) in colour development between the ambient root and ambient soil samples in the phosphorylated compound group (not visible on this graph). Cpds: abbreviation of compounds.

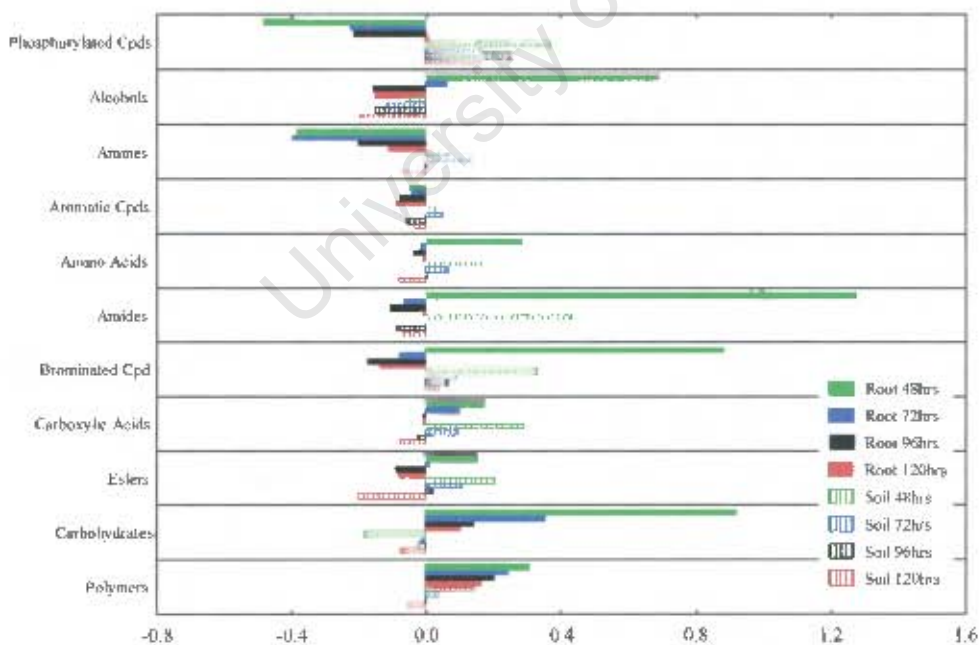


Figure 4-4: The proportional change ((Elevated colour development - Ambient colour development)/Ambient colour development) in microbial utilisation of substrate groups present on the Biolog GN plate inoculated with soil extracts from the mixed-species experiment. After 120 hours of incubation in the Amine group there was a significant difference ($p \leq 0.05$) between the root and soil responses while there was no CO_2 effect (shown in this figure). Cpds: abbreviation of compounds.

The result of interest was the consistent significant difference between ambient root and ambient soil for the phosphorylated compounds for all reading times. The assumptions of the ANOVA were violated due to the non-normality of the data ($p \leq 0.01$ for all of the Levene's test of homogeneities) but these differences were confirmed with the non-parametric Kruskal – Wallis ANOVA by ranks ($p \leq 0.05$ for 48 hours and $p \leq 0.01$ for the other three time points)

The number of wells used by the microbial population in the mixed species study are shown in Table 4-2 and one can see that the numbers are quite different to those seen for the single species experiment. Statistical analysis (Kruskal – Wallis ANOVA by ranks and multiple Mann Whitney U tests) showed no significant differences among the groups at each of the four reading times. This was largely due to the wide interquartile ranges observed in the data. Compared to the ss experiment, there were many more positive wells. This could be due to increased microbial activity resulting from the higher organic matter and moisture contents of the soils in the microcosm experiment. This is a repeat of what was seen in Figures 4-1 & 4-2, the average well colour development of the plates.

It is interesting to see that in the ms study, the interquartile ranges were very wide early in the monitoring of the plates and became narrower in later readings, even though the median number of positive wells was increasing. This clearly shows the way in which some plates developed very rapidly while others took time to develop. The final number of positive wells was quite even, however these data give no indication that it was the same substrates that were developing by the end of the reading period. Comparing between the two experiments, one can see that the ranges in the ss experiment did not change much during the course of the monitoring and that they were quite similar to those seen in the latter parts of the ms monitoring.

There was very little difference in substrate group utilisation patterns in the ms experiment (Figure 4-4). Of all the comparisons made (11 substrate groups at 4 times) only two were significant: Amines at 120 hours and Alcohols at 120 hours (ANOVA with both $p \leq 0.05$). Post hoc Tukey HSD tests on the 120 hour Alcohols group revealed that the ambient root mean was different to the elevated soil mean, again an uninterpretable difference. Investigating the 120 hours Amine group with post hoc comparisons yielded no results with a Tukey HSD test while the LSD test showed that ambient root values were lower than ambient soil, and elevated root values were lower than elevated soil samples. The means for the 4 groups were 0.610, 0.946, 0.542 and 0.885 respectively. The root values were lower than the soil values while there was no apparent CO₂ effect.

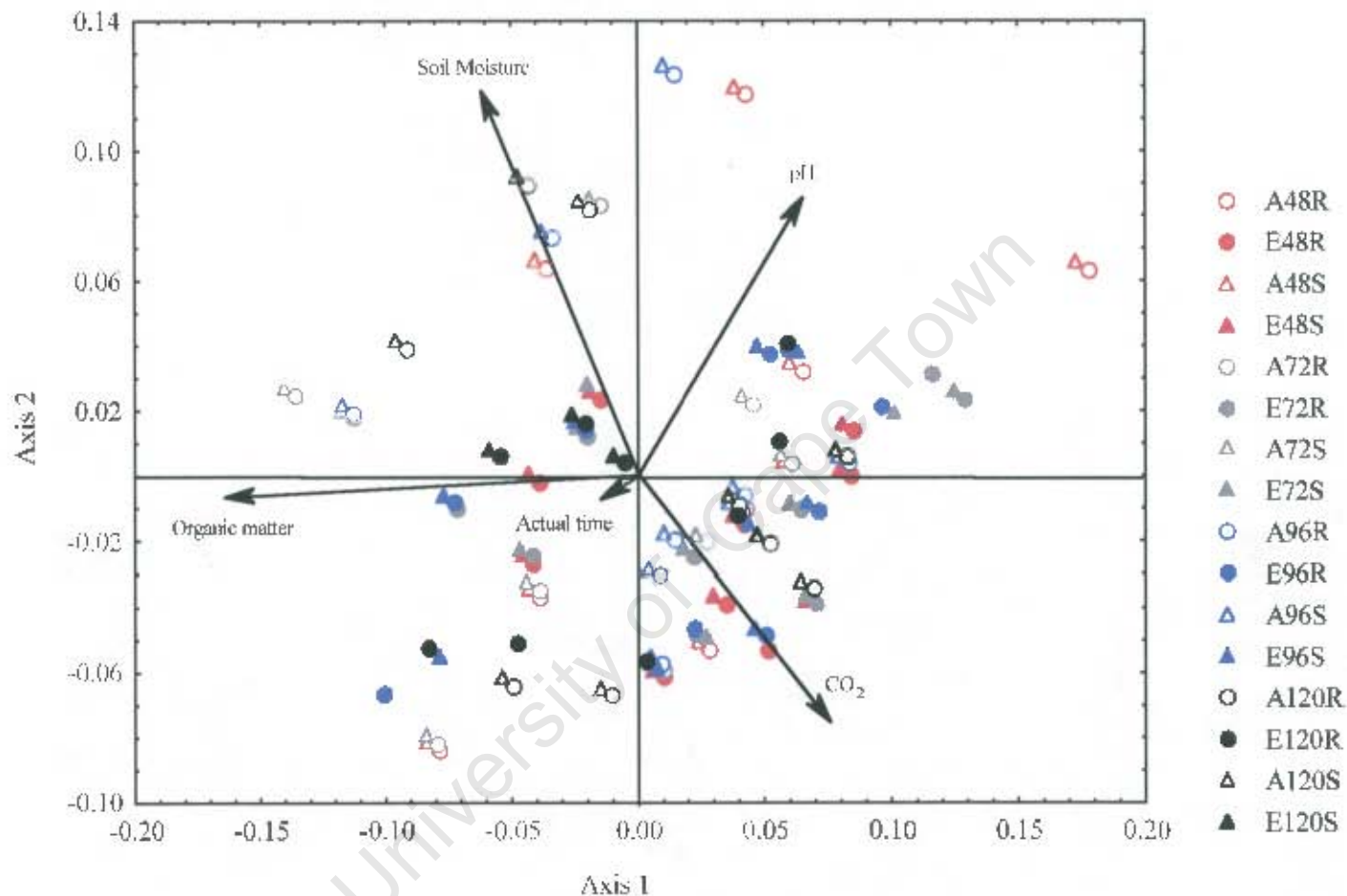


Figure 4-5: Sample score and environmental variable biplot from the CCA analysis of the root and soil samples from the *Felicia aethiopica* ssp. *aethiopica* single species experiment at all of the plate reading times. The direction of the environmental arrows gives the direction of maximum change of that variable across the diagram while its length is proportional to the rate of change in this direction (ter Braak, 1987). In this diagram, the arrow lengths have been multiplied by five to make the biplot easier to interpret. Monte Carlo permutation tests showed that none of the environmental variables were significant in separating the samples ($p > 0.05$, 199 permutations). The symbol coding is as follows: A = ambient CO₂ treatment, E = elevated CO₂, 48, 72, 96, 120 = reading time during incubation, R = root sample, S = soil sample.

Canonical Correspondence Analysis of the Biolog plate data

The biplot of sample scores and environmental variables of the single species experiment can be seen in Figure 4-5. The environmental variables investigated (actual time of plate reading, CO₂, Soil organic matter, soil pH and soil moisture content) had no significant effect on the separation of the samples in the ordination space ($p > 0.05$, 199 permutations). The Monte Carlo permutation test of the F-ratios of the first axis eigenvalue and the trace statistic (the sum of all eigenvalues) were both not significant. The eigenvalues were very low in this ordination (0.003, 0.002, 0.002, 0.001) for axes 1 to 4 respectively.

In Figure 4-5 the arrows of the environmental variables are five times longer than the original co ordinates. In this figure one can see that in most cases, soil and root samples from one pot grouped together. This shows that the substrates used by the microbial communities within one pot were more similar than those between different pots within each treatment. Although the paragraph above demonstrates that there is little support of any observed patterns with the ordination, one can see that there was some separation between the ambient treated pots in the top left of the diagram with the elevated pots (with other ambient pots) tending towards the bottom right. There was, however, no pattern in the time when the pots were read and so one cannot draw any conclusions from these patterns.

The distribution of substrate utilisation scores in relation to the environmental variables derived from the CCA seen in Figure 4-5 is shown in Figure 4-6. Substrates that significantly contribute to the separation of the pots were identified from the t-Value biplot (not shown, ter Braak & Looman, 1994). As would be expected from the lack of significance observed in Figure 4-5, only 10 of the 95 substrates were identified as being significantly affected by the investigated environmental factors (Figure 4-6 and Table 4-3).

Table 4-3: Carbon substrates on the Biolog GN MicroPlate significantly affected by the environmental variables studied in the *Felicia aethiopica* ssp. *aethiopica* single species experiment. In this table "Time" refers to the environmental variable *Actual time of plate reading*.

Substrate group	Code	Substrate	Effect	
			Positive	Negative
Carbohydrate	C3	D-psieose	Organic matter	
Carboxylic acids	D2	cis-aconitic acid		Time, Organic matter
	D5	D-galactonic acid lactone		Time
	E3	α -keto butyric acid	CO ₂	Organic matter
Amide	F2	succinamic acid	Time	
Amino Acids	F5	D-alanine	Time, Organic matter	CO ₂
	F6	L-alanine		Time
	G1	L-histidine		Time
	G10	L-threonine	Time	
Amine	H5	phenyl ethylamine		CO ₂

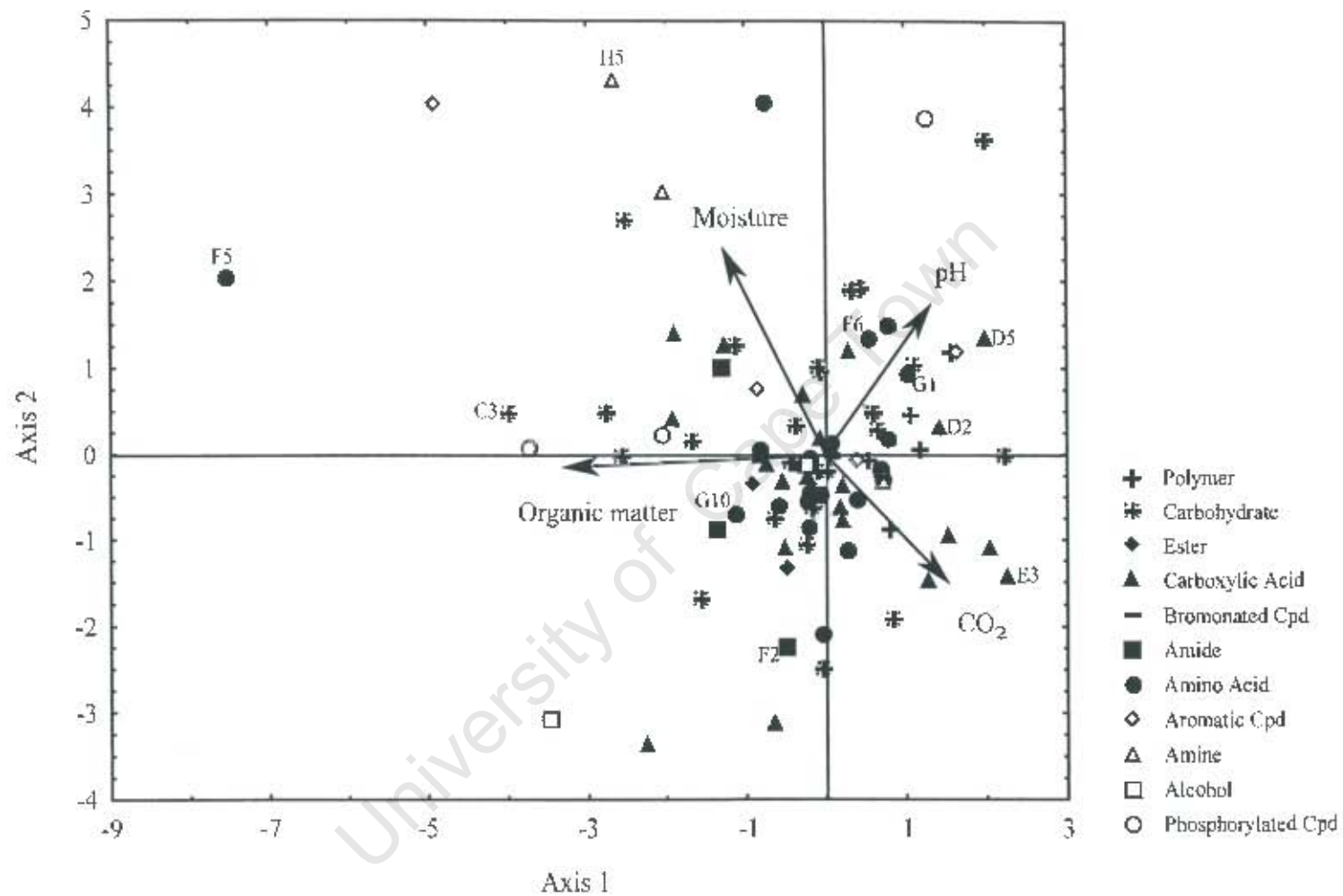


Figure 4-6: Biplot of the substrate (species) environment relation from the CCA analysis of the root and soil samples from the *Felicia aethiopica* ssp. *aethiopica* single species experiment. Substrate guilds are designated by the different point markers and substrates that were significantly affected by the environmental variables are marked with the alpha numeric code used on the Biolog GN plate (Table 4-3). The arrow lengths have been multiplied by 100. Cpd: abbreviation of compound.

The three Phosphorylated compounds that were shown to be consistently different for ambient root and ambient soil samples when analysed as a functional group (Garland and Mills, 1991) were not identified as being significantly affected by the environmental variables used in the analysis. This is because the difference was between the root and the soil samples and this is taken into account in the CCA by defining the groups of samples using co-variables.

Canonical Correspondence Analysis of the data from the mixed species study yielded very different and conclusive results (Figure 4-7). Here one can see that there was very clear separation of the two treatment groups along with close, identical clustering of the root and soil samples over the whole reading period. The environmental variable arrows are at the actual co-ordinates determined by the analysis, they have not been rescaled as was done in Figure 4-5.

As in the ss experiment, the eigenvalues were very low (0.005, 0.003, 0.002 and 0.028) for the first four axes with the first three being constrained and the last unconstrained. The cumulative variance of the species explained by the first two axes was very low (5.0 %) while the value increased to 81.5 % once the environmental variables were included in the analysis (cumulative percentage variance of species-environment relation). The Monte Carlo permutation test of the F-ratios of the first axis eigenvalue and the trace statistic were both significant ($p \leq 0.05$ for the first axis and $p \leq 0.01$ for the trace). This shows that the complete set of environmental variables adequately explains the variation in species data. The significance of the environmental variables were investigated using the automatic forward selection procedure and Monte Carlo permutation tests option in *Canoco*. This investigation showed that CO₂ and soil organic matter were significant in explaining variation in the substrate utilisation data ($p \leq 0.01$, 9999 permutations) while soil moisture content was not significant ($p > 0.05$, 9999 permutations).

Substrates that significantly contribute to the separation of the chambers were identified from the t-Value biplot (not shown, ter Braak & Looman, 1994). The distribution of carbon substrates in relation to the environmental variables derived from the CCA seen in Figure 4-7 is shown in Figure 4-8 and Table 4-4. Thirty six substrates reacted significantly to the environmental variables, CO₂ treatment and soil organic matter content. There was no significant effect of soil moisture content on any of the substrates.

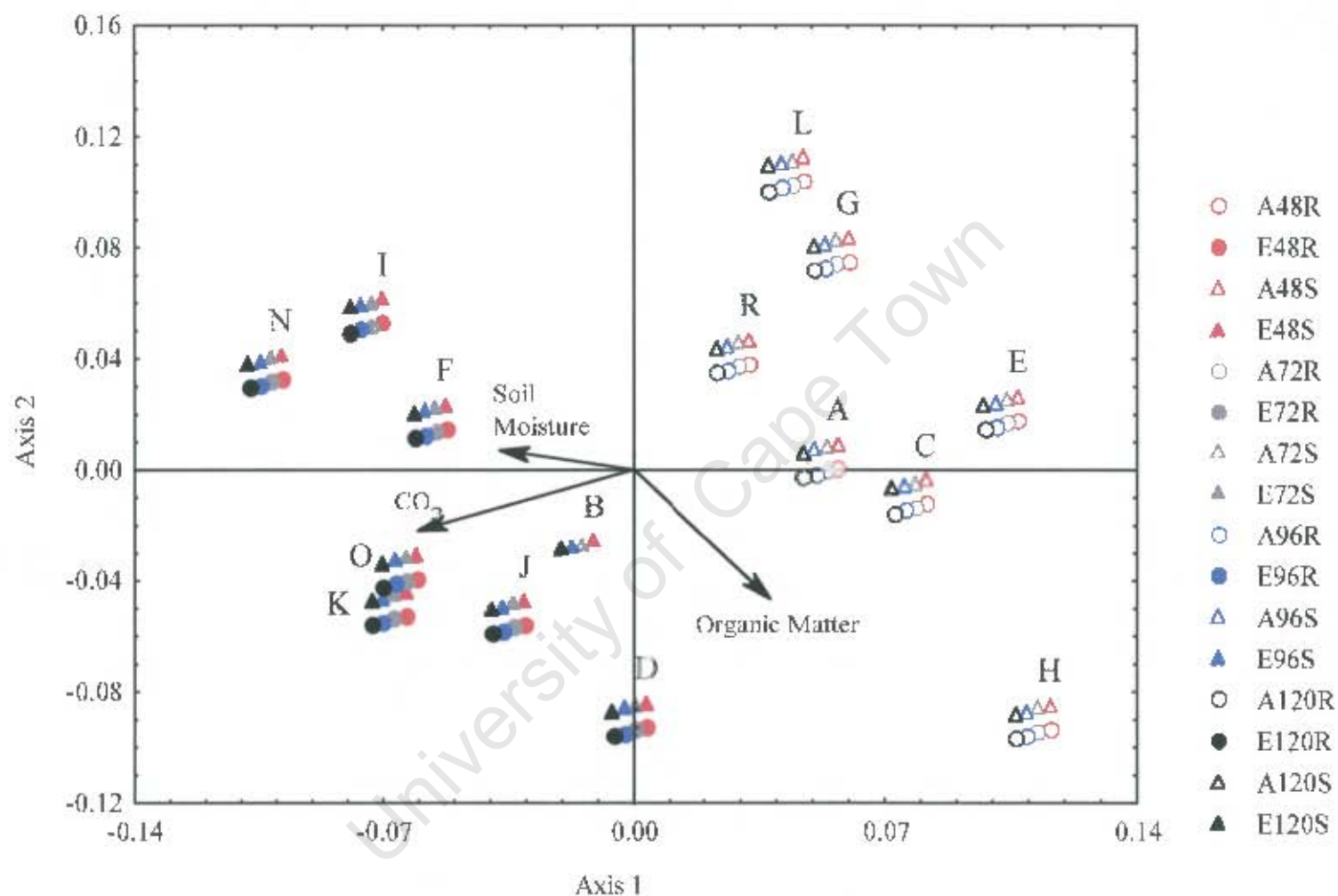


Figure 4-7: Sample score and environmental variable biplot from the CCA analysis of the root and soil samples from the mixed species study at all of the plate reading times. The letters are the codes of the open topped chambers used. Monte Carlo permutation tests showed that CO₂ and organic matter were significant in separating the samples ($p \leq 0.01$, 9999 permutations) while soil moisture content was not significant ($p > 0.05$, 999 permutations). The symbol coding is as follows: A = ambient CO₂ treatment, E = elevated CO₂, 48, 72, 96, 120 = reading time during incubation, R = root sample, S = soil sample.

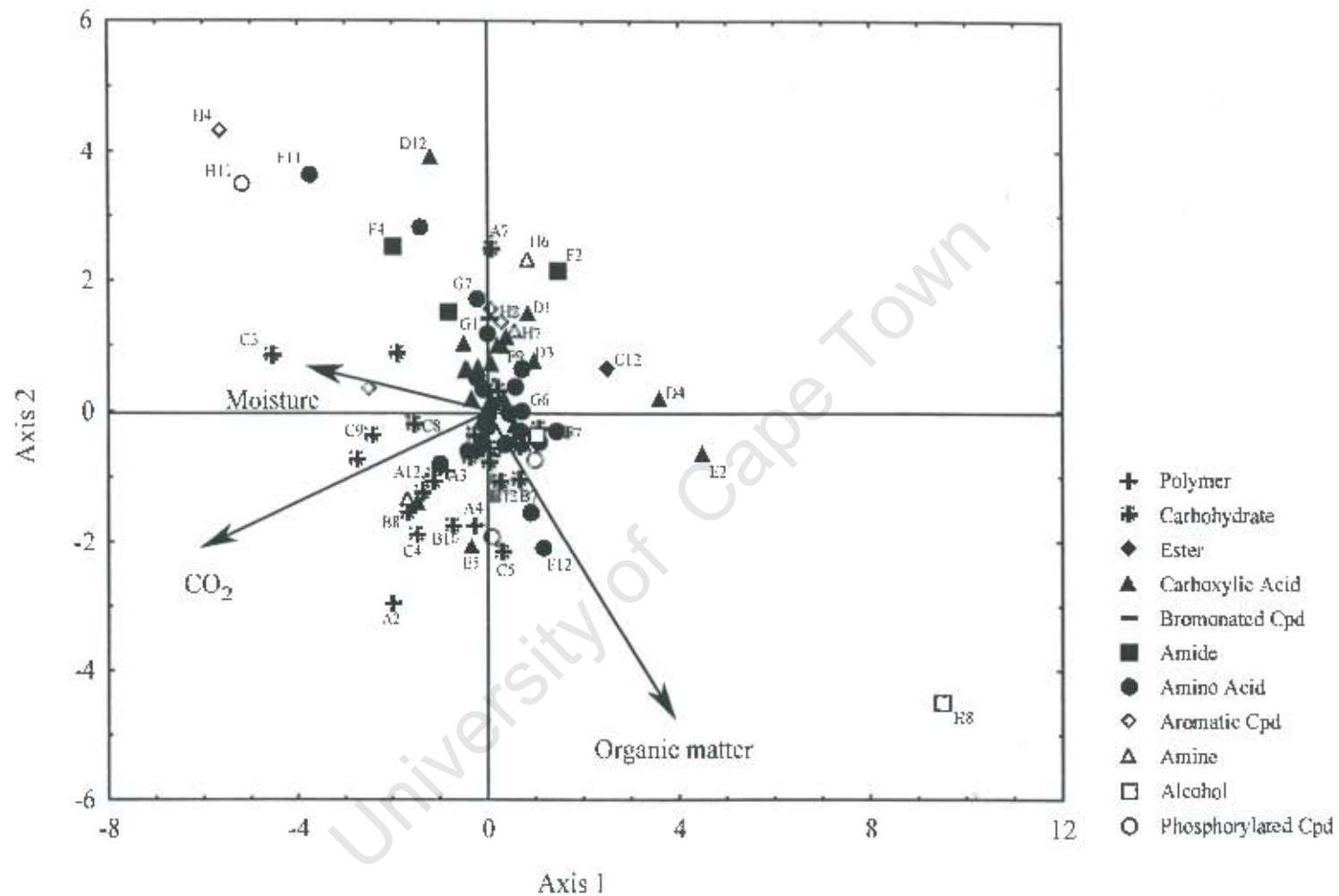


Figure 4-8: Biplot of the substrate (species) environment relation from the CCA analysis of the root and soil samples from the mixed species study. Substrate guilds are designated by the different point markers and substrates that were significantly affected by the environmental variables are marked with the alpha numeric code used on the Biolog GN plate (Table 4-4). The arrow lengths in this diagram have been multiplied by 100. Cpd: abbreviation of compound.

Table 4-4: Carbon substrates on the Biolog GN MicroPlate significantly affected by the environmental variables studied in the mixed species experiment.

Substrate group	Code	Substrate	Effect	
			Positive	Negative
Polymers	A2	α -cyclodextrin	CO ₂ , Organic matter	
	A3	dextrin	CO ₂	
	A4	glycogen	Organic matter	
Carbohydrates	A7	N-acetyl-D-galactosamine		CO ₂ , Organic matter
	A12	cellobiose	CO ₂	
	B7	m-inositol	Organic matter	
	B8	α -D-lactose	CO ₂	
	B10	maltose	CO ₂ , Organic matter	
	B12	D-mannose	Organic matter	
	C3	D-psicose	CO ₂	Organic matter
	C4	D-raffinose	CO ₂	
	C5	L-rhamnose	Organic matter	
	C8	D-trehalose	CO ₂	
C9	turanose	CO ₂		
Ester	C12	mono-methyl succinate		CO ₂
Carboxylic Acids	D1	acetic acid		CO ₂
	D3	citric acid		CO ₂
	D4	formic acid		CO ₂
	D12	γ -hydroxybutyric acid		Organic matter
	E2	itaconic acid		CO ₂
	E5	α -keto valeric acid	CO ₂ , Organic matter	
Amides	F2	succinamic acid		CO ₂ , Organic matter
	F4	alaninamide		Organic matter
Amino Acids	F7	L-alanyl-glycine		CO ₂
	F9	L-aspartic acid		CO ₂
	F11	glycyl-L-aspartic acid		Organic matter
	F12	glycyl-L-glutamic acid	Organic matter	
	G1	L-histidine		Organic matter
	G6	L-proline		CO ₂
	G7	L-pyroglutamic acid		CO ₂ , Organic matter
Aromatic Compounds	H2	inosine		Organic matter
	H4	thymidine		Organic matter
Amines	H6	putrescine		CO ₂ , Organic matter
	H7	2-amino ethanol		CO ₂ , Organic matter
Alcohol	H8	2,3-butanediol	Organic matter	
Phosphorylated compound	H11	glucose-1-phosphate		Organic matter

Discussion

Most studies that use the Biolog Bacterial Identification System investigate scenarios where one would expect to see dramatic changes in the potential substrate utilisation of microbial communities. Examples of this include the original Garland and Mills (1991) paper that compared soil extracts, environmental water samples and hydroponic nutrient solution and Bossio and Scow (1995) who investigated the effects of flooding and carbon addition, in the form of rice straw incorporation, on soil microbial communities. In fact, it would be even more remarkable if there were no observed changes in the utilisation patterns. These studies are useful, however, because they demonstrate the use and effectiveness of this relatively simple system to investigate microbial ecological processes.

Many predictions state that there would be an expected increase in carbon exudation from the roots of plants exposed to elevated atmospheric CO₂ concentrations and it is this change in root exudation that would have an effect on soil microbial processes (reviewed in Sadowsky & Schortemeyer, 1997). The *Felicia aethiopica* ssp. *aethiopica* plants in the single species study did not show any growth response to CO₂ treatment (Chapter 2). There was a decrease in the exudation of carbon from the roots (Chapter 5) and a non-significant decrease in soil microbial biomass (Chapter 3). From this one can see that there is evidence supporting the expectation that the microbial communities may change under a changed atmosphere but these changes are likely to be subtle in comparison to the changes seen in other studies.

The degree of replication in the two sections of this investigation was good using 72 plates in total for the ss experiment (2 Biolog plates per root or soil sample taken from 3 plants from each of 3 chambers from the two treatments) and 96 plates in total for the ms study (3 plates used for each root or soil sample taken from the 8 ambient or 8 elevated chambers). Lowit *et al.* (2000) complimented Bossio & Scow (1995) for their strong statistical approach of using four replicate treatments with three Biolog plates inoculated from each treatment although they did say that such a number of replicate plates may not have added to the power of the analysis. One would expect soil samples to be more variable than the well mixed water samples used by Lowit *et al.* (2000) but one can still be confident that the current analysis was powerful enough to detect differences among the samples.

The CCA gave very low eigenvalues in the analysis. This indicates that there was very little species (substrate) turnover among the samples. Despite the differences being small there was a discernable and consistent difference between the two treatment groups.

This confirms the assertion that these experiments were investigating the subtle effects caused by elevated CO₂ on wild plant species. The low eigenvalues indicate that these data were showing a linear response (short gradients) as opposed to a unimodal one and so one could analyse them with a linear method such as redundancy analysis (RDA). Although it is subjective and depends largely on the particulars of the study at hand, Kent and Coker (1992, p 235) feel that for data sets that contain good environmental and species data, CCA is the “best” ordination method available. If detailed environmental data are not available, then detrended correspondence analysis (DCA) is the most appropriate choice of analysis.

This study was concerned with the imposition of a defined treatment on a single microbial community and CCA has proved to be a useful analytical tool in controlled experiments (Pyšek & Lepš, 1991) and in this case, between the microbial community properties and specific environmental variables (Bossio & Scow, 1995). CCA also allows one to define experimental blocks which makes a much more rigorous analysis possible.

The soil used in laboratory based experiments is usually highly disturbed during the setting up of the experiment. It is important that it is given sufficient time to settle and mature to allow the reestablishment of soil food webs prior to analysis (Hu *et al.*, 1999, Klironomos *et al.*, 1996). This is the major failing of short-term experiments. Both the ss and ms experiments described here ran for more than year (whole seasonal cycle) and so they had probably gone through this settling period. A problem with the ss experiment would be the potential root restriction with impacts on root exudation during the settling period. The only way to control for this would be to analyse the pots over the course of the experiment to separate between these two processes. Intermediate duration experiments such as these discussed here are also important as they begin to answer the critical question of whether observed changes in the short-term will persist indefinitely (Hu *et al.*, 1999)

Table 4-5: Differences between the *Felicia aethiopica* ssp. *aethiopica* single species experiment and the mixed species study that used *Felicia* as one of the component species.

Single species experiment	Mixed species study
A single, independent plant and species	Multiple individuals and species present
Potential root restriction from pot effects	Practically unlimited root penetration
Plants analysed when physically small	Analysed plants physically bigger
Very low soil organic matter content	Higher organic matter content
Roots exposed to a wider range of temperatures	Roots in a more natural temperature environment
Soil dries out more rapidly	Slower soil drying
Plants watered with de ionised water	Plants watered with tap water

The single species experiment gave no clear indication of a CO₂ effect on the potential carbon utilisation while scaling the experiment up to the more complex mixed species study yielded clear and interesting Biolog results. Reasons for this difference need to be considered and one also needs to think about what would occur if one were to scale up again to a natural plant community or ecosystem. Differences between the two experiments are listed in Table 4-5. Two clear differences exist between these systems: pot size effects and soil nutrient effects resulting from the addition of the *Fynbos mix* soil. In their review, Hu *et al.* (1999) showed that the nutrient status of the soil influences the response of the microbial populations and this appears to be confirmed in the current study. Cheng *et al.* (1996) showed that carbon is not limiting in the rhizosphere of plants and so one would expect the Biolog analysis of root extracts to be unaffected by soil organic matter. This appears not to be the case in the results presented here where it was found that soil organic matter significantly influenced both the root and soil extracts from the mixed species experiment. The investigations made here were not able to separate between the pot and nutrient effects but there was clear evidence that as one approached a natural system (mixed species microcosms) significant CO₂ effects were occurring. It is likely that observations from the ms study would be similar to that seen in a natural system. Some confirmatory Biolog analyses would need to be done to see if there is a similar dependence on soil organic matter content occurring in nature.

Reviewing studies of microbial responses to elevated atmospheric CO₂ yield a variety of conflicting results. The Biolog system has been used to investigate CO₂ effects in alpine grasslands (Mayr *et al.*, 1999^b), artificial tropical ecosystems (Insam *et al.*, 1999), a potted Chaparral species, *Gutierrezia sarothrae* (Rillig *et al.*, 1997) and a grass monoculture in a Mediterranean model ecosystem (Dhillion *et al.*, 1996). Mayr *et al.* (1999^b) found that the community level physiological profiles of the soils were affected by the chambers as well as the low and high nutrient treatments in the CO₂ treated chambers. This was despite seeing no effect on the microbial biomass. Rillig *et al.* (1997) found that there were significant CO₂ effects on the soils while nitrogen addition did not influence the Biolog profile. The contrasting nutrient effects between these two studies emphasises the need for further investigation of CO₂ effects on microbes that influence nutrient cycling. Dhillion *et al.* (1996) used a standardised inoculum density but the Biolog data analysis was somewhat rudimentary and so although there were significant changes in the other measurements (hyphal lengths, enzyme activity, etc.) there was little change in the Biolog pattern while Insam *et al.* (1999) found no CO₂ effect using either Biolog analysis or PLFA.

Three PLFA studies showed no consistent CO₂ effects on the soils; artificial tropical ecosystem (Insam *et al.*, 1999), model terrestrial ecosystem (Kandeler *et al.*, 1998) and potted *Populus grandidentata* plants (Zak *et al.*, 1996). In this last study, a change in microbial biomass was observed but the PLFA analysis gave no result (Zak *et al.*, 1996). The study by Kandeler *et al.* (1998) only studied the PLFA data at a very coarse level (total bacterial to fungal ratios) and so it was really only assessing soil microbial biomass rather than soil microbial community structure.

Other techniques have been used to assess plant mediated, elevated atmospheric CO₂ effects on soil microbial populations. A clear change in the genetic structure and competitive ability of *Rhizobium* strains was observed in a European pasture grown under temperate conditions (Montealegre *et al.*, 2000) while no changes were seen in Griffiths *et al.* (1998) who investigated microcosms of either Ryegrass or wheat using broad-scale DNA profiles. Little CO₂ effect was seen in the study conducted by Schortemeyer *et al.* (1996) except for a doubling in the *Rhizobium* population around the rhizosphere of the white clover and Bruce *et al.* (2000) could find no CO₂ effect on bacterial community structure during 3 plant generations in an artificial temperate ecosystem.

A reason for these conflicts would be the time dependent nature of many of the responses (Hu *et al.*, 1999, Grayston *et al.*, 2001). The diversity of results also reflects the wide range of ecosystems and experimental conditions being investigated. The authors continue by stating that the duration of most experiments is not long enough to permit the shifts from one response (such as positive feedback) to another (negative feedback). This was demonstrated by Sadowsky & Schortemeyer (1997) who found big differences in microbial numbers in spring and autumn samplings of the same experiment. In this current investigation this time factor could be a component in the difference in the results between the two sections of the study due to the two experiments being analysed in different seasons and after different growth periods. Both components had been grown for a long period thus allowing new equilibria to be established after the initial disturbance at the start of the experiments. Better results may have been obtained if the systems were monitored regularly during the experiment.

A fundamental question related to these observed differences in Biolog profile is how can these results be interpreted in a global change context? One of the major shortcomings of this method is that it is difficult to interpret the meaning of observed differences due to the fact that this technique is measuring the potential of the microbes to metabolise the carbon sources in an aerobic environment (Garland, 1997 and Buyer & Drinkwater, 1997). This

means that it bears little relationship with what is occurring in the soil and so one is unable to say that any particular carbon species is present or absent in the treated system. The value of this technique lies in its power to make rapid, broad scale preliminary investigations into the effects that a treatment is having on a microbial population. Once a difference has been identified, one is then in a position to do a more thorough and detailed investigation to learn more about the mechanism of the response.

As was pointed out by Garland (1997), meaningful advances and hypotheses have been made in macroecology by investigating a portion of the overall community (plants, invertebrates, birds, etcetera). The same is true with microbial ecology where indirect methods such as Biolog can give valuable insights into the processes that are occurring. In microbial ecology, workers currently have the choice of using rapid, rather crude methods to allow them to perform broad scale survey work or more detailed, but much more time consuming methods, such as those used by Klironomos *et al.* (1996). What this study has revealed is that there are clear CO₂ effects on the soil microbial communities of Cape Fynbos plants. This has the potential to change the nutrient cycling patterns, carbon sequestration, litter decomposition, plant pathological responses and even competitive interactions of the plants that the microbes are associated with. It has been highlighted that a change in litter decomposition could have a dramatic effect on the uptake of carbon from the atmosphere (GCTE & COST, 2000) with the cascade of decreased decomposition leading to decreased plant growth resulting in a lower uptake of CO₂. There is still disagreement on this hypothesis due to a lack of good evidence but a change in microbial community structure gives an indication that something is happening and closer investigation is needed.

These Biolog results should also alert modelers to the fact that below-ground processes should not be included in models in too simplistic a way. Aspects of below-ground and microbial ecology may well confound predictions made by models that approach below-ground factors in a "black box" way. Modelers are aware of these issues and are concerned with addressing them (Smith, 1998). Hu *et al.* (1999) present a series of diagrams showing how soil microbial processes can influence carbon flows which can cause positive or negative feedbacks to plant growth.

Recently there has been considerable debate over the best way to analyse the large amounts of data gathered from the Biolog plates (Howard, 1999^a, Garland & Mills, 1999^a, Garland & Mills 1999^b, Insam & Hitzl, 1999, Howard, 1999^b, and Howard, 1997). These discussions revolve around the best scaling method to minimise distortion of the data. In these two experiments these arguments were not relevant because the plates were shown to be

so similar in degree of coloration and volume of original sample that one could analyse the data from the point of view of a response per unit of environmental sample. In this *Felicia* case normalisation or scaling was not necessary (Garland, 1997). The one artefact introduced into these data was the conversion of negative values to zero. Howard (1999^a) pointed out that it is impossible to get a negative dehydrogenase activity and that this could be due to the tetrazolium having an inhibitory effect on the microbes. He criticises authors for merely discarding these potentially informative sites. Due to the algorithms used one cannot have negative values in the data set. In this case it was felt that the effect of discarding negative results would not unduly affect the analysis because of the fact that the negative values were small in comparison to the range of positive values. As an example: in the mixed species study, the minimum absorbance value from the whole data set was -0.179 while the maximum value was 1.444.

These debates are very important because they are necessary to refine the technique and allow better interpretation of the patterns observed. One should, however, be careful not to reinvent the wheel and rather draw from the vast knowledge base present in community ecological analysis. Ordination is a complex field that requires a degree of mastery before one can use it successfully (see Kent and Coker, 1992 and ter Braak and Šmilauer, 1998).

Some authors recommend that the functional groups on the Biolog plate be analysed (Garland and Mills, 1991, Zak *et al.*, 1994 Rillig, 1997). This can be useful, but in this experiment no clear differences were seen. It is also important to remember that each of the groups contained vastly different numbers of substrates; 28 carbohydrates and only two alcohols and one brominated chemical. Some of the groups were therefore much more sensitive to outliers than other groups. One should therefore be wary of drawing too many conclusions from differences seen in the under-represented substrates. Looking at the ordination of substrate utilisation scores (Figures 4-6 and 4-8) one can see that there was no trend of any particular substrate guild being associated with an environmental variable.

Drawbacks of the Biolog Bacterial Identification System have been discussed in the introduction to this chapter. Steps have been taken in the present experiments to maximise the value of the data and results. Further improvements could be made by taking the following points into consideration. The problem of the low level of replication compared to the number of response variables (carbon substrates)(Lowit *et al.*, 2000, Insam & Hitzl, 1999, Hitzl *et al.*, 1997 and Howard, 1997) has now been overcome with the introduction of the Biolog ECO plates that have three sets of 31 carbon sources which increases the replication of Biolog assessments along with reducing the number of descriptors (C sources, Insam,

1997). Garland (1997) recommended that a standardised inoculum density be used, or the developing Biolog plate should be read frequently so that plates of similar AWCD can be compared as opposed to analysing plates of similar incubation time. Verschuere *et al.* (1997) suggested using the Gompertz equation to obtain kinetic parameters of the growth curve for each well on each plate. This yields results which are independent of incubation time but it is important to have enough plate readings to allow this detailed analysis. Lindstrom *et al.* (1998) presented another kinetic approach to Biolog analysis while Mayr *et al.* (1999^b) calculated the Riemann's sum from plates that had been inoculated with a standardised density of 50 000 cells per well. Garland (1997) warned that kinetic analyses of Biolog data are also reliant on the initial inoculum density and so standardisation is recommended. Hitzl *et al.* (1997) presented a two step approach to gathering functional diversity measurements of bacterial populations followed by rigorous analytical methods that allow for better hypothesis testing on the data. There are indications (Insam *pers. comm.*) that these new approaches (integrals and curve parameters) do not yield more information than that obtainable from a carefully executed comparison of plates with similar AWCD.

As has been discussed by Hu *et al.* (1999), significant progress has been made in the study of the impacts of elevated atmospheric CO₂ on subterranean microbial processes, as the current investigation has done, however the big challenge now is to take these results and predict if and how they will impact on overall ecosystem functioning (Schimel and Gullede, 1998). This is where the weakness in the Biolog Bacterial Identification system lies: significant differences have been observed but the effect of them on soil microbial population functioning is currently difficult to interpret. There is, however, evidence accumulating showing a relationship between environmental substrate supply and associated Biolog well response (Grayston *et al.*, 2001, Schutter & Dick, 2001).

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THE INFLUENCE OF ELEVATED ATMOSPHERIC CO₂ CONCENTRATIONS ON THE ALLOCATION OF RECENTLY FIXED CARBON WITHIN *FELICIA AETHIOPICA* SSP. *AETHIOPICA*.

Introduction

A common theme in elevated atmospheric carbon dioxide research has been attempts to predict the carbon sequestration potential of terrestrial ecosystems (Ward and Strain, 1999). It is generally accepted that increased CO₂ levels in the atmosphere results in an increase in total carbon input to the rhizosphere (Cheng, 1999). Most of the studies undertaken to date have been limited to agricultural grass species (mainly wheat) with only one investigation of a temperate tree species, Sweet Chestnut (Rouhier *et al.*, 1996). In contrast to the generally accepted response, some studies have shown no effect of CO₂ on root exudation (Paterson *et al.*, 1996). Knowledge of how natural, shrub dominated ecosystems will respond to the predicted change in atmospheric CO₂ is thus urgently needed (Midgley *et al.*, 1995 and Midgley *et al.*, 1999). These systems are often exposed to resource limitations such as water and nutrients.

Paterson *et al.* (1996) highlight the importance of investigating soil microbial responses mediated through plant response to elevated atmospheric CO₂. This is especially true when there are clear CO₂ effects on the plants themselves. If the carbon being released into the soil by plant roots is partitioned differently then dramatic effects on carbon cycling and sequestration, nutrient cycling, symbiotic and pathogenic relations would occur (Cheng, 1999, Paterson *et al.*, 1996, Vose *et al.*, 1997). In theory it is simple to understand the different pools of carbon and their potential interactions but in practice it is very difficult to study (Meharg, 1994).

The distinction between root respiration and microbial respiration is an important aspect of soil biology but is difficult to achieve due to the very close physical association between the two processes (Killham & Yeomans, 2001, Cheng *et al.*, 1993 Swinnen, 1994). Sources of organic carbon within the soil ecosystem come from decayed plants and animals,

mobilization of protected soil organic matter and inputs from living plant roots (Meharg, 1994) while the CO₂ that is released from soil can come from one of three sources: root respiration, microbial respiration using root derived materials and microbial respiration using carbon that was originally in the soil (Cheng *et al.*, 1994). Root deposits can take the form of exudates, secretions, mucilages, sloughed root cap cells, decaying root hairs and cortical cells. Direct root respiration does not contribute to soil organic matter formation while rhizosphere microbial respiration relies on root exudates which are an intermediate between the root and the microbes. Changes in this relationship would have an impact on soil ecological processes which may affect soil organic matter accumulation or loss (Swinnen *et al.*, 1994).

Swinnen (1994) discusses a method to separate root and rhizosphere microbial CO₂ sources using two similar plants and differing ¹⁴C labelling. The author obtained values of between 11 and 25 % of the substrate being used by the soil microbes showing that a much higher proportion of the respiration was coming from root sources.

Cheng *et al.* (1993, 1994) discuss the novel use of the isotopic trapping method that was developed by Wolf (1964). The theoretical background is explained in detail by Cheng *et al.* (1993). Briefly, the difference between root and microbial respiration can be elucidated by labelling plants with ¹⁴C and then monitoring the release of the radiation from the roots of the plants. If the soil is flooded with unlabelled substrate (¹²C glucose), the ¹⁴C release from the microbial component will be reduced due to competition from this unlabelled substrate, while the root respiration will be unaffected. Using a proportionality equation and three glucose concentrations one is able to calculate the contribution to the overall rhizosphere respiration made by the roots. The key advantage of using this method is that rhizosphere studies can be performed on intact potted plants growing in soil, which allows one to make better predictions of what may happen in natural situations.

This study combines the two research areas of below-ground respiration and plant responses to elevated atmospheric carbon dioxide. It is also unusual in that it is using a herbaceous perennial, wild plant species. It is therefore difficult to compare with other studies that used crop plants that are quite different to this species, *Felicia aethiopica* ssp. *aethiopica*. Cheng and Johnson (1998) used isotopic trapping methods to investigate the input of carbon into below-ground systems by wheat grown in elevated atmospheric CO₂ concentrations while Verburg *et al.* (1998) investigated the response of one year old heather, *Calluna vulgaris*, to CO₂ treatment for 11 weeks.

The aim of this study was to investigate the ^{14}C allocation of recently fixed carbon in ambient and elevated atmospheric CO_2 treated *Felicia aethiopica* ssp. *aethiopica* plants. Isotopic trapping techniques (Cheng *et al.*, 1993) were also applied to try to distinguish between root and microbial respiration in this plant.

Materials and Methods

Plant material

Felicia aethiopica ssp. *aethiopica* (Kirstenbosch Botanical Gardens accession number: 775/88) plants were germinated from seed in 850 g (dry weight) Fynbos soil in PVC planting tubes (7.0 cm i/d, 20 cm long with an air inlet tube at the bottom and outlet at the top) in a phytotron with a 12 hour, 20°C/8°C day/night cycle. The daytime light intensity was 139 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. The pH, nitrogen (Kjeldahl digestion followed by colorimetry) and phosphorus (Tri-acid digestion followed by Murphy and Riley colorimetric determination) contents of the soil was 3.48, 0.76 $\text{mg}\cdot\text{g}^{-1}$ and 35.08 $\mu\text{g}\cdot\text{g}^{-1}$ respectively. Three seeds were planted in each tube and then watered with 2 ml of *Kirstenbosch Instant Smoke Plus Seed Primer* (National Botanical Institute, Claremont, South Africa) and covered with a petri dish to maintain humidity. This cover was removed once the cotyledons on the seedling were established. After 40 days of growth (11 March 1996) the pots of seedlings were randomly assigned to open-top chambers situated in the University of Cape Town Botany Department's new glasshouse. One month later, planting tubes containing dead plants were replaced with tubes containing plants that had been grown in the phytotron chamber. After 107 days of growth the pots were thinned down to one plant per pot. These plants were removed by cutting them at the soil level and leaving the roots in the soil that ensured minimum disturbance to the remaining seedling. The plants were watered regularly with deionised water and were never allowed to become water stressed. The plants were not fertilised during the experimental period and they were shuffled three times within each chamber to eliminate position effects on their growth. The plants were harvested at 810 days after the start of the experiment and occurred on three distinct days; 21 April 1998, 5 May 1998 and 18 May 1998. The plants were watered to saturation either 4 or 5 days before the day of the harvest. Due to the seedling variation and previous harvests, 15 plants were used from each treatment regardless of the chamber that they had been growing in.

Growth chambers and experimental conditions

The open-top chambers consisted of a plastic rubbish bin (50 cm in diameter) with an 80 cm high, removable polyethylene cylinder on top. A fan drew air from outside the glasshouse through a 16.0 cm diameter manifold before being divided into six 7.0 cm i/d PVC tubes that led into the centre of the chamber. The air was deflected horizontally by a 10 cm diameter PVC baffle 3 cm above the opening of the tube. Airflows were high enough to ensure at least 3 replacements of the chamber air every minute. Pure CO₂ was bled into the individual 16 cm diameter pipes that led off the manifold before the diameter was reduced to the 7.0 cm pipe. Six chambers were used, 3 ambient and 3 elevated (ambient + 342 μmol.mol⁻¹ CO₂). The CO₂ concentration in the chambers was monitored for periods of 24 hours at different times throughout the experiment using an ADC LCA2 infrared gas analyser (The Analytical Development Co. Ltd., Hoddesdon, Herts, England) attached to a monitoring computer.

¹⁴C Labelling

Due to the limited size of the labelling chamber, each glucose concentration was labelled and analysed separately. Each labelling run contained 5 plants grown under ambient atmospheric CO₂ conditions and 5 plants exposed to elevated levels of CO₂.

On the day before the ¹⁴C labelling, the plants were moved into a phytotron chamber and all of the dead leaves and any basal green leaves were gently removed from the plants. This gave a clear stem at the base of the plant to allow the soil surface to be sealed. The surface of the soil was scraped clean of any moss that had developed and the planting tube was filled up to above the air outlet tube with autoclaved Fynbos soil (121°C for 15 minutes). The inside edge of the planting tube was scored with a sharp metal point, cleaned with acetone and then a ring of *Prestik* (an oil based polybutene sealant, Genkem (Pty) Ltd under licence from Bostik Ltd, England) was attached to the wall of the tube about 0.5 cm above the soil level. The soil surface was then sealed with a layer of pure *Vaseline* (petroleum jelly) that was poured at 47 °C and then allowed to cool. Each of the ten pots was attached to a compressed air supply and the air flow rate was set to 100 ml per minute. Each of the pots was then tested for an airtight seal by immersion in water. Early the following day, the pots were again tested for an airtight seal and the flow rate was checked and, if necessary, adjusted back to 100 ml per minute. Solutions of 100 ml of D-glucose solution (0.5 g.C.l⁻¹ and 6.8 g.C.l⁻¹) or a water control were sucked into the pots by means of a vacuum pump. During the ¹⁴C labelling and subsequent chase period the surface of the *Vaseline* was covered

with water to check for leaks. Periodically some crushed ice was added to the pool of water in order to keep the *Vaseline* cool, which prevented the development of leaks. The pots were placed inside a *Perspex* labelling tank of a similar design to that seen in Cheng *et al.* (1993). Two differences with this design were: 3ml of 2M NaOH in a liquid scintillation vial was used to trap the CO₂ and concentrated lactic acid was used to release the ¹⁴CO₂ from the sodium (¹⁴C) bicarbonate.

Once the chamber was set up and sealed, the lights were turned off and the ¹⁴CO₂ was released into the chamber system by adding 15ml concentrated lactic acid to 1ml 0.5M sodium (¹⁴C) bicarbonate (1.85 Mbq, Amersham, UK, 1Bq = 1 disintegration per second) that had been diluted with 10ml of distilled water. The CO₂ concentration in the chamber was monitored using an ADC 225 MkII infra red gas analyser (The Analytical Development Co. Ltd.) and allowed to stabilise which took about 15 minutes and resulted in an approximate doubling of the CO₂ concentration in the chamber. The lights were then switched on and the plants were allowed to absorb the label until the CO₂ concentration had returned to the starting levels. This took between 35 and 50 minutes. The temperature inside the chamber was monitored with a thermocouple and it started at 24°C and rose to about 30°C by the end of the labelling period. The remaining CO₂ was flushed out of the tank by opening a vent on one side of the tank while a vacuum pump attached to the opposite side sucked the air through a bottle filled with 1M NaOH. After three minutes the top of the chamber was removed and the chase period commenced. For the 0.5 gC.l⁻¹ treatment, the 3ml 2M NaOH traps were replaced every 20 minutes for 6 hours and 40 minutes while in the 0 gC.l⁻¹ and 6.8 gC.l⁻¹ experiments the traps were replaced every 30 minutes for a period of 10 hours. One large aliquot of 15ml 2M NaOH was then used to trap CO₂ released overnight before plant harvesting commenced.

A 5ml aliquot from each 2M NaOH trap was taken, mixed with 5ml 1M BaCl₂, 4 drops of 1% w/w phenolphthalein in ethanol (Dalal, 1979) and then titrated against 0.1M HCl. The amount of CO₂ released from the pots was calculated according to Anderson (1982).

Harvesting

The plants were cut at soil level and the top parts were separated into green leaves, dead leaves and stems. This material was dried at 60 °C. Once dry, all of the samples were weighed on an analytical balance and then the leaf and root material was ground in a mortar

and pestle using liquid nitrogen while the stem material was ground in a Wiley mill (20 mesh).

After removing the top parts of the plants, the soil was tipped out into a tray and the roots were gently separated from the soil. Having been saturated with either glucose solution or water, the soil was very wet which made separation of the roots and rhizosphere soil difficult. Soil that was loosely adhering to the roots was shaken off and then the whole root-rhizosphere sample was dried and, once dry, the soil was shaken off and defined as rhizosphere soil. A sample of the moist bulk soil was passed through a 2 mm analytical sieve and 20g each was used for duplicate 50 ml 0.5M K_2SO_4 extracts and 80g was used for a moisture determination (by means of oven drying at 107 °C for 24 hours). A sample of the bulk soil was dried at 80°C. The equipment used in this processing was washed between each sample to prevent contamination.

This harvesting procedure resulted in 6 distinct compartments or pools of carbon that could then be analysed further. These pools were: green leaves, stems, roots, rhizosphere soil, bulk soil and K_2SO_4 extractable carbon.

^{14}C analysis

The $^{14}CO_2$ that had been trapped during the chase period was analysed for radioactivity by adding 10ml *Hionic Fluor* (Packard Instrument Company) to the vial containing the 3ml 2M NaOH, shaking and then reading in a liquid scintillation counter (Beckman LS 5000 TD)

The K_2SO_4 samples were frozen at -20°C for about one and a half months before they were analysed. Once defrosted, the samples were shaken and then filtered through Whatman GF/B glass microfibre filters. Radioactivity was assessed by taking 9 ml of sample, mixed with 10 ml of *Hionic Fluor* and then measuring it with a liquid scintillation counter (Beckman LS 5000 TD)

The dry material (leaves, stems, roots, rhizosphere soil and bulk soil) was weighed into duplicate 20 mg samples and the radioactivity was measured by liquid scintillation counting (Packard Tri-Carb 1900CA) after combustion in a Packard Tri-Carb Sample Oxidiser. A protocol of 45 seconds of combustion in O_2 with the liberated CO_2 trapped in 8ml of *Carbosorb* and then mixed with 12 ml *Permafluor V* (Packard Instrument Company) was used. Packard High Performance Vials were used in all of the radioactivity determinations.

Data analysis and statistics

The $^{14}\text{CO}_2$ released from the pots during the chase period of the experiment was expressed as fractions of the total ^{14}C recovered using the formula

$$\left[\frac{{}^{14}\text{CO}_2 \text{ evolution in Bq}}{\text{total } {}^{14}\text{C recovered}} \right] \times 10^5 \quad (\text{Cheng et al., 1994}).$$

Once the pots have been treated with glucose solution and the radiation released from soil respiration has been measured, the proportional equation:

$$\frac{Z_G - R}{Z_W - R} = \frac{C_S}{C_S + C_G} \quad (\text{Cheng et al., 1994})$$

can be established where

Z_G = $^{14}\text{CO}_2$ evolution rate from the glucose treated container.

Z_W = $^{14}\text{CO}_2$ evolution rate from the water treated control container.

R = $^{14}\text{CO}_2$ evolution rate due to root respiration.

C_S = soluble carbon concentration in the rhizosphere (g C l^{-1})

C_G = added glucose carbon concentration (g C l^{-1})

The two unknown variables are R and C_S which can be ascertained by establishing a family of simultaneous equations for two different added glucose concentrations along with the water control.

Statistical analysis was performed using *STATISTICA for Windows*, release 5.1 (StatSoft, Inc, 1984 - 1996) All data were tested for normality and homoscedascity. Pots that developed irreparable leaks in the *Vaseline* seal were excluded from the $^{14}\text{CO}_2$ release analyses. These pots, along with pots that showed negative soil ^{14}C values due to the very low radiation values, were excluded from the percent allocation investigations. Two-way analysis of variance was performed using CO_2 and glucose as the two independent variables. Some of the calculations called for the data to be expressed in terms of percentages. To statistically analyse these results the data were arcsine transformed using the formula:

$$p' = \frac{1}{2} \left[\arcsin \sqrt{\frac{X}{n+1}} + \arcsin \sqrt{\frac{X+1}{n+1}} \right] \quad (\text{Zar, 1984})$$

Where the actual proportions are expressed as X/n , the ^{14}C value of the pool/total ^{14}C in the plant.

Table 5-1: Labelling chamber CO₂ concentrations and total amounts of radiation absorbed by the *Felicia aethiopica* ssp. *aethiopica* plants along with the amount that was recovered. The values are in $\mu\text{mol}\cdot\text{mol}^{-1}$, Becquerel (1Bq = 1 disintegration per second) or percentage. 1.85 MBq ¹⁴CO₂ was added to each chamber. No statistical analysis could be performed on these data because there was no replication of the glucose treatments.

	Glucose treatment		
	0 g C.l ⁻¹	0.5 g C.l ⁻¹	6.8 g C.l ⁻¹
Initial [CO ₂]	342 $\mu\text{mol}\cdot\text{mol}^{-1}$	375 $\mu\text{mol}\cdot\text{mol}^{-1}$	345 $\mu\text{mol}\cdot\text{mol}^{-1}$
Start of labeling [CO ₂]	586 $\mu\text{mol}\cdot\text{mol}^{-1}$	620 $\mu\text{mol}\cdot\text{mol}^{-1}$	586 $\mu\text{mol}\cdot\text{mol}^{-1}$
Final [CO ₂]	275 $\mu\text{mol}\cdot\text{mol}^{-1}$	272 $\mu\text{mol}\cdot\text{mol}^{-1}$	276 $\mu\text{mol}\cdot\text{mol}^{-1}$
Total radiation absorbed	9.82x10 ⁵ Bq	1.04x10 ⁶ Bq	9.78x10 ⁵ Bq
Radiation recovered	5.96x10 ⁵ Bq	5.00x10 ⁵ Bq	5.97x10 ⁵ Bq
Percent recovery	61 %	48 %	61 %

Results

The 0.5 g glucose C.l⁻¹ subsection of the experiment was the first one performed in the study, the chase period ran for about 8 hours after the start of the labelling. It was found that this period of time was too short and so the next two chase periods were extended to 11 hours after the commencement of labelling. On subsequent investigation it was found that the amount of radiation recovered during the analysis of the 0.5 gC.l⁻¹ plants was very much lower than that of the other two sections of the study (Table 5-1). These plants also had an anomalous pattern of below-ground ¹⁴CO₂ release during the chase period with the elevated CO₂ treated plants showing a pattern that was similar to the pattern seen in the 6.8 g glucose C.l⁻¹ treatment while the ambient treated plants exhibited a release pattern that was similar to the 0 gC.l⁻¹ water control pots (data not shown). It was for these three reasons that it was decided to exclude these data from further analyses and discussion.

Measurements of overall plant growth showed a non-significant ($p > 0.1$) CO₂ treatment effect (Figure 5-1). As was explained in the methods, some of the green leaf material had to be removed prior to labelling resulting in the dry mass of green leaf material and other plant parts exposed to the ¹⁴CO₂ labelling seen in Table 5-2.

Figure 5-2 shows the ¹⁴CO₂ release from the pots during the chase period. It took 2 to 3 hours for the label to appear in the below-ground CO₂ stream and there was no distinct peak in ¹⁴CO₂ release in the 0 g glucose C.l⁻¹. There was just a general increase, with the appearance of a plateau after 10 hours. For the 6.8 g glucose C.l⁻¹ treatment there was only a very gently increase in ¹⁴CO₂ release with no evidence of a plateau.

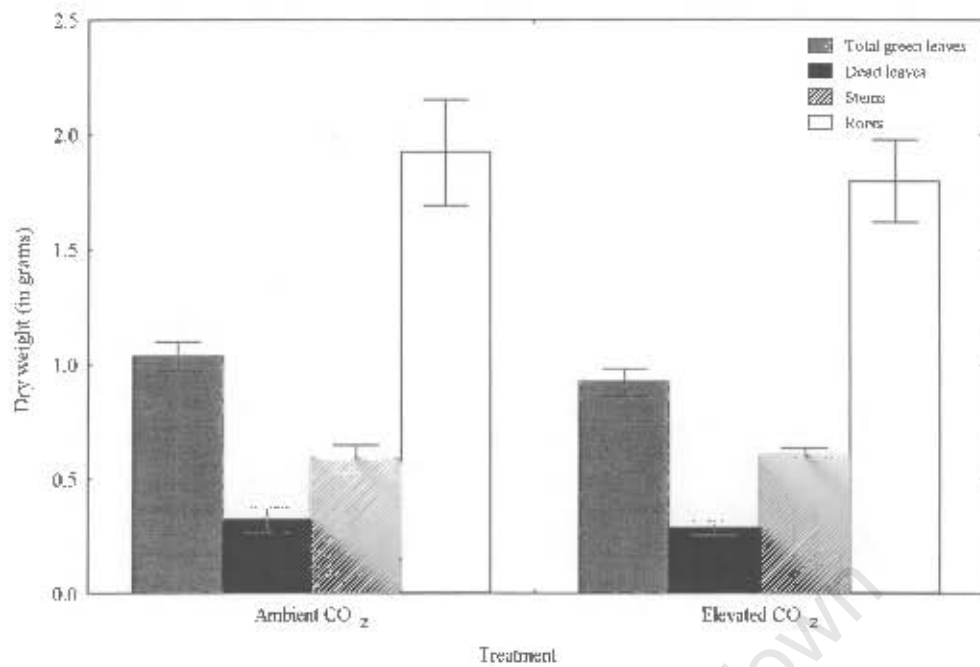


Figure 5-1: Mean dry weights (in g) of *Felicia aethiopica* ssp. *aethiopica* plant parts at the beginning of the labelling experiment (n=10). The bars are means \pm se.

Table 5-2: Mean dry weight of green leaves, stems and roots of the *Felicia aethiopica* ssp. *aethiopica* plants exposed to ¹⁴CO₂ labelling. The values are in grams \pm standard error (n = 5). R/S is the root to shoot ratio. ns Not Significant, *p \leq 0.05.

Treatment		Green leaves	Stems	Roots	R/S
0 g C.l ⁻¹	Ambient CO ₂	0.946 \pm 0.100	0.566 \pm 0.059	2.340 \pm 0.266	1.571 \pm 0.141
	Elevated CO ₂	0.854 \pm 0.090	0.640 \pm 0.036	1.739 \pm 0.264	1.149 \pm 0.110
6.8g C.l ⁻¹	Ambient CO ₂	0.925 \pm 0.071	0.609 \pm 0.112	1.505 \pm 0.281	1.006 \pm 0.207
	Elevated CO ₂	0.784 \pm 0.063	0.567 \pm 0.043	1.860 \pm 0.268	1.393 \pm 0.219
Statistics ^a					
Glucose effect		ns	ns	ns	ns
CO ₂ effect		ns	ns	ns	ns
Glucose x CO ₂		ns	ns	ns	*

a: Two-way ANOVA

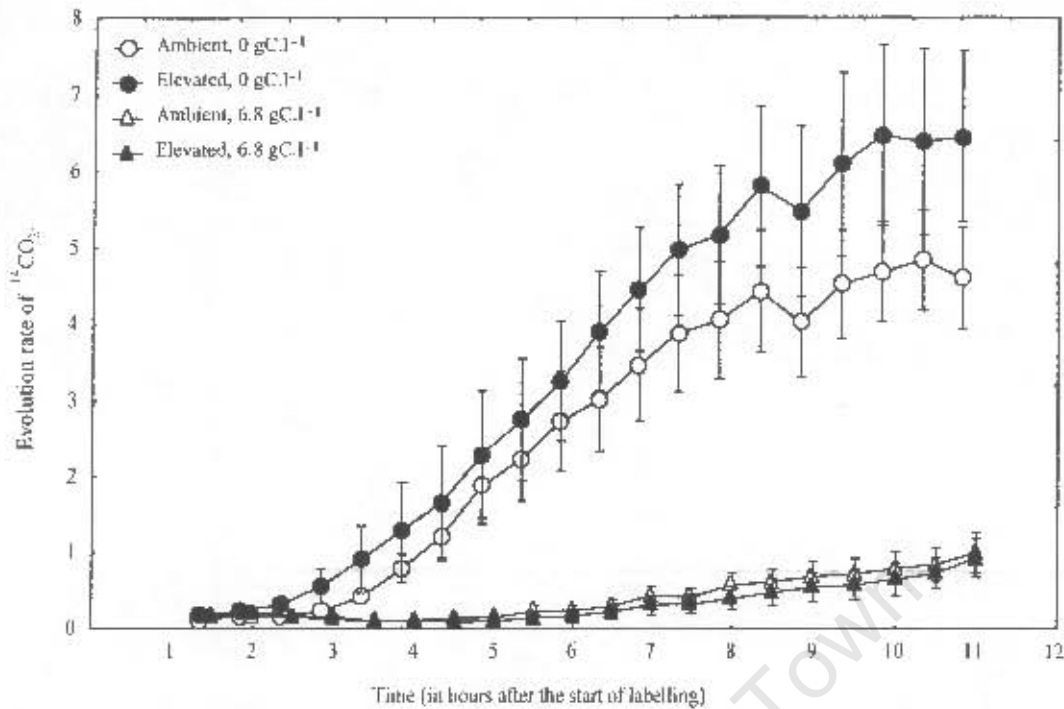


Figure 5-2: Rates of $^{14}\text{CO}_2$ evolution ($(^{14}\text{CO}_2$ evolution in Bq/total ^{14}C recovered)* 10^5) from the soil surrounding *Felicia aethiopica* ssp. *aethiopica* plants after pulse labelling the shoots with $^{14}\text{CO}_2$. $n=5, 4, 3, 3$ for the 0gC.l^{-1} ambient and elevated CO_2 and 6.8gC.l^{-1} ambient and elevated CO_2 treatments respectively). The points are means \pm se.

There was no difference between the ambient and elevated atmospheric CO_2 treated plants while the very significant effects of glucose on $^{14}\text{CO}_2$ release was shown by the substantial reduction in $^{14}\text{CO}_2$ release of the 6.8gC.l^{-1} line.

The overnight CO_2 trap was analysed in two ways. The radioactivity of the sample was measured and the total amount of CO_2 released from soil respiration was measured by titration with 0.1M HCl . The average values of CO_2 release, in $\text{mgCO}_2.\text{hr}^{-1}.\text{pot}^{-1}$ were 4.00 and 3.89 for the $0\text{g Glucose C.l}^{-1}$ ambient and elevated treatments respectively (Figure 5-3) while the $6.8\text{g Glucose C.l}^{-1}$ treatment yielded values of 7.24 and $7.35\text{ mgCO}_2.\text{hr}^{-1}.\text{pot}^{-1}$ for the ambient and elevated treatments respectively. Here it can be seen that there was no effect of CO_2 treatment on the soil respiration rate but there was a very dramatic increase in the soil respiration as a result of the glucose addition ($p \leq 0.001$).

The ^{14}C activity of the overnight sample was added to the cumulative value of the daytime $^{14}\text{CO}_2$ release to yield a total below-ground respiration value that was used in further comparisons. The four values for the $0\text{g glucose C.l}^{-1}$ ambient and elevated CO_2 treatment and $6.8\text{g glucose C.l}^{-1}$ ambient and elevated CO_2 treatment are $1332.7, 1178.0, 256.01$ and 170.54 Bq respectively.

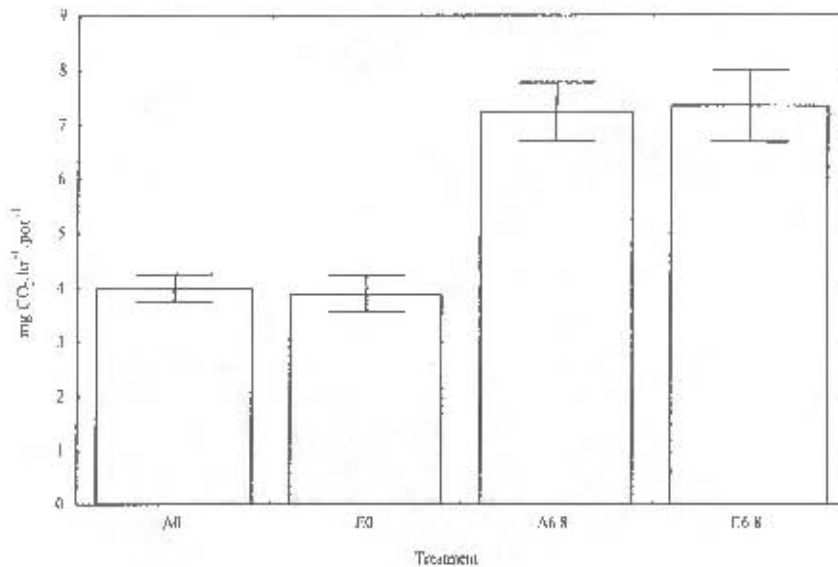


Figure 5-3: Total overnight soil respiration of *Felicia aethiopica* ssp. *aethiopica* plants exposed to elevated atmospheric CO₂ concentrations and glucose additions to the soil. The bars are means \pm s.e. Codes used are as follows: A0 = 0 g glucose C.l⁻¹ and ambient CO₂ treatment (n=5), E0 = 0 g glucose C.l⁻¹ and elevated CO₂ treatment (n=5), A6.8 = 6.8 g glucose C.l⁻¹ and ambient CO₂ treatment (n=4), E6.8 = 6.8 g glucose C.l⁻¹ and elevated CO₂ treatment (n=3).

Investigating the CO₂ treatment effect on plant carbon allocation revealed that there was a significant reduction in ¹⁴C present (expressed as total Bq) in the leaves of the *Felicia aethiopica* ssp. *aethiopica* plants grown at elevated CO₂ concentrations (Table 5-3). This table also shows the reductions through the rest of the plants with significant differences occurring in the roots and K₂SO₄ extractable carbon. These results show that there was a down regulation of the photosynthetic machinery that resulted in less ¹⁴CO₂ being taken up by the plant and consequently less being released by the roots into the soil.

To correct for potential differences in plant sizes and slightly different labelling conditions within the chamber during the two glucose treatments, the allocation of the absorbed ¹⁴C around the plant is expressed in terms of the percentage of the total ¹⁴C recovered from the plant. This allows the investigation of allocation within the plant. It was found that there were no significant differences and so it can be concluded that the plants were taking up less carbon but proportional allocation around the plant remained constant (Table 5-4). The one exception occurred for the soluble carbon that showed a reduction in the percentage of the total that was allocated to this pool.

Table 5-3: Total radiation (in Bq) in the different plant parts of *Felicia aethiopica* ssp. *aethiopica* exposed to elevated atmospheric carbon dioxide concentrations. A signifies ambient carbon dioxide treatment while E signifies elevated CO₂ conditions. The values are means ± standard error (n=5, 5, 4, 3 for the 0gC.l⁻¹ ambient and elevated CO₂ and 6.8gC.l⁻¹ ambient and elevated CO₂ treatments respectively). ns Not Significant, *p≤0.05

Treatment		Leaves	Stems	Roots	Rhizosphere	Bulk Soil	Soluble ¹⁴ C
0 g C.l ⁻¹	A CO ₂	50076 ±15903	12167 ±6143	5939 ±912	565 ±270	2114 ±522	199 ±69
	E CO ₂	32032 ±8985	7427 ±1492	3459 ±939	431 ±243	3135 ±1361	77 ±23
6.8g C.l ⁻¹	A CO ₂	55171 ±6703	14813 ±2227	4959 ±527	296 ±62	1467 ±571	179 ±61
	E CO ₂	30279 ±2082	7832 ±214	3473 ±414	180 ±36	587 ±386	56 ±6
Statistics ^a							
Glucose effect		ns	ns	ns	ns	ns	ns
CO ₂ effect		*	ns	*	ns	ns	*
Glucose x CO ₂		ns	ns	ns	ns	ns	ns

a: Two-way ANOVA

Looking at the glucose effects on plant carbon allocation: to allow for comparison, these data have been presented in a similar manner to that seen in Cheng *et al.* (1994). The statistical analyses however are very different. In Cheng *et al.* (1994) multiple t-tests were performed on the untransformed data. The use of multiple t-tests can result in spurious significant differences (an increase in type II error) while percentages form a binomial distribution (Zar, 1984) so the data need to be transformed to allow parametric statistical analysis. In this *Felicia* experiment the percentage data was arcsine transformed (see formula in the methods) followed by a two way analysis of variance. The results can be seen in Figures 5-4 and 5-5 while the output of the statistical analyses are summarised in Table 5-4. Here it can be seen that there was no glucose effect on the allocation of carbon through the plant while significant effects occurred in the soil with a significant reduction in the rhizosphere value and reduced ¹⁴CO₂ release from the soil.

One of the aims of this experiment was to separate between root and microbial respiration within the rhizosphere along with calculating the rhizosphere soluble carbon concentration. Due to the elimination of the 0.5 g glucose C.l⁻¹ data one is not able to compile the simultaneous equations needed for the calculation. An approximation can however be made by using the assumption that root respiration was approximately equal to the 6.8 g glucose C.l⁻¹ treatment (Cheng *et al.*, 1994). The percentage contribution made by the roots of *Felicia aethiopica* ssp. *aethiopica* plants to the total rhizosphere respiration was 14% for the ambient CO₂ and 9% for the plants exposed to elevated CO₂ treatment.

Table 5-4: Statistical analysis of the ^{14}C allocation patterns in the labelled *Felicia aethiopica* ssp. *aethiopica* plants. The percentage data was arcsine transformed and then subjected to a two way analysis of variance (n=5, 4, 3, 3 for the 0gC.l⁻¹ ambient and elevated CO₂ and 6.8gC.l⁻¹ ambient and elevated CO₂ treatments respectively). ns Not Significant, *p≤0.05, ** p≤0.01 and ***p≤0.001.

	Effect		
	Glucose	CO ₂	Glucose x CO ₂
Green Leaves	ns	ns	ns
Stems	ns	ns	ns
Roots	ns	ns	ns
Respiration	***	ns	ns
Rhizosphere	**	ns	ns
Bulk soil	ns	ns	ns
K ₂ SO ₄ extractable carbon	ns	*	ns
Insoluble carbon	ns	ns	ns

It is difficult to attach statistical significance to these numbers due to fact that the glucose treatments were not in matched pairs. Only the means of the two groups could be used in the calculation. It can be assumed that there was no statistical significance between the ambient and elevated root respiration values due to the lack of significance between the two groups in other calculations such as $^{14}\text{CO}_2$ release and carbon allocation within the plant.

By measuring the ^{14}C in the K₂SO₄ extracts of the soil one was able to separate between insoluble carbon and soluble carbon in the soil. In this experiment it was shown that the soluble carbon component was affected by the CO₂ environment of the plants while the insoluble fraction was unaffected by either CO₂ treatment or glucose treatment. The distribution of the bulk soil carbon between these two pools can be seen in Table 5-5.

Table 5-5: Distribution of the bulk soil ^{14}C between the K₂SO₄ soluble and insoluble fractions. The results are expressed as percentages of the total ^{14}C recovered and the statistical analyses were performed on the arcsine transformed data. (n=5, 4, 3, 3 for the 0gC.l⁻¹ ambient and elevated CO₂ and 6.8gC.l⁻¹ ambient and elevated CO₂ treatments respectively). ns Not Significant, *p≤0.05

Treatment		Soluble ^{14}C	Insoluble ^{14}C
0 g C.l ⁻¹	Ambient CO ₂	0.280	2.927
	Elevated CO ₂	0.187	6.705
6.8g C.l ⁻¹	Ambient CO ₂	0.299	1.943
	Elevated CO ₂	0.112	2.916
Statistics ^a			
Glucose effect		ns	ns
CO ₂ effect		*	ns
Glucose x CO ₂		ns	ns

a: Two-way ANOVA

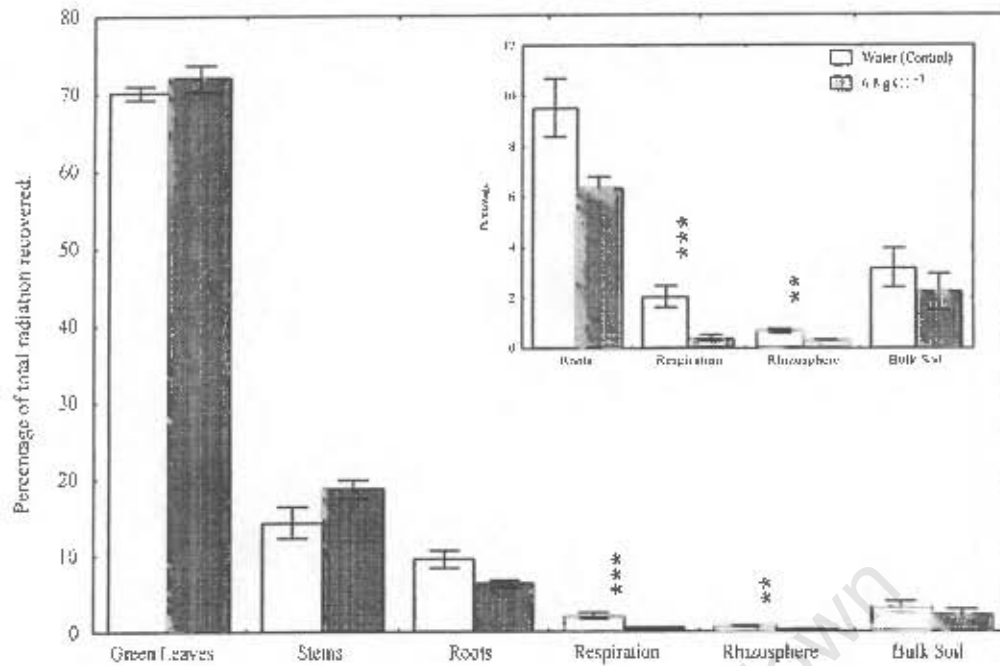


Figure 5-4: Distribution of ^{14}C in plant and soil pools after pulse labelling *Felicia aethiopica* ssp. *aethiopica* plants that had been exposed to ambient atmospheric CO_2 concentrations for 810 days. Measurements made after 20 hours of tracing. ($n = 5$ for water control and $n = 3$ for 6.8 g glucose C.l^{-1}) The bars are means \pm se of the percentage allocation as was presented by Cheng *et al.* (1994) while the statistics were performed on arcsine transformed data. ** $p < 0.01$ and *** $p < 0.001$.

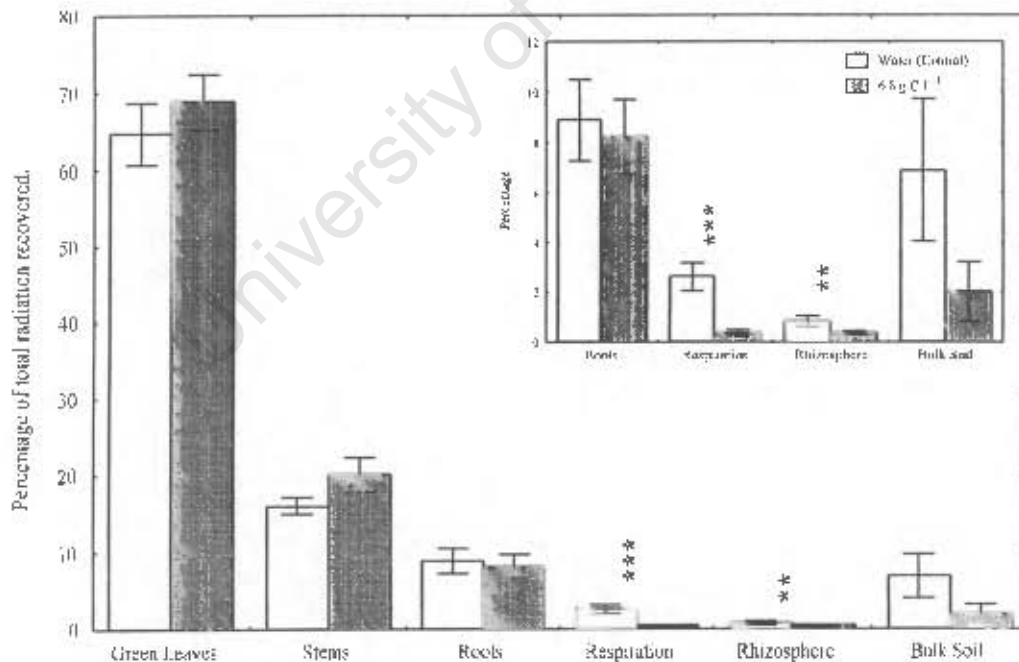


Figure 5-5: Distribution of ^{14}C in plant and soil pools after pulse labelling *Felicia aethiopica* ssp. *aethiopica* plants that had been exposed to elevated atmospheric CO_2 concentrations for 810 days. Measurements made after 20 hours of tracing. ($n = 4$ for water control and $n = 3$ for 6.8 g glucose C.l^{-1}) The bars are means \pm se of the percentage allocation as was presented by Cheng *et al.* (1994) while the statistics were performed on arcsine transformed data. ** $p \leq 0.01$ and *** $p \leq 0.001$.

Discussion

The rate of carbon transfer through *Felicia aethiopica* ssp. *aethiopica* was considerably slower than in wheat (*Triticum aestivum*), tall fescue (*Festuca arundinaceae*) and buffalo gourd (*Cucurbita foetidissima*) that were investigated by Cheng *et al.* (1993 and 1994). In *Felicia* it took two to three hours for the label to first appear in the soil air and the peak $^{14}\text{CO}_2$ release was at about 10 hours after the start of labelling (Figure 5-1). The other plant species studied had times of between 30 and 60 minutes for the label to first appear while the peaks ranged from two hours for wheat to 4 hours with no decline for the tall fescue (Cheng *et al.*, 1993 and 1994). In both of these measurements it can be seen that the *Felicia* plants were between two and five times slower to respond to the addition of the ^{14}C label. This plant is a small leaved herbaceous perennial that is adapted to a low nutrient Mediterranean environment (Bond & Goldblatt, 1984). Due to the nutrient limitations one would expect the plant to use a larger proportion of the captured carbon in maintenance of tissues rather than growth. A possible soil mediated reason for a delay in the appearance of $^{14}\text{CO}_2$ is the sorption of organic molecules to the soil particles making them unavailable to soil microbes (Jones and Edwards, 1998). In this experiment the soil used was very sandy with low organic matter and clay content and thus one would not expect this sorption effect to be significant.

Comparing Figures 5-4 and 5-5 to those seen in Cheng *et al.* (1994) one can see that similar patterns of ^{14}C allocation were occurring. The significant reduction in respiration seen for wheat and tall fescue are again seen in this study. With glucose treatment, Cheng *et al.* (1994) showed significant increases of ^{14}C in the rhizoplane of wheat and tall fescue with no change in buffalo gourd while in this, *Felicia*, case the rhizosphere values were depressed. The slower rate of translocation and allocation in *Felicia* can again be seen by the greater proportion of the ^{14}C remaining in the leaves and stems (80 to 90%) as compared to the 45, 60 and 60% occurring in the shoots of wheat, tall fescue and buffalo gourd respectively (Cheng *et al.*, 1994).

The isotopic trapping method relies on three assumptions for the calculations to be valid (Cheng *et al.*, 1993, Cheng *et al.*, 1994). These are that the addition of glucose to the root-soil column only dilutes the root exudates and does not produce short-term effects on the overall plant's physiology, that glucose is compatible with the root exudate in terms of substrate specificity, assimilation efficiency and absorption kinetics for the rhizosphere flora

and that addition of the glucose does not stimulate or suppress the microbial activities within the rhizosphere.

In Barley plants, the osmotic effect of glucose treatment on the carbon partitioning was shown to be transient, lasting less than three hours (Williams *et al.*, 1991) indicating little influence of the added osmoticum on the isotopic trapping experiments. Low molecular weight sugars are easily absorbed by intact plant roots at concentrations very much lower than the ones used in this isotopic trapping experiment (Hendrix 1984, Jones & Darrah, 1992) while Cramer and Lewis (1993) showed that significant amounts of HCO_3^- can be absorbed by roots which could result in $^{14}\text{CO}_2$ resorption by the plant roots. None of these studies were performed in natural soil (Hendrix, 1984, Jones & Darrah, 1992, Cramer and Lewis, 1993) and so carbon uptake was measured in the absence of competition from microbes that may influence the rates considerably. Cheng *et al.* (1996) showed that carbon in the rhizosphere was not limiting to the microbes and so one can conclude that there would be the potential for carbon resorption by the plant.

One effect that could confound the experiment would be the occurrence of isotopic trapping elsewhere in the plant as a result of the soil addition of glucose upsetting the ^{14}C allocation within the plant. There was weak ($p=0.06$) support of the glucose effect on the 4% increase in allocation of ^{14}C to the stems which could indicate some trapping occurring in this region. There was, however, no effect on the root allocation of ^{14}C . It is possible that with the addition of the glucose to the soil, the sink strength from the microbes was reduced while the demand from the roots remained the same and so the differences between root and microbial respiration would still be valid. Support for this conclusion comes from Jones and Darrah (1992) where the authors clearly demonstrated that flowing culture solutions caused significantly more root exudation when compared to static cultures. In this current case one can interpret the addition of glucose to the soil in terms of changing a flowing system (flow of carbon from the root into the growth and respiration of microbes) to a static system where the demand from the microbes is now being fulfilled by the added glucose solution. The above lines of evidence support the conclusion that the physiology of the *Felicia* plants was not significantly upset by the addition of the glucose solution to the soil.

Harris and Paul (1991) accepted that not taking the carbon recycling by carboxylase enzymes into account is a simplifying assumption but studies such as the current one produce an integrated estimate of CO_2 release that is still a useful figure when one is considering whole plant or ecosystem level carbon flows.

On the question of whether glucose is a good surrogate for root exudates Swinnen (1994) investigated the problem by using three forms of labelled carbon supply, glucose, dried root extract and root cell wall material. The author showed that glucose was the most rapidly respired additive and so in this current, short-term, experiment glucose was the most suitable substrate to add.

Direct evidence of isotopic trapping is given by observing the increase in ^{14}C in the soluble extract taken from the soil along with an increase in the labelling of the soil itself (Cheng *et al.*, 1993 and 1994). In this experiment there was no increase in these two values but there was a very dramatic decrease in the net below-ground $^{14}\text{CO}_2$ release expected with the addition of unlabelled glucose to the soil. Considering there is no compelling evidence to the contrary, one can conclude that the soil microbes were indeed being affected by the addition of glucose.

As happened in Cheng *et al.* (1994) the intermediate glucose treatment data were not usable in the calculations of root respiration and rhizosphere carbon concentration. This is despite the reduction of added glucose concentration from 1.2 to 0.5 g glucose C.l^{-1} .

Another clear effect of the glucose addition to the soil is the substrate induced respiration (SIR) shown by total rate of CO_2 release (Figure 5-3). It is important to remember that this SIR was measuring the entire soil volume while the ^{14}C investigations are more limited to the rhizosphere region of the soil. It has been shown (Cheng *et al.*, 1996) that carbon is not limiting in the rhizosphere and so this induced respiration should not have had any effect on the labelling results. It is interesting to note that the two CO_2 treatments showed no difference in total respiration after the addition of glucose clearly indicating that there was no reserve respiration capacity available that was not being manifested due to carbon limitations.

Large variability of the data was encountered in this experiment. This stems from two sources: (i) the plants were seed grown from wild populations and so one would expect to find a reasonable amount of variability in the plants that germinate and (ii) during the analysis of the labelled material, some of the samples had very low levels of radiation that were not much higher than the background measure. This was particularly pronounced in the soil samples where small initial differences were multiplied up to the total soil mass of 850g. In addition to this, the samples that were combusted in the sample oxidiser were very small (20mg) and so when measuring out soil samples it was difficult to ensure that the samples were homogeneous. These two factors were responsible for the lack of statistical support for the apparent difference in insoluble ^{14}C seen in Table 5-5. Cheng *et al.* (1994)

encountered similar variability problems in buffalo gourd. This led the authors to suggest that this method of isotopic trapping is more suitable to plants that have a high exudation potential meaning one would have to do some preliminary assessments of this potential prior to the trapping experiment.

Natural seed variability may mean that ecosystem responses to global changes may not be as catastrophic as would be predicted from results of pure strain, agricultural models. Competition among species is another factor that has the potential to confound single species based predictions.

The labelling of the plants with $^{14}\text{CO}_2$ involved placing ambient and elevated treated plants into similar conditions within the labelling chamber. During the labelling period the CO_2 concentration was reduced from about $600 \mu\text{mol}\cdot\text{mol}^{-1}$ to about $340 \mu\text{mol}\cdot\text{mol}^{-1}$. These conditions resulted in the elevated CO_2 treated plants absorbing less ^{14}C than the ambient plants. The significant reduction can be explained by the combination of the non-significant reduction in leaf weight and the ns reduction in leaf nitrogen content seen in Chapter 2. This shows that there was acclimation in the plants in response to the CO_2 treatment. This down regulation of carbon uptake is not unique for habitats that are characterised by nutrient-poor soils (Ward and Strain, 1999).

Insam *et al.* (1999) extensively studied many aspects of below-ground biology in an artificial tropical ecosystem. Very few significant effects of elevated atmospheric CO_2 resulted which was attributed to the soils having a low organic matter content and low nutrient supply. This lack of response also appeared to occur in this *Felicia* experiment. Insam *et al.* (1999) did find a reduction of water extractable organic C which was repeated in the current study with the K_2SO_4 soluble ^{14}C measurement. Initially most elevated atmospheric CO_2 concentration studies looked at systems that contained plentiful resources but it is being realised that many natural systems are not as responsive as they were expected to be as a result of other resource limitations (Insam *et al.*, 1999)

By looking at the proportion allocation of the absorbed carbon (Table 5-4 and Figures 5-4 and 5-5) one can see that the CO_2 treatment did not affect the plants except for increasing the soluble carbon in the soil. This observation that CO_2 did not affect the pattern of allocation, just the total amount of carbon, lends further support to Ward and Strain (1999) who discussed studies that used allometric techniques to show that CO_2 rarely alters the allocation of biomass between root and shoot. Cheng and Johnson (1998) also found that CO_2 treatment did not affect the pattern of allocation within wheat plants while, in contrast, Hodge and Millard (1998) grew *Plantago lanceolata* seedlings in sand culture for 43 days

and found that there was a proportional increase in carbon allocation to the roots but the release of total organic carbon into the soil was significantly reduced.

Paterson *et al.* (1996) investigated the allocation of radiolabelled carbon around wheat, rye grass and Bermuda grass plants that had been exposed to elevated CO₂ conditions. The plants used in the study were young (21 days old) and the chase period was five hours which was shown to be long enough to allow 45% of the label to be translocated to the roots. Overall growth of the wheat and rye grass was enhanced by CO₂ but the allocation of the label around the plants was unchanged while there was a significant increase in carbon allocation to the rhizosphere of the wheat. There was no difference between the rye grass and Bermuda grass plants (Paterson *et al.*, 1996). From this one can conclude that there is no generalised rhizosphere response to applied CO₂ treatments. Continuing from this, Norby *et al.* (1987) found that root exudation from *Pinus echinata* seedlings exposed to elevated CO₂ concentrations was increased at 31 weeks but by 41 weeks this increase was no longer seen showing that temporal effects also play a role. These studies show the care that one needs to exercise before making generalised statements about the responses of plants to applied CO₂ treatments. There are both species and temporal effects in force.

The primary aim of this experiment was to ascertain the proportional split of the total rhizosphere respiration between the roots and the microbial biomass associated with the roots. The results of this calculation are 14% for the plants exposed to ambient CO₂ conditions and 9% for the plants in the elevated atmospheric CO₂ concentrations. These values are slight over statements of the actual root respiration due to the use of the 6.8 g glucose C.l¹ treatment as an estimate of root respiration (Cheng *et al.*, 1994). Compared to other results in the literature these values for the root respiration are very low. Cheng *et al.* (1993) obtained a root respiration value of 40.6% of the total rhizosphere respiration for wheat while Cheng *et al.* (1994) obtained values of 55.4% and 68.8% for root respiration of tall fescue and buffalo gourd respectively. Swinnen (1994) obtained values of 91 to 96% for wheat and 87 to 95% for barley in pots while field values were at least 75%. The method used was completely different to the one used here but the tremendous difference between the values is notable. The one advantage of the model rhizodeposition technique as used by Swinnen (1994) is that it gives a longer term, more integrated estimate of respiration rather than the very short-term (hours) estimate that results from the isotopic trapping method used here. A disadvantage of the rhizodeposition technique is that the model substrate is injected into the soil and one needs to assume that it is approximating the processes that are occurring around the root. In isotopic trapping the label is delivered directly to the actively exuding

regions of the roots (Cheng *et al.*, 1996). Using a modeling method, Rouhier *et al.* (1996) calculated that between 70 and 90% of the CO₂ was coming from root respiration but there was no difference in the percentage ¹⁴C respired between two CO₂ treatments. When the values were expressed as radiation released per gram of root material there was a significant increase in root respiration under the influence of CO₂ treatment. Although there is little statistical support, there was an intriguing difference between the calculated root respiration of the *Felicia* plants between the two CO₂ treatments. It is possible that the composition of the exudate changed making it more easily used by the microbes which would result in less carbon being stored in the soil.

Cheng and Johnson (1998) used both ¹⁴C and δ¹³C techniques to investigate carbon input to the soil by wheat plants that had been exposed to elevated atmospheric CO₂ concentrations. The authors included a nitrogen treatment along with the CO₂ treatment. They found that CO₂ treatment greatly enhanced growth and by using δ¹³C techniques, rhizosphere respiration was shown to increase. Cheng and Johnson (1998) also found that nitrogen strongly influenced many of the responses of the plants to CO₂. Under the low nitrogen treatment, there was no stimulation of total below-ground respiration. Few significant results arose out of the ¹⁴C isotopic trapping investigations. The contribution of root respiration to the total rhizosphere respiration was unchanged from 51.3% under ambient conditions to 52.3% in the CO₂ treated plants. Again these values are very much higher than the ones observed in the current experiment. After discussing the many conflicting results seen in the literature, Cheng and Johnson (1998) concluded that the change in decomposition of soil organic matter as a result of CO₂ treatment was dependant on the plant-soil system being studied and that the effects were not unidirectional.

When treated to a short exposure period, the heather *Calluna vulgaris* did not respond to CO₂ but did respond to the concurrent nitrogen fertilisation (Verburg *et al.*, 1998). Plant biomass was unchanged and the percent allocation of ¹⁴C was also not affected by CO₂ treatment. There were differences in the uptake of ¹⁴CO₂ by the leaves which was then carried through to significant increases in bulk soil and soil respiration values. The rate of decomposition appeared slower in the elevated CO₂ treatment. Along with the increase in rhizodeposition there are indications that this slow growing, woody species may have the potential to store additional carbon in below-ground pools.

This experiment with *Felicia aethiopica* ssp. *aethiopica* revealed that a large amount of the respiration in the rhizosphere was coming from the soil bacteria surrounding the root.

This shows that much of the below-ground carbon was being released into the soil pool before being respired by the microbes, indicating the potential for change in soil storage and sequestration of carbon if the quality or quantity of exudates changes. In this experiment it was revealed that root exudation was decreased but there was the potential of an increase in the proportion contribution of microbes to the rhizosphere respiration under the influence of elevated atmospheric CO₂ concentrations. These two observations could be linked in that the increased respiration resulted in less extractable carbon from the soil. If this effect persists in other species native to the Fynbos then there is the potential for significant reductions in the sequestration of carbon in the soils of this biome under current global change scenarios. The system is unfortunately not as simple as this and so other plant species would have to be investigated to see if they respond in similar ways and the effects of temperature and changed rainfall would also need to be accounted for when investigating effects on the carbon storage potential of this system under global change scenarios.

Swinnen *et al.* (1994) warn of errors arising from root losses occurring during the isolation of the roots from the soil which would increase soil values while decreasing the root values. In this experiment the roots were not washed and so no carbon would have been leached in this step. The analysed soil samples were quite carefully picked of roots although complete removal was not possible.

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TOWARDS AN INTEGRATED UNDERSTANDING OF CARBON FLOWS
THROUGH TWO SOUTHERN AFRICAN ECOSYSTEMS EXPOSED TO
ELEVATED ATMOSPHERIC CO₂ : FYNBOS SHRUBLANDS AND
C₄ SUBTROPICAL GRASSLANDS.

Introduction

This chapter synthesises the information presented in earlier chapters (chapter 2 to 5) concerning the overall flow of carbon in the Fynbos and grassland systems investigated. Such a synthesis enables one to assess whether the ecosystems investigated have the potential to act as sinks of carbon in the face of elevated atmospheric CO₂ concentrations. To structure the diverse data that were collected, a model of the system is used. This model is based on that presented by Cardon (1996) and was adapted for application to the current study (Figure 6-1).

Nearly all biological and biologically regulated processes occurring in the soil can be influenced by increasing carbon dioxide levels through the mediation of above-ground plant biomass (Berntson & Bazzaz, 1996). In the model presented in Figure 6-1, one can see the interdependence of plant responses, soil microbial reaction to the fixed carbon and the ultimate sequestration of the carbon in various parts of the system. The series of experiments performed in this project attempts to tease out many of the processes occurring in this scheme. Although these processes are all interrelated this chapter splits the model up into 5 components for convenience and the results of the experiments are discussed according to this five part scheme (Figures 6-2 to 6-6). The single species (ss) Fynbos study forms the bulk of the discussion with the results obtained from the mixed species (ms) Fynbos study and the C₄ Natal grassland only introduced later to try to identify areas of commonality at a larger scale.

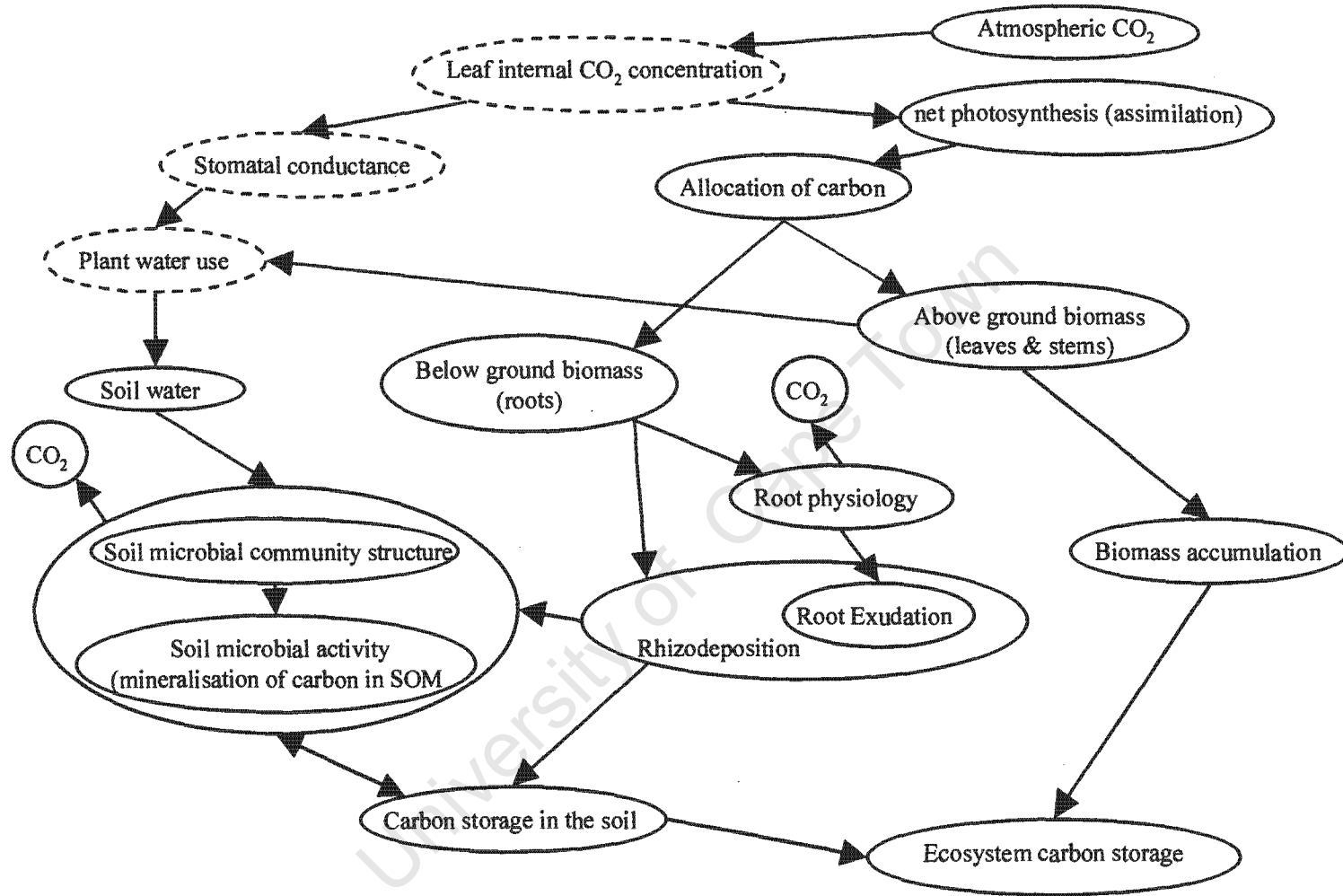


Figure 6-1 : Model of the carbon flows through a plant soil system showing the interactions that can occur among the different compartments, modified from Cardon (1996). SOM = soil organic matter.

The two-part Fynbos study allows the comparison of both spatial and temporal scales of CO₂ response. The experiments looked at either one species (*Felicia aethiopica* ssp. *aethiopica*) growing individually in a pot (ss experiment) or arrays of five different species grown in microcosms (ms study). The next level of response would be at the landscape level and although experiments were not conducted at this level, predicted responses will be discussed assuming that some the more robust of the microcosm results can be scaled up. In the ss experiment two harvests were made, one at 568 days after planting and the second at 810 days after planting. This difference gives indications of how the responses of the plants could change with time and as growing conditions change.

The species chosen for the ss study (*Felicia aethiopica* ssp. *aethiopica*) exhibited very slow growth and development with an average of only 3.7 g of dry matter accumulating in almost three years of growth. This study thus complements earlier studies of fast-growing species (Poorter et al. 1993) and provides insights into how slow growing shrubby plants will respond to changes in the atmospheric CO₂ concentrations.

The grassland study site was natural grassland surrounding a CO₂ source (Harris et al., 1997) that is known to have been fumigating the area for at least 70 years and possibly longer. Added to this, a controlled fumigation experiment was established away from the primary source that was investigated for two years (1997-1998). The site thus yielded data from a 70+ year treatment, a two year treatment, a one year treatment and control plots. The different time-scales of CO₂ exposure allows for the comparison of short-term studies with long-term outcomes. Many earlier elevated CO₂ studies were compromised by the very short-term experimental treatments. These problems have been overcome by the above design.

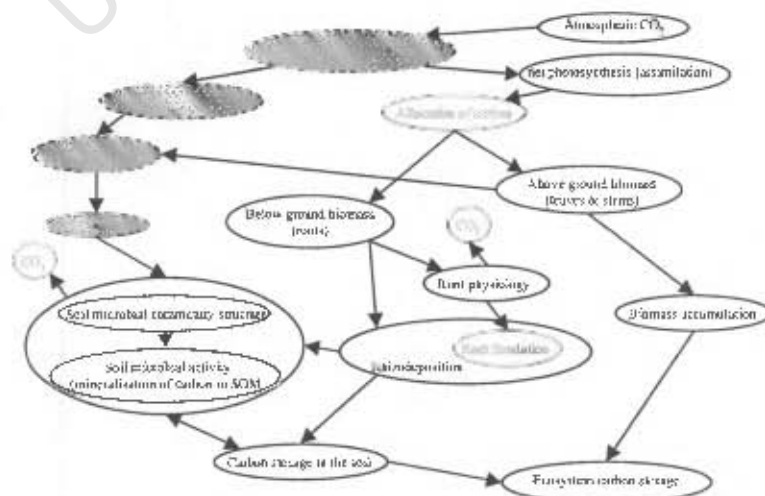


Figure 6-2 : Leaf physiology, plant water use and soil water content.

Part A: Leaf physiology, plant water use and soil water (Figure 6-2).

This section of the model does not contain very much information, as can be seen from the dashed lines. Through these linked processes one can see the way in which the soil water content can be affected by elevated atmospheric CO₂ concentrations although investigations of this effect were not within the scope of the current project. In both Chapter 3 and Chapter 4 it was found that there was no significant change in the soil moisture content. This was largely due to the experimental design where it was ensured that the plants were well watered in order to prevent soil moisture effects altering the CO₂ effects being studied.

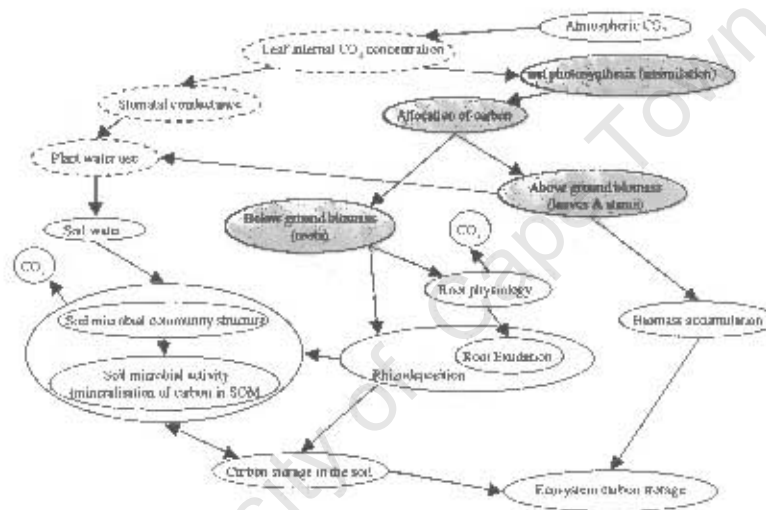


Figure 6-3 : Photosynthesis and biomass allocation.

Part B: Photosynthesis and biomass allocation (Figure 6-3).

No direct measurements of the photosynthetic rates were made; however a surrogate measure, namely the uptake of ¹⁴C by the plants, showed that the plants acclimated to the changed atmospheric environment through the significant decrease in the amount of carbon being absorbed by the CO₂ treated plants when exposed to the conditions present in the ¹⁴C pulse-chase experiment (Chapter 5). This conclusion is supported by the decrease in the leaf nitrogen content of the plants (Chapter 2). The decreased ¹⁴C uptake resulted in other plant parts showing reduced radioactivity as well. There was little change in the gross allocation of biomass around the plant with the plants becoming slightly smaller with no change in the root to shoot ratio (Chapters 2 and 5).

Nutrient and ¹⁴C allocation data can be expressed either as total amounts of material or as percentages of the overall size of the pool within the plant. These two portrayals reveal

different information on the plant response. Looking at the total amount of substance, for example the ^{14}C absorbed by the leaves, one is able to investigate the overall carbon budgets of the system while the underlying physiological reasons for the differences may be hidden. The reduction in absorbed radiation could be due to a reduction in the leaf area or by a physiological down-regulation of the photosynthetic machinery. This subtlety is only revealed if the data are expressed in terms of percentage of the total radiation recovered or some other feature of the plant that takes out bias such as leaf area or weight. Presenting the data in one of these ways reveals physiological responses of the plant, whether there are changes in the processing of the substance or a change in the allocation patterns of the product around the plant.

When the carbon labelling data were expressed as a percentage of the amount of ^{14}C absorbed it was found that the allocation patterns around the plant and $^{14}\text{CO}_2$ release from the soil were unchanged. The only significant change was the soluble carbon concentration in the soil, which will be discussed in a later section. This shows that the response of the plant was limited to one of acclimation and so the next task is to explain reasons for this lack of response.

The reduction in the root to shoot ratio from the 568 day harvest to the 810 day harvest could be interpreted as a pot effect influencing the growth of the plants (Arp, 1991). When one considers the fact that there was less than 2 g of root material in pots containing 850 g soil in 770 cm^3 of volume it would be surprising for the plant to be pot limited. Furthermore, the above-ground leaves and shoots continued to grow with no evidence of the pot influencing the aerial growth of the plants (Chapter 2).

In a recent meta-analysis of the growth responses to elevated CO_2 of plants exposed to sub-optimal growth conditions, Poorter & Pérez-Soba (2001) showed that low soil nutrient supply reduced the proportional growth stimulation by CO_2 treatment. The soil used in this Fynbos experiment was natural and unfertilised and so one can conclude that the nutrient limitation that is characteristic of this soil type (Specht & Moll, 1983) was restricting the response of the treated plants.

Comparing the results of these experiments with other studies performed in Mediterranean climates is difficult because of the very different experimental protocols used. Dhillon *et al.* (1996) grew the weedy annual grass *Bromus madritensis* for 5 months in soil monoliths and found that root growth was significantly stimulated by CO_2 treatment. They concluded that this allowed the plant to mine larger volumes of soil for water and nutrients which would assist the plant during times of drought or nutrient stress. This increased root

growth also increases the amount of carbon exudation into the soil for microbial and fungal utilisation.

The *Felicia aethiopicæ* ssp. *aethiopicæ* plants grew very slowly in these experiments and appeared to show no difference in growth rate under the two CO₂ treatments. This is consistent with the ideas of Van Noordwijk *et al.* (1998) who suggested that inherently slow growing plants may not be able to increase their growth in resource enhanced environments because of limited internal sinks for carbohydrates. This would mean that the plants were not carbon limited and growth was controlled by a shortage of nutrients (Oren, 2001, Scholes & van Breemen, 1997, Gorissen, 1996).

There was a significant increase in the herbivory of the plants grown under elevated CO₂ conditions. The biomass allocation patterns showed that the two groups of plants were the same size. This means that although the CO₂ treated plants were being damaged by the insects they were still maintaining their size when compared to the ambient plants. The CO₂ treated plants were thus acquiring sufficient extra carbon to maintain their size relative to the control group. The ¹⁴C studies showed a reduction in the absorption of CO₂ but it needs to be remembered that the plants were labelled in identical elevated CO₂ conditions. In a similar CO₂ environment, the elevated treated plants would photosynthesise at a lower rate than ambient plants. However, in the glasshouse environment, there were two CO₂ concentrations with the plants exposed to the elevated CO₂ treatment photosynthesising more than the ambient plants.

Part C: Root physiology and Rhizodeposition (Figure 6-4).

The root/soil interface is a critical region of the model because the carbon flows occurring here drive the majority of below-ground ecosystem processes (Killham & Yeomans, 2001) and this topic formed the main focus of this project. Root exudation is a relatively minor contributor to total below-ground carbon transfer, relative to processes such as root turnover, but it is a very important process due to the nature of the exudates that can influence microbial responses and acquisition of nutrients (Cardon, 1996). The idea that elevated atmospheric CO₂ can enhance the exudation of carbon compounds from the roots and lead to significant microbial responses is key to our understanding of the long-term effects of elevated CO₂ on soil:plant interrelationships (Hu *et al.*, 1999).

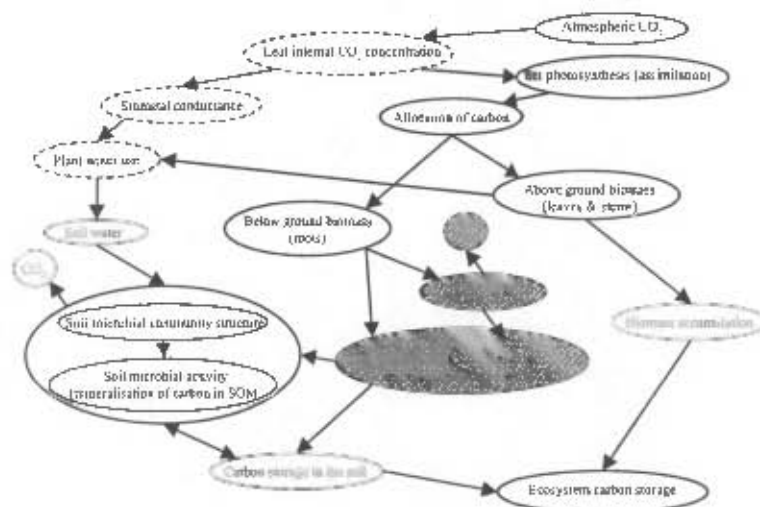


Figure 6-4 : Root physiology and rhizodeposition.

In this project, Chloroform Fumigation Extraction (CFE) techniques were used to elucidate changes in the microbial biomass and to measure the soluble carbon content of the soil (Chapter 3) while $^{14}\text{CO}_2$ labelling was used to follow recently fixed carbon from the leaves into the soil along with making direct measures of the soluble carbon release into the soil (Chapter 5).

Elevated atmospheric CO_2 treatment reduced the release of soluble carbon from the roots of *F. aethiopica* while the root biomass remained constant. This indicates that there was less carbon released per unit root weight demonstrating a physiological change in the plants. This reduction would be likely to have the effect of reducing the size of the rhizosphere, the volume of soil influenced by the plant root. This prediction is confirmed by the weight of the rhizosphere soil in the elevated CO_2 treated plants shrinking by a non-significant 51 percent at the first harvest (Chapter 2). This reduction in the size of the rhizosphere would potentially make the plant less able to absorb nutrients from the surrounding soil which could cause it to become less competitive.

Prior to labelling, there was no difference in total soil respiration (Chapter 5). This measure is an integrated soil release measure that is not differentiating among root activity, rhizosphere microbial activity, and bulk soil microbial activity.

The CFE experiment revealed that there was significantly more soluble carbon in the soil after growth in elevated atmospheric CO_2 while the labelling experiment yielded contradictory results showing a significant decrease in the total soluble radiation in the soil as well as a decrease in the allocation of carbon to this pool by the plant. Reasons for this apparent contradiction need to be sought.

In the CFE experiment the measure of soluble carbon is an integrated measure resulting from root exudation that has occurred over a period of time while the $^{14}\text{CO}_2$ labelling experiment investigated release of recently fixed carbon. No "historical" carbon was measured. Data from Chapter 2 shows a non-significant reduction in green leaf mass along with a significant increase in leaf herbivory which resulted in less carbon being available for release from the roots. This was shown by the ^{14}C experiment (Chapter 5). On the other hand, Chapter 3 showed that there was a non-significant 17% drop in the soil microbial biomass along with a decrease in the size of the rhizosphere (the region of highest microbial activity in the soil). This would result in less root exudate being utilised by the organisms that would allow the accumulation of soluble carbon in the soil. This is how it is possible for less exudate to be released from the soil while allowing for the measured increase in soluble C in the soil. The above example demonstrates how important it is in below-ground investigations to clearly understand the analyses that are being performed. This allows one to identify differences in methods that initially appear to be measuring the same process.

Darrah (1996) used a model to show that there can be significant re-uptake of carbon exudates from roots in non-sterile soils. This observation can influence the interpretation of root exudation studies. From the ^{14}C experiment very little carbon was absorbed by the roots indicated by the lack of any isotopic trapping in the root samples (Chapter 5).

Part D: Soil Microbial responses (Figure 6-5).

Three important changes can occur in the soil microbial populations: the biomass can change, the activity of the organisms can change or the community structure of the microbes can change.

Reasons for the slight reduction in microbial biomass (Chapter 3) in spite of the significant increase in soluble carbon in the soil need to be sought. Again, as with the plant growth, it is possible that the soil organisms were not carbon limited as was found in the rhizosphere by Cheng *et al.* (1996). The quality of the exudate may also have changed making it more difficult for the microbes to utilise which would have reduced their biomass along with allowing the accumulation of soluble carbon in the soil (Lekkerkerk *et al.*, 1990).

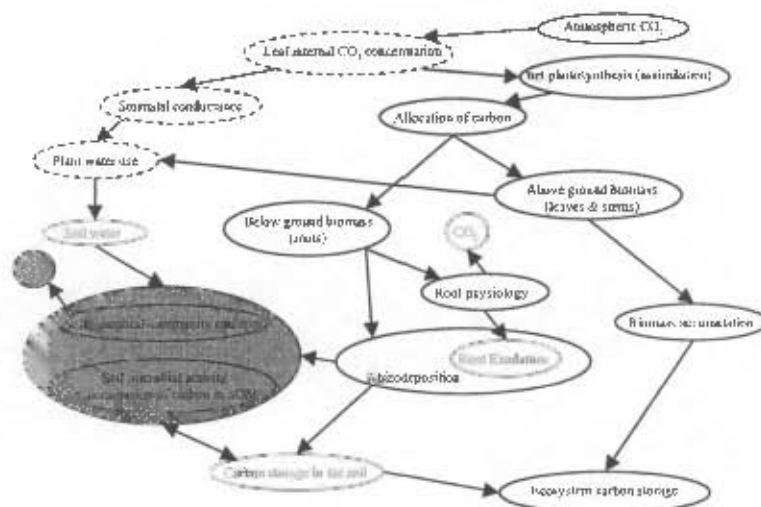


Figure 6-5 : Soil Microbial responses.

The AWCD (average well colour development) of the Biolog™ plates showed that the potential activity of the microbes was also unchanged with CO₂ treatment (Chapter 4). Any dormancy due to moisture, nutrient- or carbon-supply would have been broken by the Biolog™ analysis. Sadowsky & Schortemeyer (1997) warn that indirect measurements of microbial activity rarely mimic what is occurring under unperturbed conditions.

The ¹⁴C labelling experiment allowed the calculation of the proportion of the respired carbon coming directly from the plant's roots versus the amount that was originating from the soil microbes (Chapter 5). This is an important difference because CO₂ released by the microbes needs to pass through the soil in the form of exudates allowing greater possibilities for the carbon cycling to change under changed environments. The experiment also provided another measure of the activity of the soil microbes associated with the *Felicia* roots. It was shown that a large percentage of the recently fixed CO₂ released from the soil was being generated by the soil microbes. In the ambient group, 86% of the ¹⁴CO₂ was coming from the microbes while in the elevated treatment the value had increased slightly to 91%. There was little evidence for any statistical support for this observed difference. From these high values one can see that small changes in the root and microbial interactions have the potential to have large effects on the overall carbon flows within the soil.

Further data analyses from the Biolog™ system (Chapter 4) revealed that both the richness of substrate utilisation (number of positive wells on the plate) and the responses of the 11 different substrates were unchanged. Analysis at this level of detail begins to reveal microbial responses to CO₂ at the community response level. Dhillon *et al.* (1996) showed differences in the use of amines and amides by the soil microbes in their Mediterranean grass

study and interpreted this as an indication that the soil microbial communities may be susceptible to shifts in the quality of the organic matter input. In this *Felicia* experiment, it was surprising to see how similar the microbial reactions were in the bulk soil and at the root surfaces considering the very different substrate environment one would encounter in these two regions of the soil.

The most detailed analysis done on the data collected from the Biolog™ plates was a Canonical Correspondence Analysis (CCA) which allowed the assessment of changes in the structure of the microbial community in the soil. This analysis showed that samples from within each pot (root surface and bulk soil) generally grouped together and that there was weak separation between the two treatment groups (ambient and elevated). There was no statistical support for the difference. These results show that the potential carbon source utilisation of the microbial community was unchanged, indicating that the microbial populations were functionally unchanged. A key disadvantage of using the Biolog™ bacterial identification system in assessing microbial changes in environmental samples is that the link between an observed substrate utilisation and what is actually occurring in the soil is not possible. What this analysis did indicate though, was that in this single species experiment there was likely to be little change in the function of the microbial community which means that current carbon flows within the soil would continue under a CO₂ enriched world.

Even though there was a slight reduction in the gross microbial biomass within the soil there is clear evidence that the overall activity and community functioning was unaffected by the CO₂ treatment. There is an urgent need for data on CO₂ induced microbial responses in mature or nutrient limited systems (Hu *et al.*, 1999) and the investigations discussed above fulfils some of this need.

Part E: Carbon sequestration (Figure 6-6)

The chapter so far has shown how *Felicia aethiopica* ssp. *aethiopica* plants responded to elevated atmospheric CO₂ concentrations and these findings can now be used to make predictions on how the above- and below-ground carbon storage patterns may be influenced. It is difficult to have absolute certainty in predicting C storage patterns because of the tenuous link between changes in short-term biological carbon uptake and long-term carbon sequestration (Hungate *et al.*, 1996).

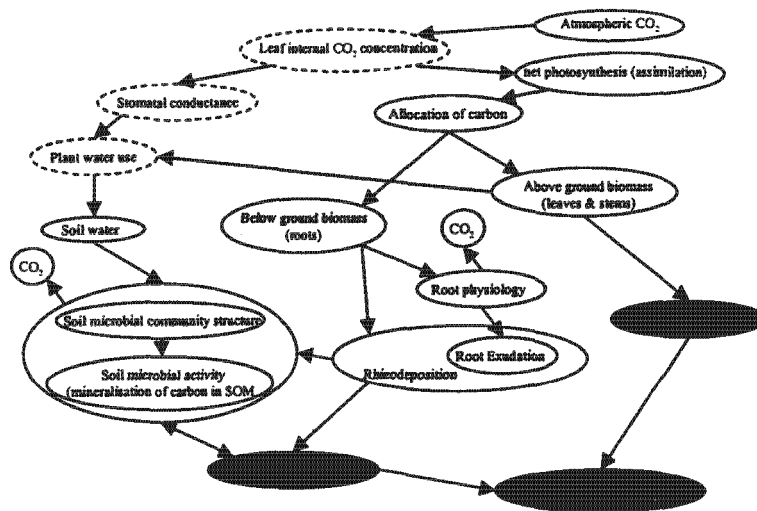


Figure 6-6 : Carbon sequestration.

Turnover rates of carbon in the system are important too, because it is only the small fraction of the fixed carbon that enters the slowly metabolised, recalcitrant pools that can be considered stored carbon (Drake *et al.*, 1996).

In an investigation of agricultural soils of the Corn Belt (East – Central USA), Collins *et al.* (2000) found that the slow turnover pools of soil organic carbon accounted for between 50% and 65% of the carbon in the soil and had mean residence times of between 12 and 80 years depending on the soil type and location.

Investigating ecosystem carbon storage runs into the problem that changes at this level take a long time to manifest themselves due to the large pool size and the slow turnover rate of storage forms of carbon (Hungate *et al.*, 1996, Canadell *et al.*, 1996). Furthermore, when using a single species, pot experiment such as the one used here it is difficult to make predictions about how a complex system would react to changes. This latter problem was investigated in the mixed species microcosm study that was performed alongside the ss experiment.

Total plant weight and above ground accumulation of biomass were unchanged by CO₂ treatment (Chapter 2). There was a certain amount of dead leaf material attached to the plant and it was found that at 568 days there was a non-significant, 36 % increase in dead leaf material on the elevated CO₂ plants. By 810 days this potential difference was not present. These plants are small perennials (Bond & Goldblatt, 1984) and so it is unlikely that any significant amounts of carbon would be stored in the above-ground plant parts. It is possible that the accumulation of recalcitrant litter could sequester carbon but it was found in Chapter 2 that the nitrogen content of the dead leaves was significantly increased by 15% while the

phosphorus content increased by a non-significant 28%, indicating that microbial decomposition of the litter would be increased. The Fynbos is a fire prone environment (Grubb, 1992, Deacon *et al.*, 1992) and so there would be little possibility for above-ground, long-term accumulation of carbon due to the fact that it is periodically removed by combustion. Another important consideration is that the Fynbos is a low stature vegetation type growing in low nutrient and low organic matter soils and so the physical ability of it to store carbon in meaningful quantities is questionable. Scholes and Bailey (1996) suggest that the Savanna ecosystem is the only natural system in South Africa that would be able to sequester significant amounts of carbon from the atmosphere.

Changes in soil carbon storage are entirely mediated by plant responses to the atmospheric conditions. The amount of root in the soil was unchanged showing that the net amount of carbon being added to the soil from root death would be the same under elevated CO₂ but the nutrient changes observed (a reduction in P while N remained unchanged) could influence root decomposition by the soil microbes. This would govern the turnover rate of the recently fixed carbon. Although not measured in this study, Gorissen *et al.* (1995) showed reductions in decomposition of root material from ryegrass plants grown at elevated atmospheric CO₂ concentrations

After looking at total soil carbon, microbial quotient, ¹⁴C input and total soil respiration, the results show that in the period of this experiment there were no discernable changes occurring in the carbon storage and release patterns in the soil. One can conclude that in this single species, pot experiment, there were no indications of carbon storage change around the plant in the face of an atmosphere containing elevated levels of carbon dioxide. The next step would be to see if this is in fact a general response for Fynbos plants and these results would then allow one to decide whether there will be a change of ecosystem carbon storage. Reason for this lack of change could be due to nutrient limitations as was shown by Oren *et al.* (2001) who looked at wood sequestration of carbon for a mid-latitude pine forest.

Hungate *et al.* (1996) showed that statistical power in measuring changes in soil carbon under changed climate conditions is low. In their analysis of Californian sandstone-derived soils in the Jasper Ridge region, a 35 % stimulation of carbon addition to the soil would take 9 years before being statistically measurable while a 7% stimulation would never become statistically significant, even though a change of this magnitude would dramatically affect ecosystem carbon cycling. For the serpentine soils in the same region it was found that none of the stimulation scenarios investigated yielded statistically detectable changes in the soil carbon pools. Dhillon *et al.* (1996) reported no change in the soil carbon content of

their Mediterranean model ecosystem. This is not surprising considering the fact that the experiment was only run for a period of 5 months. Another challenge facing scientists trying to predict changes in long-term soil carbon storage is finding acceptable ways of extrapolating trends gathered from short-term investigations (Hyvönen *et al.*, 1998). Hyvönen and coworkers (1998) used continuous-quality theory in their scaling-up attempts because this model requires a limited number of parameters. Results showed that 5% errors in parameters such as initial litter properties and metabolic rate of decomposers had little effect on the predicted soil carbon store (over 10 000 years) while other factors such as rate of change in litter quality and the parameter describing interactions with the soil (both of which are currently difficult to measure) can cause substantial, up to three fold differences, in model outcomes with as little as a 5% variation in the initial values.

There is evidence accumulating that soil carbon stores will reach a saturation point where further carbon gain by the system is not possible. This could be due to P limitation (Gifford *et al.*, 1996) or non linear responses in carbon storage as one moves from pre-industrial to elevated CO₂ concentrations (Gill *et al.*, 2002). This issue of soil nutrient limitations restricting carbon storage was also reviewed by Schimel (1995) who discussed evidence that the storage will be much smaller than that predicted from short-term photosynthetic measurements or pot-based plant growth studies. Janzen *et al.* (1998) took a more general view and describe soil-stored carbon in terms of steady states where changes in soil organic matter, either gain or loss, is of finite duration and magnitude. Soils that are shown to be sinks of carbon will only remain so while a new equilibrium between primary production and decomposition is established.

Single species to mixed species

As has been highlighted in the earlier discussion, the bulk of the investigations made in this thesis were concerned with a single species grown individually in pots. In order to make predictions on how relevant the conclusions are to larger scale, more natural systems, the next level of complexity within the Fynbos investigations was established (Stock *et al.*, 1997). Arrangements of 5 representative Fynbos species were grown in competitive arrays in microcosm chambers.

Analysis of the soil surrounding the *Felicia aethiopica* plants revealed that the CO₂ effect was frequently smaller in the mixed species situation. As an example, CO₂ treatment caused the soluble carbon in the soil to increased by 85% in the ss study while it only

changed a non-significant 10% in the ms case. This reduction in response was also seen in soil microbial biomass and microbial quotient. These results indicate that the ms microcosm was more robust due to the soil volume and possibly the increase in species number, and less likely to be affected by perturbations. There was a certain degree of bias in the ms data because the soil samples were collected near to the roots of the *Felicia aethiopica* ssp. *aethiopica* plants and so it is possible that different responses were occurring in other parts of the chamber. Bertson & Bazzaz (1996) point out that even within a single ecosystem there is likely to be much variation in potential growth responses of the different components and so to make predictions of an ecosystem response one needs to have a detailed understanding of the functioning of many of the parts of the system. Poorter & Pérez-Soba (2001) demonstrated that the variable and conflicting results obtained in elevated CO₂ studies could be a result of experimental design with low levels of replication (the studies investigated had a median replication of 5 plants per treatment) combined with the inherent variability present in natural plant populations. The authors therefore suggest that predictions should be derived from an average response from a number of experiments.

Investigations of the soil microbial population structure using the Biolog™ Bacterial Identification System revealed a very interesting difference between the two experiments. As was mentioned earlier, there were only vague differences between the control and the treatment in the ss experiment while in the ms study Biolog™ analysis clearly and significantly distinguished between the two treatment groups. This indicates that in the more complex, and thus natural, system there was a clear shift in the soil microbial population structure with respect to its potential to utilise carbon sources. The unfortunate drawback of the method is its inability to make predictions on how the differences would manifest themselves. As was discussed in Chapter 4, the value in this technique lies in its power to make rapid, broad-scale, preliminary investigations into treatment effects on a microbial population. Once a difference has been identified, one is then in a position to do a more thorough and detailed investigation to learn more about the mechanism and consequences of the response. Garland (1997) pointed out that meaningful advances and hypotheses have been made in macroecology by investigating a portion of the overall community (plants, invertebrates, birds, etcetera). The same is true with microbial ecology where indirect methods such as Biolog™ can give valuable insights into the processes that are occurring and these will direct future work that will develop a more complete picture of the interactions and processes occurring.

Schimel & Gullledge (1998) reviewed how changes in microbial community structure caused by global change could impact biogeochemical processes. Global changes could have two effects on the microbial populations. The functioning of existing organisms could change or the community could be restructured. In the second case the rates of biological processes could change along with fundamental controls of these rates. The Biolog™ results in this instance indicated that the second scenario, community restructuring, was in operation. Although not observed, this could have significant effects on the carbon cycling in the soil due to the very high percentage of the exuded carbon passing through the soil system to be respired by the microbial community (Chapter 5). Schimel & Gullledge (1998) concluded by suggesting that, although there was conflicting evidence, changes in microbial populations in the soil would have an impact at the global scale but the current state of the science was not advanced enough to be able to predict how changes in the microbial functioning would change the atmosphere.

From this simple comparison between these two studies it is likely that as one moves from the mixed species, microcosm study up to a natural Fynbos ecosystem it is possible that the differences would again be slight and difficult to measure. Körner (1995) sounds a clear warning that one should use very great care when extrapolating from small-scale experimental studies up to more “natural” systems. It is important to remember that many of the responses being investigated are quite subtle and the techniques that were used were being applied in a situation where the differences would be expected to be smaller than in the situations where they were developed or routinely used.

The question of the potential of the Fynbos system’s ability to sequester significant additional CO₂ from the atmosphere remains to be answered. Reich *et al.* (2001) found that in their grassland system the higher the species diversity, after passing a threshold of four species per plot, the more carbon was taken up by the system. It may be possible that Fynbos also has a threshold after which CO₂ effects would appear but this would only be revealed by a large study similar to Reich *et al.* (2001). These investigators were however only looking at plant biomass measures and not extending the study to flux rates and long-term pools such as soil organic matter. Nijs *et al.* (2000) used ecosystem gas exchange measures to monitor carbon flows in an early successional Mediterranean ecosystem established in old-field soil monoliths. Their experiment was run for three years using four herbaceous species, including two members of the Asteraceae. By the third year of the experiment one of the assemblages was a net carbon sink while another two had reached equilibrium and the last assemblage remained a net CO₂ source. The sink effect was largely

caused by a reduction in below-ground respiration. Whether there was enough of a difference in the Fynbos study to result in meaningful changes in the carbon flows is difficult to predict largely due to the arguments of Hungate *et al.* (1996) concerning the statistical power of the investigations currently used. It is important to remember, however, that relatively small, and thus difficult to measure, changes in vegetation response multiplied over the whole biosphere could have very large impacts on the global carbon budgets (e.g. Arnone & Körner, 1995, Goudriaan, 1995). Gifford (1994) showed that a 0.03% to 0.2% increase in the world's soil storage of carbon could negate the "missing carbon sink" of 0.5 to 4 Gt C per annum.

It is likely that the Fynbos might not be a large C sink, because of its limited aerial extent and soil storage capacity, nevertheless it deserves attention due to its significance as a centre of biodiversity (Myers *et al.*, 2000, Bond & Goldblatt, 1984) along with its vulnerability to the global changes that are currently occurring (Sala *et al.*, 2000).

The C₄ Grassland

The main focus of this thesis has been on the effects of elevated atmospheric CO₂ on below-ground functioning. The bulk of the investigations were made on the oligotrophic Fynbos system. In an attempt to understand CO₂ effects on soil biological process in other South African systems, the investigations were extended to a natural sub-tropical, C₄ dominated grassland situated around a natural CO₂ spring (Chapter 3). Hungate *et al.* (1996) highlighted the importance of soil carbon storage in grassland systems due to their non woody nature and hence the system's inability to store carbon for long periods in phytomass. Many factors in this system are very different to the Fynbos investigations (See Table 1) and so one would not expect the two systems to behave in the same way.

Table 6-1: Comparison between the physical characteristics of the Fynbos system and C₄ dominated grassland studied in this thesis.

Fynbos	C ₄ grassland
Mediterranean climate	Sub – tropical climate
Nutrient-poor, sandy soil	Richer in nutrient, more clay rich soil
Sclerophyllous shrubs	Grasses dominate
Pots and microcosms in open top chamber	Point source and fumigation ring
Glasshouse based	Natural hillside

It was found that there was a significant soil moisture effect under elevated atmospheric CO₂ while there were no changes in any of the carbon pools investigated bar a small increase in total soil carbon in the area that had received the very long-term (70+ years) treatment. An investigation of a temperate ryegrass grassland showed that after 2.5 years of treatment there was a significant accumulation of carbon in the roots and larger (and thus more recent) soil fraction while the smaller size fractions had not been affected yet (Loiseau & Soussana, 1999). This could be as a result of the observed reduction in root decomposition rate for ryegrass plants grown in elevated atmospheric CO₂ conditions (Gorissen *et al.*, 1995). The magnitude of carbon sequestration may be less than predicted from plant biomass responses and even measurements of “new” carbon being added to the soil, as shown by Loiseau & Soussana, (1999), as sequestration relies on slowly turned over fractions. Niklaus *et al.* (2001) found that after six years of treatment on a calcareous grassland, more carbon was being added to the soil but it was going into rapidly turned over pools as opposed to recalcitrant fractions. This increase in the rate of carbon cycling despite a net increase in input was also found by Hungate *et al.* (1997) and Gill *et al.* (2002).

In a four year enrichment experiment on *Danthonia richardsonii* swards, Lutze & Gifford (1998) showed that there was a net microcosm and soil carbon gain even in the face of severe nitrogen limitation. The microcosm gain ranged from 15 to 34% depending on the nitrogen supply and the authors suggested that if these rates of increased sequestration were present in other ecosystems then the “missing carbon sink” would be accounted for. Although not significant, there is some evidence that the Natal grassland could be contributing to this sink.

Paustian *et al.* (1996) used a modeling approach to investigate the effects of global change and management practices on soil carbon of the agricultural regions of the semi-arid central Great Plains of the USA. These researchers found that over a 50 year period soil-stored carbon would increase slightly under elevated atmospheric CO₂ scenarios. More importantly however, they found that the management of the system had a greater effect on carbon storage than any of the projected climate changes. This is similar to the C₄ grassland where rainfall and soil moisture appear to play a role in the soil carbon dynamics (Chapter 2). Similarly, Nijs *et al.* (2000) showed that soil saturation with water in the early part of the growing season exerted a strong influence over the carbon flows in the system. These practical observations are supported by the theoretical treatment of global change and root function made by Van Noordwijk *et al.* (1998) where they showed that a doubling of atmospheric CO₂ modified the external above- to below-ground resource ratio by a factor of 2

while all plants experienced far wider changes in this ratio due to changes in other resources such as soil moisture availability. Gill *et al.* (2002) showed that responses of grassland are non-linear with more significant reactions to atmospheric CO₂ occurring from pre-industrial to present levels than what would occur in the future. A reason for this reduction in responsiveness was found to be nitrogen limitation which is contrary to the findings of Lütze & Gifford (1998).

Gifford *et al.* (1996) warned that uncertainties in both data and functional relationships makes modeling more suited to qualitative evaluation rather than quantitative prediction while Schimel & Gullledge (1998) extended this caution by suggesting that modeling of soils is currently impossible because hysteresis may occur in CO₂ fluxes when unsaturated soils undergo drying and wetting cycles. Hysteresis is the process whereby the response obtained in one direction (say soil respiration on wetting) is different to when the process is reversed (respiration changes on drying of the soil).

The question of time

Another dimension that is frequently not considered in global change experimental work is the duration of the experimental treatment (Hu *et al.*, 1999). There is strong evidence to show that there are often dramatic short-term effects of enriched CO₂ atmospheres followed by a period of acclimation at a new response level (Canadell & Mooney, 1999, Drake *et al.*, 1996, Canadell, 1996). If measurements are made in the initial, exponential growth phase then predictions based on these data can turn out to be spurious as the system settles to a new equilibrium state (Arnone & Körner, 1995). This highlights the importance of running enrichment experiments for as long as possible so as to allow enough time for ecosystem maturation. The experiments described in this thesis were run for a considerable period of time. In the case of the Fynbos investigations, 568, 810 and 700 days in the ss first harvest, ss second harvest and ms experiment respectively.

The study conducted in the C₄ grassland surrounding the natural CO₂ spring was constructed so that it would be possible to investigate temporal effects on the responses of the plants. After a long-term exposure, in excess of 70 years, this system showed a small increase in the soil storage of carbon while there was no evidence of change in total soil carbon in the short-term components of the study. There seemed to be no time dimension in microbial biomass C and soluble carbon response, seen from the variable values obtained. It is more likely that these more dynamic pools of carbon could change on a seasonal basis.

An inter-annual sampling would allow investigation of this, as was shown by Hu *et al.* (2001) with a strong temporal effect in the Jasper ridge soils and Dalal (1998) who showed a peak of microbial activity during maximal root activity. A review by Wardle (1998), however, showed that there was little seasonal effect on microbes in soils from warm temperate and tropical regions indicating that there may indeed be no pattern in the microbial and soluble carbon pools although this would only be revealed by better sampling.

To understand the processes occurring around soil organic matter turnover, one needs lengthy experiments. It is also necessary to develop an understanding of whether the predictability provided by the more common, shorter term laboratory studies allows one to extrapolate the results to more natural, longer term situations. This shows the great value of natural, field experiments such as the Natal study where one has a baseline long-term treatment along with more controlled, short-term investigations.

Conclusion

Investigations of soil processes are difficult and frequently hampered by crude analytical methods. The importance of below-ground activities in the functioning of ecosystems is now appreciated and this has spurred researchers to develop more elegant and powerful tools to study these processes. This thesis showed that there were a number of responses to elevated atmospheric CO₂ but many of the effects were subtle, making it difficult to predict how natural systems would behave in the face of global changes. This is very different to the large scale, gross responses frequently seen in agricultural situations and of resource-rich systems. It is important, however, to persevere with these investigations. There is a great need to develop theories of how ecosystems will respond to anthropogenic changes so that the maximum biological diversity can be maintained in order to maintain the stability of the biosphere.

There are many international bodies concerned with the looming threat of global catastrophe created by one of its inhabitants and these issues have now become serious enough to be placed on the political agendas of governments. The *Amsterdam Declaration on Global Change* is a document that was produced in recognition of the challenges facing scientists in particular and the world's population in general (Canadell & Noble, 2001). Of relevance to this thesis, the declaration highlights the fact that global changes cannot be understood by simple cause and effect models. They are multidimensional, acting at many spatial and temporal scales across entire regions of the earth.

One of the big confounding problems of soil science is the huge soil heterogeneity that causes difficulty in statistical analyses of soil data sets. The designs of the experiments in this thesis were standard for such investigations, with a reasonable amount of replication considering the practical constraints of the project: manpower and money. Poorter & Pérez-Soba (2001) found that the median number of replicates used in CO₂ x environment investigations was 5 with a low number of 4 and high number of 10 (the 20th and 80th percentiles). Although authors such as Körner (1995) criticise the use of pots, the experiments performed here were run for a long period of time and they used natural, unamended soils. It was necessary to use pots to give better replication along with allowing detailed manipulation of the root environment. There may be philosophical problems with the methods but this study yielded valuable insights into the soil system that is very poorly understood.

Further investigations should be highly targeted, addressing specific questions rather than grand investigations of general responses. Many of the gross responses, such as plant size and carbon deposition, do not yield much useful information in natural systems. It is the small changes such as in soil microbial population structure that will have the significant, long-term influence on the ecosystem response.

Canadell & Noble (2001) sounded a warning that we may not be able to rely on carbon sinks to mitigate the rise in atmospheric CO₂ concentrations due to human impacts on these sinks as well. The usefulness of these sinks is to give the human population an opportunity to make fundamental changes to their consumption of fossil fuel reserves and agricultural practices so that the long-term survival of the human species, and many others too, can be ensured.

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