CHAPTER I

REVIEW OF THE LITERATURE

An appreciation of the habit and growth of the Genus *Panicum* is a prerequisite for the understanding of their reaction to a changing environment affecting their morphological and anatomical structure and associated physiology. The first part of this review provides a brief summary of current knowledge of the anatomical and physiological make up of those *Panicum* species which are significant tropical forage grasses. The second part of this review is concerned with the effects of high carbon dioxide concentrations on our environment in general and their environmental pressure on C₃, C₄ and C₃/C₄ intermediate plants. This part of the review also provides comparisons of the *Panicum* grasses with other plants and their corresponding response to high CO₂ concentrations. Emphasis has been placed on studies of the epidermis with its stomata and protective epicuticular wax layer which is of special interest concerning altered water use efficiency and leaf reflectance.

1.1. THE GENUS *PANICUM*

*Panicum* grasses are annuals or perennials of various habit growing up to to 3 metres tall. The *Panicum* grasses discussed here are representatives of a large genus of over 300 species of annuals and perennials found in tropical and subtropical regions of both hemispheres and extending into warm temperate areas. *Panicum* occurs in all states of Australia, 28 species being native and 9 introduced. There are 20 species in New South Wales. Some
species are valuable for grain and forage production and as pasture plants, others are weeds and several species are implicated in photosensitization of grazing animals. Seeds of several *Panicum* species were used by the Aborigines for food (Vickery, 1975; Wheeler et al., 1982).

The occurrence of C₃, C₄ and C₃/C₄ intermediate species in closely related groups of one family (*Pancicoideae*) like the *Panicum* genus is rare in the Gramineae (Poaceae).

For this study the perennial forage grasses *P. tricanthum* Nees (C₃), *P. decipiens* Nees ex Trin. (C₃/C₄) and *P. antidotale* Retz (C₄) were chosen.

### 1.2 PLANT RESPONSES TO A CHANGING ENVIRONMENT: HIGH CARBON DIOXIDE AND ITS EFFECTS ON THE WORLD FLORA

#### 1.2.1 CARBON DIOXIDE CONCENTRATIONS

The biosphere, lithosphere, hydrosphere, and atmosphere of the earth are in a dynamic equilibrium; each is changing and yet each sustains the others in the sequence of naturally occurring events. However, human activities of landscape modification, resource exploitation, and chemical contamination have reached sufficient magnitude to perturb the global ecosystem for an indefinite time into the future. One of the main contaminants is the colorless, odorless simple gas carbon dioxide, currently the most significant greenhouse gas.

The primary cause of the increase of atmospheric carbon dioxide is the imbalance between the release of CO₂ from fossil fuel combustion and deforestation and the uptake of CO₂ by the oceans and the biosphere (Bolin, 1986; Houghton et al., 1990).

Carbon dioxide has the important property, shared by all polyatomic molecules, of strongly absorbing and emitting reradiation in the infrared. The
Earth's surface and atmosphere remain at temperature equilibrium by absorbing incoming sunlight and reradiation of infrared energy to the cosmic cold of outer space. Anything interfering with this flow of energy, from sun to earth and earth to outer space, changes the atmospheric temperature and climate. Carbon dioxide gas, by being transparent to visible light and partially opaque to the infrared, thus interferes with the earth's energy budget.

There is still considerable uncertainty surrounding the magnitude of the fluxes of CO$_2$ between the ocean, the biosphere and the atmosphere. However, a growing body of evidence indicates that the biosphere is acting as a significant sink for atmospheric CO$_2$. Models suggested by Taylor and Lloyd (1992) indicate that the tropical rainforest may be the major component of the "missing sink" of atmospheric CO$_2$. If this is the case, then current IPCC (Intergovernmental Panel on Climate Change) modelling will significantly underestimate the growth of atmospheric carbon dioxide concentrations and, as a result, the rate of climate change.
1.2.2. PLANT RESPONSES TO HIGH CARBON DIOXIDE CONCENTRATIONS:

There has been little attention given to the impact of global environmental change on Australia’s grazing industries, i.e. to possible effects on economically important forages and crops. Research, mainly carried out in the 1970s and early 1980s focussed largely on temperate crop and forest species, predominantly representative C$_3$ species.

The impact of global climate change on summer rainfall and subtropical based grasslands could be different according the involvement of C$_3$/C$_4$ and C$_4$ species, which are thought to be less responsive to high CO$_2$ than C$_3$ species. Global warming, which might accompany any atmospheric CO$_2$ increments could result in increased aridity in major agronomic areas in the world. Plants with the C$_4$ photosynthetic pathway are usually better adapted to arid conditions than are C$_3$ plants and might be favoured by increased aridity (Patterson et al., 1984). Whether plants with an intermediate pathway are likely to change to a more favourable physiological pathway, either the C$_3$ or to the C$_4$ has yet to be verified.

Calculations carried out by Idso (1980) and confirmed recently by CSIRO in Australia, raise questions about the extent of global warming that may accompany a doubling of the atmospheric carbon dioxide concentration. In any case a change in weed/crop interactions can be expected. In regard to changes in productivity and composition of important forage grasses like Panicum, in response to anticipated climate change one could expect a decrease in digestibility, i.e. a diminished pasture quality, which would affect the grazing industry and land use patterns. Generally this would be due to temporal and spatial changes in carbon allocation at an elevated carbon dioxide concentrations. This might take part at the so-called whole plant concentrations (root length to leaf ratio) or at the leaf concentrations (greater allocation to cellulose in cell walls and to lignin).
Numerous studies since at least the 1920s have shown that an increase in the concentration of CO₂ will result in greater photosynthetic rates and biomass accumulation in C₃ plants, since at current CO₂ concentrations the photosynthetic metabolism in these plants is relatively inefficient (Keys, 1986; Lawlor and Keys, 1993). From a physiological point of view the main consequence of an increased atmospheric CO₂ concentration for photosynthesis is an increase in the effective concentration of CO₂ at the active site of RUBISCO and a decrease in the competition of atmospheric O₂ with CO₂ for RuBP (Tolbert, 1980; Kimball, 1983; Cure and Acock, 1986; Lawlor and Keys, 1993; Delgado et al., 1994). Reviews by Kimball (1983), Cure and Acock (1986) and Poorter (1993) of experiments, mainly on crop species, carried out under a wide range of conditions show that doubling of the atmospheric CO₂ concentrations from ca. 330 to 660 ppm increases the biomass productivity of C₃ plants by 33% on average.

It is generally agreed that productivity losses occur from higher temperatures and changes in water availability which can be compensated for by the effects of CO₂, with small net gains in productivity (Allen, 1990).

Despite the advantages of elevated CO₂, many authors have shown that the potential for increased rates of carbon assimilation may not be realized for all species (e.g. tomato, Besford et al., 1990). Leaves may respond to elevated CO₂ either by reducing the rate of photosynthesis without changing leaf composition or photosynthetic capacity (so-called “downregulation” or “fine control”, Idso and Kimball, 1991), or by decreasing amounts of photosynthetic components with a loss of photosynthetic competence and capacity (so called “acclimation” or “coarse control”, Yelle et al., 1989 (I) and (II); Sage et al, 1989; Stitt, 1991). This type of acclimation of the plant in response to high carbon dioxide has been observed under conditions in which carbohydrates accumulate due to inadequate sink demand; in cereals, for example, such accumulation may result from poor development of tillers under conditions of nutrient deficiency or high temperature. On the other hand, carbohydrate accumulation and hence acclimation could be partially
under genotypic control with regard to expression of enzymes concerned with sucrose synthesis (Foyer et al., 1995; Galtier et al., 1995).

1.3. EFFECTS OF ELEVATED CO$_2$ ON THE CUTICULAR STRUCTURE OF C$_3$, C$_4$ AND C$_3$/C$_4$ PLANTS:

1.3.1. EFFECTS OF ELEVATED CARBON DIOXIDE CONCENTRATIONS ON THE STOMATAL APPARATUS:

Stomata control the entry of CO$_2$ into a leaf for photosynthesis and, at the same time, the exit of water vapour which evaporates from the cell walls in contact with air spaces within the leaf (Raschke, 1975). Stomata open when exposed to light but usually become narrower or close, i.e., stomatal conductance decreases, when the carbon dioxide concentration in the air increases.

The mechanism by which the concentration of carbon dioxide determines whether stomata tend to open or close is not known, but high CO$_2$ leads to stomatal closure, while, as CO$_2$ decreases, stomata open. The stomata respond to the intercellular CO$_2$ concentration of the guard cells which is, in part, affected by the intercellular and ambient CO$_2$ concentration.

It is also evident that high CO$_2$ concentrations affect membrane permeabilities and that carbon dioxide concentrations surrounding guard cells are therefore important in controlling stomatal functioning (Heath and Russell, 1954). Stomata tend to have reduced apertures at elevated CO$_2$ concentrations, thereby reducing conductance to water vapour and transpiration, and therefore enhancing growth and water-use efficiency (Farquhar and Sharkey, 1982).

Stomatal opening is the result of an accumulation of potassium salts in the guard cells. The counter-ions to K$^+$ are Cl$^-$ and organic anions, particularly
malate. The organic anions are produced within the guard cells after the mobilization of starch. It is not known whether expulsion of H$^+$ by the guard cells (in exchange for K$^+$) or anion transport into the vacuoles is the primary transport process involved in increasing the osmotic pressure in these cells. The increase in osmotic pressure causes an increase in turgor. The guard cells swell, and the stomatal pore opens. A reversal of the process just described leads to stomatal closure, K$^+$ and Cl$^-$ are released from the guard cells and starch is reformed (Willmer, 1983).

Stomata of C$_4$ species appear to be more sensitive to CO$_2$ than those of C$_3$ species, and closure in C$_4$ species occurs at much lower CO$_2$ concentrations than observed in C$_3$ species (Willmer, 1983). Akita and Moss (1972) found the stomata of C$_3$ species (Triticum aestivum; Hordeum vulgare; Taraxacum officinale) were less prone to closure than were stomata of C$_4$ species (Zea mays; Setaria viridis; Amaranthus retroflexus) as the CO$_2$ concentration increased to 800 ppm. Akita and Moss (1972) also recorded a smaller growth response to elevated CO$_2$ (0 ppm to 800 ppm CO$_2$) of the dicot Glycine max in comparison to the moncot Triticum aestivum. They suggested that the difference was due to a greater reduction in stomatal resistances of C$_4$ plants rather than in C$_3$ plants. These authors observed a lack of response in Zea mays to elevated carbon dioxide concentrations which was thought to be due to early closure of stomata following changes in CO$_2$ concentration. There are no data on stomatal frequencies of C$_3$/ C$_4$ stomata under high carbon dioxide. Different CO$_2$ concentrations are also thought to be involved in the morphogenesis of the stomata and especially seem to affect stomatal frequencies (Ciha and Bruhn, 1975). Microscopic investigations on leaf sections from plants grown under enriched CO$_2$ conditions (450 ppm -1000 ppm) did not reveal significantly higher stomatal index, density and epidermal density in the C$_3$ species Fragaria virginiana, Glycine max, Phaseolus vulgaris, Liqidambar styraciflua, Eleusine indica, Pinus taeda, Digitaria sanguinalis, Lolium perenne and
Setaria faberi and the C₄ Zea mays (Cave et al., 1981; Thomas and Harvey, 1983; Radougou and Jarvis, 1992). Plants like tomato grown under high CO₂ showed reduced stomatal frequencies (Bristow, 1969; Madsen, 1973; Woodward, 1987). This reduction in stomatal frequency is ascribed to overall larger leaves owing to the formation of bigger cells under high CO₂. Interestingly, Thomas and Harvey (1983) found an increase of stomatal indices and frequencies in soybean (Glycine max), grown under > 600 ppm CO₂.

1.3.2. EFFECTS OF ELEVATED CARBON DIOXIDE CONCENTRATIONS ON THE EPICUTICULAR WAX LAYER:

1.3.2.1 THE CUTICLE AND ITS ASSOCIATED EPICUTICULAR WAX

The aerial surfaces of plants are covered by a non-living, lipid membrane referred to as the cuticle (Brongiart, 1830, 1834). The cuticle and its associated epicuticular wax have been the subject of many reviews (Martin and Juniper, 1970; Bukovac et al., 1981; Juniper and Jeffree, 1983).

Epicuticular waxes are a complex mixture of substances, but the advent of improved chromatographic and spectrophotometric methods has enabled detailed identification of wax constituents (Tulloch, 1975; Tulloch and Hogge, 1978). The plant cuticle is composed of an inert polymer, ‘cutin’, which dissolves in strong alkalis to produce fatty acids and hydroxy acids (C-16-18 chains) (Baker et al, 1964a; Holloway et al, 1977; Kolattukudy, 1980; Cutler et al., 1982; Juniper and Jeffree, 1983). The major classes are free primary alcohols, fatty acids, alkyl esters, and long chain hydrocarbons (Baker et al, 1964; Juniper and Jeffree, 1983). The compounds vary in the number and position of functional groups, and in the degree of branching and unsaturation (Eglington and Hamilton, 1967).
Several techniques have been used to determine the components of the plant waxes quantitatively. The standard fraction (Silva Fernandez et al, 1964) and colometric methods (Ebercon et al, 1977) are unsuitable for routine use because of their comparative slowness. Thin layer chromatography (TLC) (Holloway and Challen, 1966), gas-liquid chromatography (GLC) (Tulloch, 1975) and GLC-mass spectrometry (GLC-MS) (Tulloch and Hogge, 1978) are all useful tools in the elucidation of wax constituents. More recently, proton magnetic resonance (PMR) has been discussed as a rapid and accurate alternative (Johnson et al., 1984).
1.3.2.2 FACTORS AFFECTING THE CUTICLE AND THE EPICUTICULAR WAX

Environment, stage of development and heredity are all factors that affect the formation and composition of the cuticle and epicuticular wax (Kolattakudy, 1970).

The cuticle acts as the interface between the plant and its habitat. It prevents excessive water loss by the plant and protects against attack by insects and disease-causing organisms. It can also protect against the effects of ultraviolet radiation.

There is considerable debate about how waxes are affected by the environment. High temperatures are thought to influence certain properties of wax, for example formation of hard wax is due to an increase in temperature (Schieferstein and Loomis, 1956; Kollattakudy, 1970). The production of precursor substances for specific chemical reactions (Baker, 1974) and the rate of crystallisation of the extruded wax (Juniper and Bradley, 1968; Whitecross and Armstrong, 1972) are also affected by temperature and humidity.

Both temperature and humidity regulate the quantity of wax formed (Baker, 1974). Light has also been found to be an important determinant of wax production. Juniper (1960) found that for normal wax synthesis light intensities of at least 20% full daylight were required. These observations raised questions on the means by which wax emerges onto the cuticular surface and this remains a controversial area of research.

McNair (1931) investigated wax composition in different climates. Wax hydrocarbons, alcohols, and fatty acids in plants grown in the tropics have greater molecular weight and lower melting points than those of wax, for example, from fruits in the temperate zone. The opposite relationship holds for fats and alkaloids. Temperature cannot be the sole determinant of wax composition because temperature also affects substrate availability, the rate of biosynthesis and the mobility of wax and its precursors (McNair, 1931).
The change in wax structures observed in *Brassica napus* was related to the effect of temperature on the wax constituents and to the rate of wax crystallization (Whitecross and Armstrong, 1972).

Daly (1964) reported a negative correlation between the abundance of leaf surface wax of *Poa colensoi* and rainfall. He also observed a weak positive correlation between surface wax and mean temperature. Leaves grown in direct sunlight are reported to have heavy cuticles with higher wax content than leaves grown in shaded areas (Skoss, 1955). Giese (1975) suggested that light acts on the biosynthesis of wax rather than on wax composition. Excessive wind or exposed growing conditions result in dense and compact wax layers (Juniper and Bradley, 1958).

Tissue age may affect plant waxes as well (Markley and Sando 1931; 1933). Changes in the wax composition may affect the physical properties of the coating and this, in turn, affects the permeability of the plant waxes to gases and liquids (Huelin and Gallop, 1951a and b).

1.3.2.3. **THE FORMATION OF EPICUTICULAR WAXES**

The quantity of wax varies from species to species (Kurtz, 1950; Martin and Batt, 1958; Roberts et al., 1959; Baker et al., 1964b; Baker and Martin, 1967; Tulloch, 1974).

Wax was proposed to be transported to the surface of the cuticle via cuticular pores, pits, and channels (Scott et al., 1948) and was thought to be excreted as a liquid which hardened on the plant surface (Kreger, 1948). Mueller et al. (1954) suggested that wax production occurs throughout the period of organ development by extrusion of a soft paste under pressure through wax-extruding cuticular pores, but upon examination the pores did not appear to extend through the cuticle.

Schieferstein and Loomis (1956), and Juniper (1960) found no evidence of the proposed wax channels, or pores, or their involvement in wax formation. In observing wax deposits Schieferstein and Loomis (1956) discovered no
correlation between the amount of wax and plant adaption to the environment. They proposed that wax structure depends on individual cuticular properties and their specific chemical composition.

Hall (1966) proposed that wax is extruded through the numerous pores found in the epicuticle. The observation of these pores was difficult because of the low resolving power of the replica technique used. Hall (1967a; 1967b) confirmed the presence of these pores and he claimed that they were instrumental in wax extrusion and in determining the microstructure of the wax. Hallam (1970) found no evidence of pores on the surface of Eucalyptus after removing the epicuticular wax. Structural changes in wax formation were observed under different light intensities and as a result of mechanical injury. He suggested that the final wax morphologies were more likely to result from chemical oxidation, polymerization and recrystallization of the wax and its precursors than from an extrusion process. The distribution of microchannels in the cuticular lamellae was proposed to be either genetically (von Wettstein-Knowles, 1974) or environmentally determined (von Wettstein-Knowles, 1974; Baker, 1974).

Fisher and Bayer (1972) detected channels in the outer walls of the epidermal cells, but these channels did not appear to penetrate the cell wall. The birefringent properties of these channels in polarized light disappeared upon heating. This observation led to the proposal that the channels were functional in the transportation of wax to the epidermal cell surface. Chafe and Wardrop (1973) observed similar electron dense strands during development and while the cuticle was forming.
1.3.2.4. THE CUTICULAR PATTERN OF EPICUTICULAR WAXES AND THEIR DETERMINATION

The distinctive cuticular patterns of wax on many species often reflect the characteristic features of the underlying epidermal cells (Cutter, 1978; Bukovac et al., 1971, 1981; Carr et al., 1985) and the determination of the wax structure itself seems to be under genetic control. Both were studied in relation to taxonomic classification (Freeman and Turner, 1985).

According to Davis (1971) the different theories on the origin of wax are a reflection of the variety of techniques used for microscopy and of differences in the developmental stage of the plant. Recrystallisation of waxes from volatile solvents using a wick-feed technique resulted in wax structures similar to those found on plant surfaces (Jeffree et al., 1975). The results of this work implied that the structure of the epicuticular wax is not a reflection of the inherent properties of the supporting tissue, and provided evidence that pores in the cuticular membrane may not contribute to the resulting epicuticular wax structure.

On the other hand, the structure of the wax may influence the properties of the plant surface (Cutter, 1978). The carbon replica technique was the first method used to examine the structure of wax. It was found that there are marked structural differences between plant surfaces that are easy to wet (i.e., smooth) or difficult to wet (i.e., rough), (Juniper and Bradley, 1958). Using the carbon replica technique, it was found that wax characteristics changed rapidly in response to a changing environment. The conspicuous deposit of wax on the plant cuticle is referred to as ‘bloom’. The bloom can be deceptive because it gives no indication of the amount of wax present—some deposits are non-reflective. The types of wax structure observed include rods, plates, granules, tubes and sheets (Baker and Holloway, 1971; Cutter, 1978; Juniper and Jeffree, 1983). The discovery of such structures was made possible by the advent of the first transmission electron microscope (TEM), and later the scanning electron microscope (SEM), which
is a quicker method giving a better resolution (van Volkenburgh and Davies, 1977).

1.4. EFFECTS OF ELEVATED CARBON DIOXIDE CONCENTRATIONS ON MORPHOLOGICAL PARAMETERS OF C₃, C₄ AND C₃/C₄ PLANTS:

1.4.1. LEAF AREA:

Leaf area is an important parameter of plant productivity, and increases may occur in both the rate of leaf expansion and the final leaf area attained in plants developing under CO₂ enrichment (Wong, 1990; Tolley and Sionit, 1983). Increased leaf area allows increased net photosynthesis, which becomes compounded into significantly more leaf area per plant in some species (Sionit, 1983), but not in others (Tolley and Strain, 1984).

Many authors have observed an increase in leaf area under elevated CO₂.

The decrease of leaf area in Glycine max with increasing atmospheric CO₂ has been related to additional cell layers of the leaf (Hofstra and Hesketh, 1975). This has also been observed in Glycine max, Liquidambar styraciflua and Pinus taeda (Thomas and Harvey, 1983) and wheat plants grown between 350 ppm and 800 ppm CO₂ (Neales and Nichols, 1978).

Investigations on Glycine max and Abutilon theophrasti also indicate that leaf area is more affected by CO₂ in the plants with the Benson-Calvin pathway (C₃) than in plants with a dicarboxylic-acid pathway (C₄) (Patterson and Flint, 1980).

Since leaf area expansion results from cell wall expansion (Nelson and McAdam, 1989), an increase in leaf area may cause an increase in photosynthesis, which consequently leads to an increase in carbohydrate metabolism. Differences in the leaf area as well as in leaf thickness may be
related to differences in photosynthetic accumulation per unit leaf area (Patterson and Flint 1980; Sionit and Patterson, 1984).

1.4.2. PLANT DRY MATTER:

An early study of some 30 species found that the C₃ group but not the C₄ group had substantial dry matter gains when they were grown in an CO₂ enriched environment (Akita and Tanaka, 1973). A compilation of data for crop species indicated that the mean net CO₂ exchange rate of C₃ species was initially 52% greater than that of their ambient CO₂ grown counterparts, but declined to 29% (Cure and Acock, 1986). Despite this acclimation in photosynthesis, enriched plants accumulated on average 30% more biomass and had a 41% greater yield. Another survey concluded that yields of C₃ species could increase by roughly 33% (Kimball, 1983). Exceptions are also known, such as young ryegrass (Lolium perenne), where elevated CO₂ increased photosynthesis without affecting dry matter production (Ryle et al., 1992).

Patterson et al. (1984) reported in Sorghum halepens (Johnsongrass) (C₄) and Glycine max (C₃) that CO₂ enrichment to 600 ppm and 1000 ppm had the effect of decreasing biomass partitioning into the leaves. The distribution of leaf biomass as leaf area also declined, which resulted in a significant decrease (from 21-28% when CO₂ was increased from 350-675 ppm) in the production of leaf weight ratio and specific leaf area, and of the leaf area ratio. Neales and Nichols (1978) found that the decrease in specific leaf area was related to dry matter in the leaves of the C₃ plant wheat, and also noted that a response to high carbon dioxide concentrations depended on plant age. Conversely, previous studies on Glycine max by Clough et al. (1981) had found a significant enhancement of dry weight at 1000 ppm CO₂. The
annual C₄ grass *Eragrostis orcuttiana* also showed a 150% dry weight increment under elevated carbon dioxide concentrations (Smith et al., 1987).
1.4.3. STEM HEIGHT AND WEIGHT:

Plants grown under CO₂ enriched conditions often show higher rates of extension, and greater final heights (Sionit et al., 1985). Perennial plants and annual plants with indeterminate growth (Paez et al, 1984) became taller at carbon dioxide concentrations above 350 ppm than at normal concentrations. Determinate annuals have been shown to increase in height at a faster rate at high CO₂, although there was no difference in final height (Paez, 1984).

Increase in stem height is proportionally less than the concomitant increase in dry weight (Ford and Thorne, 1967; Sionit, 1981). In Glycine max (soybean), under high nutrient provision, and in Cassia obtusifolia (sicklepod) and Crotalaria spectabilis (crotalaria), under low nutrient provision, growth at 675 ppm CO₂ led to a significant increase in plant height (Patterson and Flint, 1982). A similar increment in height was observed by Potvin and Strain (1985) on the two weeds Echinochloa crus-galli and Eleusine indica at 28°C and 675-1000 ppm CO₂, but the total dry weight, including the stem weight in this case, showed a similar proportional increment in comparison (Potvin and Strain, 1984). Patterson et al. (1984) also observed an increased height in Sorghum halepense Johnsongrass (C₄), but not in Glycine max (C₃) under 675 ppm CO₂. The increase in height is generally seen to occur under elevated CO₂ in both C₃ and C₄ plants.
1.4.4. FREQUENCIES OF TILLERS AND INTERNODES:

Increases in numbers of tillers and internodes in plants grown under high CO$_2$ have been recorded by various authors. In *Pueraria lobata* (Kudzu), a type of vine, branching increased by 50%, which resulted in an increase in total branch length. *Liquidambar styraciflua* produced more branches when grown under high CO$_2$, whereas *Pinus taeda* did not (Sionit et al., 1985). In *Eriophorum vaginatum* the tillering rate increased sixfold at 675 ppm CO$_2$, compared with the 350 ppm ambient treatment. Wheat grown at 1500 ppm showed a greatly increased number of tillers and heads (Sionit et al., 1980). This was also observed by Rogers et al. (1983), who found that wheat allocates much of the extra photosynthate produced at higher CO$_2$ to tiller production, and little starch accumulates in the leaf.

1.5. EFFECTS OF ELEVATED CARBON DIOXIDE CONCENTRATIONS ON THE LEAF ANATOMY OF C$_3$, C$_4$ AND C$_3$/C$_4$ PLANTS:

1.5.1. LEAF ANATOMY OF C$_3$ AND C$_4$ PLANTS:

The main types of photosynthetic CO$_2$ fixation are connected with characteristic leaf anatomy. The leaf mesophyll of C$_3$ plants is usually divided into layers of palisade parenchyma and spongy parenchyma. The cells of the palisade parenchyma are elongated and regularly orientated with their long axis at right angles to the adaxial leaf surface. The spongy parenchyma appears to be less regular and has conspicuous intracellular spaces. In grass leaves the mesophyll does not show any distinct differentiation between palisade and spongy parenchyma. Most C$_4$ plants
possess the so-called Kranz anatomy (for review see Dengler et al., 1985). Vascular bundles are surrounded by concentric layers of two types of photosynthetic tissue: the bundle sheath cells (or photosynthetic carbon reduction sheath) and the mesophyll cells, which radially surround them. Chloroplast dimorphism is exhibited: chloroplasts of the bundle sheath cells are more numerous or larger than the chloroplasts in the mesophyll (for review see Dengler et al., 1995). The distances between vascular bundles and between the leaf substomatal cavities and vascular bundles are relatively short, and the presence of a less extensive leaf air space system implies a greater tissue density per unit leaf volume than in C_3 plants (Laetsch, 1974). Stomatal density in C_4 plants is generally 1.5 times higher than in C_3 plants (Apel, 1979). Three groups of C_4 plants distinguished by their differing C_4 acid decarboxylating systems also display differences in the intracellular location of chloroplasts (centrifugal; centripetal), and the content and ultrastructure of mitochondria (Hatch et al., 1975; Hattersley and Watson, 1976; Hattersley and Browning, 1988). Panicum members have been found to belong to all three sub-groups of C_4 plants (Hatch et al., 1975; Prendergast, Hattersley and Stone, 1987; Dengler et al., 1994):

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<tr>
<td>NAD-ME</td>
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<td>PEP-CK</td>
<td><em>Panicum maximum</em></td>
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<tr>
<td>NADP-ME</td>
<td><em>Panicum antidotale</em></td>
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The attribution of photosynthetic mode to these species is not controversial (Hatch, Kagawa and Craig, 1975; Akin, Wilson and Windham, 1983; Sage and Seeman, 1993).
1.5.2. LEAF ANATOMY OF C$_3$/C$_4$ PLANTS:

The leaf anatomy of C$_3$/C$_4$ species is intermediate, resembling more or less the C$_3$ or the C$_4$ anatomy (Brown and Brown, 1975; Rathnam and Chollet, 1980). Such intermediates have been identified in seven genera across five families, including representatives of the Monocotyledonae and Dicotyledonae (Edwards and Ku, 1987). The best example of a naturally occurring intermediate plant is the tropical grass, Panicum milioides. The leaf anatomy is basically non-Kranz, but with several characteristics resembling Kranz anatomy (Kanai and Kashiwagi, 1975). The parenchyma sheath cells show non-Kranz features and are quite large with few chloroplasts, the latter appearing similar in size and shape to those of mesophyll cells. In addition, parenchyma sheath cells are thinner than those most Kranz cells, but thicker than for non-Kranz species. The chloroplasts are atypical of non-Kranz cells, but typical for one subgroup of C$_4$ species, those which have high activities of the NAD-malic enzyme. The mesophyll surrounding the parenchyma sheath cells is radial as in many Kranz plants (Brown and Brown, 1975; Morgan and Brown, 1979). In case of Panicum milioides even the CO$_2$ compensation point shows an intermediate value: 20 ppm CO$_2$ compared to values typical of the C$_4$ (< 10 ppm) and C$_3$ species (> 40 ppm) (Krenzer et al., 1975).

Another characteristic of C$_3$/C$_4$ plants is the increase in the number of organelles within the bundle sheath cells in comparison to related C$_3$ species, again a feature described as Kranz-like (Holaday et al., 1981). The mitochondria are found along the cell wall immediately adjacent to the vascular cells and they are overlain by the chloroplasts. The combined number of mitochondria and peroxisomes per unit cell area in bundle sheath cells was also four times greater than that in mesophyll cells, whereas the number of chloroplasts per unit cell area was only slightly increased (by 1.4-fold on average) in the bundle sheath cells (Brown and Hattersley, 1989).
That the anatomy is determined genetically is clearly shown by segregation of the C₃/C₄ anatomy away from a C₃/C₄ like carbon dioxide compensation point in an F₂ population derived from a C₃ x C₃/C₄ cross in the genus Panicum (Bouton et al., 1986). The anatomy can be inherited independently of other C₃/C₄ characters which influence the leaf biochemistry, and hence the compensation point. Thus the compensation point cannot be solely a consequence of the expression of these anatomical characters in a C₃/C₄ intermediate species (Rawsthorne et al., 1992). The intermediate Panicum decipiens used in this study displays Kranz anatomy, but behaves physiologically like a C₃ plant (Wilson, CSIRO BRISBANE, personal communication).

1.5.3. EFFECTS OF CO₂ CONCENTRATIONS ON THE LEAF ANATOMY OF C₃ AND C₄ PLANTS:

One response to elevated CO₂ concentrations has been identified as an increase of specific leaf weight (SLW), an indicator of leaf thickness (Hofstra and Hesketh, 1975; Neales and Nichols, 1978; Patterson and Flint, 1982). Changes in leaf thickness generally indicate the existence of more photosynthetic mesophyll cells or larger cells. On the other hand, in thicker leaves diffusion pathways for CO₂ are longer, but there might be more parallel diffusion on the larger inner leaf surface. Continuing thickening of leaf epidermis, of cell walls, cuticle and other surface structures contributes less to variation in photosynthesis.

An increase in leaf thickness of plants grown under elevated CO₂ between 350 ppm and 900 ppm is often correlated with leaf starch content, and seems to be limited to C₃ species (Downton et al., 1980, Sionit and Patterson, 1984). This was also observed in histological investigations on Glycine max, Liquidambar styraciflua and Pinus taeda, while the C₄ plant
*Zea mays* did not show an increased leaf thickness (Thomas and Harvey, 1983; Rogers et al., 1983). These authors found an increase in the number of cells within the mesophyll of all three C3 species, which was identified in *Glycine max* as a third layer of palisade cells rather than two as observed under normal atmospheric conditions. This extra leaf thickness was a result of more rapid development of leaves in high CO2 concentrations during the period of lamina differentiation and cell enlargement (Thomas and Harvey, 1983). Thicker needles in *Pinus taeda* were caused in part by a third mesophyll layer but also by a greater development of vascular bundle and transfusion tissues. The origin of this third layer is still unknown and requires further studies, but it is likely that it is initiated by cell division in the second palisade layer or in the unique paraveinal layer (Fischer, 1967; Dengler et al., 1975). The spongy mesophyll did not differ significantly. In a study on the effects of 700 ppm CO2 on four poplar clones, Radouglo and Jarvis (1992) demonstrated that leaf thickness was effected by an increase of the entire mesophyll area. This was in turn the result of greater mesophyll cell expansion and more extensive intercellular spaces. Kriedemann et al. (1976) showed on transverse sections made of *V. vinifera* grown above 500 ppm CO2 an increased depth of both palisade and spongy mesophyll tissue, which could imply a more tortuous path for CO2 molecules diffusing towards fixation sites. Overall, there were more cells per unit leaf area and less individual cell expansion under CO2 enrichment. This could lead to the assumption that the number of mesophyll cells and not the size, i.e. the cell enlargement, eventually causes a higher photosynthetic activity (Thomas and Harvey, 1983).

Mesophyll size was negatively correlated with photosynthetic rate in perennial ryegrass. In this case it was suggested that an increase in leaf area production rate via increase in final cell size would result in reduced photosynthetic rate (Wilson and Cooper, 1969).
On the other hand, increased cell expansion seems to occur at higher carbon dioxide, especially in palisade cells rather than in epidermal cells, as shown for the C₃ species *Glycine max*, *Pinus taeda* and *Liquidambar styraciflua* (Thomas and Harvey, 1983). This was attributed to the increased osmotic potential of cells associated with their higher total carbohydrate content, which causes the cells to absorb more water and thus enlarge.

Studies on the effects of other environmental factors on tropical grasses, such as high light intensities, low relative humidities or conditions conducive to high transpiration showed that there is a similar tendency to develop abundant tissue, and this also occurs in leaves exposed to strong winds. These xeromorphic changes are expressed by an increase in the vascularization thickness, and also lignification of cell walls, and the development of sclerenchyma in the leaves, i.e. in components likely to increase the proportion by weight of cell wall materials, and which constitute the less digestible constituents of the plant tissues (Metcalfe, 1960; Sinnott, 1960; Wilson, 1975).

Experiments with tomato and soybean have also demonstrated that increased CO₂ leads to thicker and heavier leaves and may cause excess starch accumulation which may directly inhibit photosynthesis (Hofstra and Hesketh, 1975; Madsen, 1968). This hypothesis of assimilate control, reviewed by Neales and Incoll (1968). It has been supported by observations of a correlation between accumulation of dry weight in vegetative tissue and the suppression of photosynthetic rates. It was thought that the dry weight increase represented starch accumulation, which has been shown in high CO₂ grown plants (Hofstra and Hesketh, 1975, Madsen, 1968). This seems to be limited to C₃ species (Dowton et al., 1980; Sionit and Patterson, 1984).
1.5.4. EFFECTS OF CO₂ CONCENTRATIONS ON THE ANATOMY

C₃ /C₄ PLANTS:

Compartmentation of phospho-enolpyruvate and ribulose bisphosphate carboxylase in the mesophyll and bundlesheath cells of C₃/C₄ species is thought to reduce photorespiration (Rathnam and Chollet, 1978) and therefore enable these to increase their photosynthetic assimilation of CO₂. Since CO₂ fixation in at least some C₃/C₄ species may involve elements of both C₃ and C₄ cycles, intermediates may be more susceptible to change by CO₂ or O₂ concentrations during growth. Because phosphoenolpyruvate carboxylase has a much greater affinity for CO₂ than does RuBP carboxylase, growth of plants at low CO₂ may favour development of C₄ cycle enzymes, which would give C₃/C₄ plants an advantage over C₃ plants. Conversely, growth of C₃/C₄ species at high CO₂ may discourage energy investment in C₄ enzymes (Byrd and Brown, 1989). Studies to examine the degree of Kranz anatomy in species intermediate to C₃ and C₄ types in *Panicum*, *Neurachne*, *Flaveria* and *Moricanda* showed that the percentages of leaf photosynthetic cell profiles partitioned to bundle sheath were higher in C₃ and C₄ species, while C₃/C₄ species tended to be in between. C₃/C₄ species partitioned more photosynthetic tissue to bundle sheath than C₃ species in *Moricanda*, but not in *Flaveria* nor in *Neurachne*, which showed a very small proportion on a total cell area basis. Also, the percentage of organelles partitioned to the bundle sheath was much greater in C₃/C₄ species in comparison to C₃ on C₄ species. The CO₂ compensation concentration was negatively correlated to the partitioning of tissue to the bundle sheath, and to the percentage of organelles in the bundle sheath. It was concluded that all of the C₃/C₄ species examined have developed some degree of Kranz anatomy and that this altered anatomy is involved in their
reduced photorespiration (Brown and Hattersley, 1989). This result confirms the previous observations on C3/C4 species mentioned above.

Studies on growth of the C3/C4 intermediate Flaveria floridana under CO2 concentrations of 100, 350 and 750 ppm showed no significant effect on CO2 exchange characteristics or leaf anatomy. Carboxylation efficiency and CO2 compensation concentrations developed under high CO2 were intermediate compared to Flaveria pringli (C3) and Flaveria trinervia (C4) (Byrd and Brown, 1989).

1.6. EFFECTS OF HIGH CARBON DIOXIDE CONCENTRATIONS ON THE PHOTOSYNTHETIC CAPACITY AND NON-STRUCTURAL CARBOHYDRATE ACCUMULATION OF C3, C4 AND C3/C4 PLANTS:

1.6.1. WHAT IS PHOTOSYNTHETIC CAPACITY?

The photosynthetic capacity of a leaf is a function of many factors, including the amount and configuration of the cells present and the CO2 exchange potential of those cells.

The source of differences in photosynthetic rate per unit leaf area is often not apparent because leaves might vary considerably in number of cells, total dry weight, and the chlorophyll, per unit leaf blade area. Changes in the amount of cellular material per unit area or in physiological capacity of the cells each might produce changes in whole leaf CO2 exchange rate. Hypothetically, even a change in the spatial relationship of cells could affect whole leaf CO2 exchange rate by altering gas diffusion pathways (Parkhurst, 1978).
1.6.2. STARCH FORMATION UNDER HIGH CARBON DIOXIDE CONCENTRATIONS

One common phenomenon among many CO₂ studies is that of greater accumulation of carbohydrates. Much of the triose-phosphate produced by photosynthesis often remains temporarily in the chloroplast and is converted to starch for subsequent metabolism and export (Geiger and Servaites, 1994). This requires partitioning of current photosynthates to both starch and sucrose; allocation of carbon for starch synthesis must be regulated without restricting the synthesis of sucrose for export (Fondy and Geiger, 1980). Many studies, carried out on crop species like Glycine max (Vu et al., 1989), and Oryza sativa, and also in the tree species Liriodendron tulipifera and Quercus alba, suggested a limitation in the allocation of carbon for sucrose synthesis relative to that of starch, which is also structurally detectable microscopically, unlike sucrose. Therefore the study described in this thesis puts its emphasis on the determination of this non-structural carbohydrate using qualitative structural and quantitative physiological analytical tools.

1.6.3. CHLOROPHYLL CONCENTRATION: A MARKER FOR PHOTOSYNTHETIC CAPACITY:

Another common observation is that the accumulation of carbohydrates in leaves of plants grown at high CO₂ is accompanied by a gradual loss of photosynthetic capacity of the leaves (reviewed by: Eamus and Jarvis, 1989; Arp, 1991; Stitt, 1991; Gunderson and Wullschleger, 1994). This was accompanied by a decline of leaf chlorophyll concentration in many plants in response to high CO₂ (DeLucia et al., 1985; Oberbauer et al., 1985; Houpt et al., 1988; Mousseau and Enoch, 1989; Norby et al., 1992; Wullschleger et al., 1992).
1.6.4.: DETERMINATION OF PHOTOSYNTHETIC CAPACITY THROUGH INVESTIGATION OF THE CHLOROPLAST STRUCTURE: THYLAKOIDS

Since photosynthesis is stimulated at higher CO$_2$ concentrations to such an extent that the extra supply of sugars cannot be used by the plant for growth, an accumulation of starch will occur and, finally, due to a feedback inhibition, there will be a decrease in the rate of photosynthesis, i.e. photosynthetic capacity (Clough, 1981; Wulff and Strain, 1981). Sometimes, this negative feedback on the rate of photosynthesis might be even stronger in cases where starch accumulation causes disruption of the chloroplasts. This detrimental effect of high starch accumulation on the thylakoid structure (reduced grana formation) was shown in electron micrographs of mature leaves of Desmodium paniculatum grown under 1000 ppm CO$_2$. This effect probably causes phenomena like chlorosis in clover (Cave 1981; Wulff and Strain, 1982).
CHAPTER 2

MORPHOLOGICAL:

EFFECTS OF HIGH CARBON DIOXIDE CONCENTRATIONS ON THE MORPHOLOGY OF PANICUM GRASS SPECIES WITH DIFFERENT PHOTOSYNTHETIC PATHWAYS

2.1. INTRODUCTION:

This chapter presents a study on the morphological responses of Panicum forage grasses to elevated CO₂ concentrations. This is of considerable importance in relation to their digestibility and therefore forage quality and growth under changing environmental conditions. The information is of considerable practical significance for an altered pattern in grassland management demanded by a future high CO₂ environment through changes in water use efficiency, nutrient uptake and energy metabolism.

Morphological parameters such as stem weight and height, total plant dry weight, node and tiller frequencies and leaf area were chosen. Special attention was given to the surface of the leaf, i.e. its outer peel and general epidermal surface. The aim of this investigation was twofold, first, to investigate if there is a morphological response of the Panicum species to high carbon dioxide concentrations, and secondly to develop a quick and reliable method to describe changes of the leaf surface structure due to high CO₂ on a microscopical level. Emphasis was given to the stomatal apparatus and the epicuticular wax layer, both representing important morphological
features concerned with water use efficiency and protection of the plant surface.

2.2. MATERIALS AND METHODS

2.2.1 PLANT MATERIAL

The *Panicum* species examined, *Panicum antidotale* (C₄), *Panicum tricanthum* (C₃) and *Panicum decipiens* (C₃/C₄), sub-family Panicoideae, were chosen to represent the full range of photosynthetic pathway variation known in the *Poaceae*. The grasses were grown in normal daylight under controlled temperature 30/22°C day/night and humidity 80/50% day/night conditions in specially designed microclimate chambers (design UWSH). They were exposed to elevated CO₂ from a clean industrial source (900 ppm, +50ppm; CIG) and ambient CO₂ (350 ppm), which also served as control. 900 ppm CO₂ was chosen because preliminary experiments (carried out at the BCRI Rydalmere) had shown the highest effect of this concentration on the plants used in regard to a visible morphological response. Grasses were sown into a 60:40 sand:peat mixture in seed trays. After 3-4 weeks the seedlings were thinned out to give at least ten plants per species for each environment. The seedlings were planted in 350 ml pots (*P. decipiens*) and in 100ml tubes (*P. antidotale* and *P. tricanthum*) containing potting mix with slow release fertilizer (Osmocote).

2.2.2 SAMPLING PROCEDURE AND GROWTH CONDITIONS:

Sampling and data collection took place within the timeframe of June 1994 until March 1995. Plants were grown between 1993 and 1995 in order to avoid the extreme seasons between summer and winter. They were harvested 8 to 9 weeks after emergence just before flowering. 10 plants per species per environment were taken at about 9.30 am for morphological
investigations on the following parameters: leaf area, leaf weight, plant dry matter, numbers of tillers and internodes, stem weight and height. The root system was not investigated due to the roots being to fine to be extracted from the potting mix and lack of appropriate equipment. Leaf area (leaf lamina and sheath) was measured using the Delta T Area Measurement System. Dry matter content was determined by selecting 10 leaf blades of each species from each environment at three different harvests, which were then oven-dried for 24h at 80°C. Data collected at each sampling date represent individual measurements and were analysed by analysis of variance (see “statistical analysis”) using a randomized complete block design with plants as blocks using the SAS statistical package (see 2.2.6 “statistical analysis”). Since it would have been difficult to recover all the fine roots from the medium, the recovery was not attempted.

2.2.3. SPECIMEN PREPARATION FOR THE SCANNING ELECTRON MICROSCOPY:

A 2 cm transverse section taken from the midpoint of 2 or 3 leaves of each species from each environment was also taken for investigations of the epicuticular waxes using scanning electron microscopy. A qualitative assessment of the response to high carbon dioxide concentrations of the structure of epicuticular waxes of the leaf surfaces of all species used was carried out using a Jeol 35 C and a Phillips 503 Scanning Electron Microscope at the University of Sydney. The micrographs selected are representative of 50 to 60 micrographs taken at each of the sampling dates.

2.2.4. PRELIMINARY EXPERIMENTS: PREPARATION FOR THE SCANNING ELECTRON MICROSCOPE

The standard leaf area investigated was the area of cuticle covering several epidermal cells, sometimes including guard cells and trichomes. Scanning electron microscopy was selected in preference to the transmission
microscope because the use of the latter requires the making of replicas. This is likely to cause damage and shrinkage to the cuticle and therefore would have given false impressions of the microphotograph. Various techniques were tested to ascertain the best methods for preparing the cuticles of the Panicum grasses for ultramicroscopic examination using SEM, and comparative studies were undertaken of cuticles of fresh material, ambient dried, freeze dried, critical point dried and frozen material kept at low temperature (LTSEM).

2.2.4.1. REPLICA TECHNIQUE:

Fresh, hydrated leaf specimen and epoxy resin replicas were used to study the epicuticular wax layer and eventual changes in the epidermal cells (Williams et al., Panicum leaf surface and left to set. A positive resin replica was made from the negative mould. The resin replicas and fresh specimens were coated with gold palladium or platinum (approximately 60A), and examined with an accelerating voltage of 15 kV.

2.2.4.2. CRITICAL POINT DRYING:

The leaf sections were fixed in 3% (v/v) glutaraldehyde, buffered at pH 7.2 with 0.05 M phosphate buffer at 4°C for 7 to 14 days. Samples were then dehydrated in 50, 70, 90 and twice in 100% ethanol (v/v) for 30 min each. The samples were critical point dried with liquid CO₂, mounted on aluminium stubs, coated with approximately 240 A gold or platinum, and observed in a Phillips 503 Jeol scanning electron microscope at 15 kV.

2.2.4.3. FRESH FROZEN MATERIAL HELD AT LOW TEMPERATURE (COLD-STUBS PROCEDURE, LTSEM):
Using a special brass stub (designed by Toni Romeo, University of Sydney), freshly cut leaf strips 3mm square were attached with carbon tincture before being plunged into liquid nitrogen for 25 seconds. The uncoated stub was then rapidly placed in the SEM (JSM JEOL 303, Phillips) and the frozen cuticles were examined at 7.5 kV following the sublimation of the surface ice (around 20 minutes) and before shrinkage commenced.

2.2.4.4. FREEZE DRYING TECHNIQUE:

Specimens were frozen to liquid nitrogen temperature, put on cold stage and then, in a special freeze dryer, were allowed to return to room temperature under vacuum in a 12 hour time frame. They were then mounted onto stubs and coated with gold or platinum at 60 A.

2.2.4.5. AIR (AMBIENT) DRIED MATERIAL

Ambient air dried leaves were attached on conventional aluminium stubs with carbon tincture, coated with gold or platinum at 60 A and viewed under the SEM.
2.2.5. CONCLUSION AND DISCUSSION OF THE DIFFERENT METHODS USED FOR THE SCANNING ELECTRON MICROSCOPE:

The visualization of leaf surface waxes is hampered by certain of their properties. The waxes are fragile, demanding care in handling, because they melt at temperatures frequently encountered in the focussed beam of the SEM, and dissolve in solvents commonly used in tissue preparation for EM (Sargent, 1983). Cuticle preservation without damage to its surface was considered to be of paramount importance and thus used the following criteria to determine the accuracy of preservation:

(i) degree of obvious damage, erosion or other alteration to the actual surface of the cuticle caused by the examination or preparation of the specimen

(ii) degree of distortion of the cuticle as indicated by the severity of cuticular wrinkling caused by procedures/solvents

(iii) maximum resolution obtained using a specific microscope (SEM)

A major problem of any biological material viewed under the SEM is the desiccating environment of the specimen. Fresh tissue at room temperature quickly loses water on the stage of a SEM, particularly under high vacuum. Another impediment to obtaining high resolution images of fresh uncoated tissue is the rapid charging which occurs at high accelerating voltages. In this study the lowest possible voltages were used (7.5 kV for the cold stubs technique). The coating with metal to overcome the charging problem involves subjecting the tissue to low pressure dessicating conditions even before it reaches the SEM. Some dehydration must also occur during the
deposition of carbon under vacuum in the replica technique. The problems associated with recognizing and reducing artefacts associated with the examination of biological material in the SEM have been well reviewed by Robards (1978).

2.2.5.1. REPLICA TECHNIQUE:

The replica technique was proved to be inappropriate for the preparation of the specimens used in this study (figures 18 G;H). The silicon-component in the hydrophilic exaflex reacted with the Spurr’s resin used and created artefacts. This phenomenon has been observed by other users of the EM unit in Sydney University (personal communication) who used plant and animal specimens.

2.2.5.2. FREEZE DRYING (FD) PROCEDURE:

Boyde and Franc (1981) have used the freeze drying procedure to overcome the worst effects of shrinkage and, provided leaf surface features are not disrupted during the freezing process, this seemed to offer an ideal method. These results could not be confirmed in this study. The FD method preserved the form of the wax structures and the wax bloom of the Panicum species investigated, but their orderly orientation seemed to be destroyed, making it nearly impossible to recognize the outlines of underlying cells (figures 15A;B and 16 C;D).

2.2.5.3. AIR DRYING (AMBIENT) TECHNIQUE:
The air dried specimens which were cut transversely showed a collapse of cells and a tearing effect on the cell margins which made the resolution unclear (see figure 17 E;F).

2.2.5.4. CRITICAL POINT DRYING (CPD)

Sargent (1983) in his studies on the cuticle of wild oat (Avena fatua) considered the damaging effect of the solvents used for CPD and stated that there is a resulting removal of waxes from the plant surfaces. In contrast, Koziol and Cowling (1981) reported that cuticular waxes from ryegrass prepared for and critical point dried showed no differences from fresh unfixed material when examined by SEM. This was also the case in the Panicum leaf surfaces of this study and a very clear resolution of the cuticle could be achieved (figures 19 I;J). The images were free from shrinkage and distorting artefacts associated with dehydration and solvent extraction.

2.2.5.5 COLD STUBS TECHNIQUE (LTSEM):

The LTSEM or cold stubs technique involves three stages of specimen manipulation: cryo fixation, cryo preparation and the examination of the prepared specimen on the cold stage of a SEM (Turner and Smith, 1974; Huang et al., 1994). Generally this technique permits extensive examination of leaf surfaces in the absence of known artefacts. The epidermal cells retain their turgid form and the epicuticular structures remain largely intact and well ordered (figures 20 K;L). Critical point drying is a much slower process than the cold stubs technique, and the latter is therefore more appropriate for future studies which have to be done in a short amount of time. Other advantages include:

(i) specimen can be fixed fresh and unfixed and are therefore lifelike
(ii) it permits extensive examination of leaf surfaces in the absence of known artefacts

(iii) distortion is likely to result only from ice crystal growth; the sizes of these crystals can be minimized by rapid initial freezing

(iv) freezing hardens soft tissues so they do not collapse under vacuum; these may also be fractured to show internal structures

(v) tissue retains its full water content and its full volume, and turgid cells therefore stand in the same relation to each other as they did during life, therefore:

(vi) the epicuticular structures remain largely intact and well ordered

(vii) water soluble substances are trapped by freezing at, or close to, their positions in the living tissue

I concluded that cryo preservation (cold-stubs technique) and the conservative critical point drying technique were the superior techniques for viewing leaf surfaces in the SEM, because they gave the best and clearest resolution of the specimens without having caused any damage to them.
2.2.6. EXTRACTION OF LEAF WAX

2.2.6.1. EXTRACTION OF THE LEAF WAX:

Two grams of fresh leaves from each species from each environment were ground in liquid nitrogen. The homogenate was transferred to a filter thimble, and placed in a Soxhlet apparatus (8 hours). Distilled chloroform (containing 1% ethanol), was used as an extracting solvent. The chloroform fractions were concentrated to dryness using a rotary evaporator (Buchi, Rotavapor-R, Switzerland) and weighed.

2.2.7. QUANTITATIVE DETERMINATION OF THE EXTRACTED WAX: THIN LAYER CHROMATOGRAPHY

Isolated waxes were examined for classes of constituents and amount of constituents by thin-layer-chromatography on silica-gel (Merck, Darmstadt, FRG). The preparative plates were pre-run in chloroform:methanol and stored at 110°Celsius. After the plates had been loaded with both standard and wax extracts by microliter-pipettes (Microcaps Drummond Scientific Co., USA), the plates were run with chloroform as the mobile solvent. Spots were detected by spraying with 50% (aq.) sulphuric acid and charrring at 110°C for 10 minutes.

2.2.8. STATISTICAL ANALYSIS

The aim of the investigations was to determine the influence of two different carbon dioxide concentrations (350 ppm and 900 ppm CO₂) on the three Panicum species used in this thesis. This was accomplished by doing a two-way analysis of variance using the JMP procedures of SAS (SAS Inst. Inc.).
An important assumption in an analysis of variance is that the variances are equal. Where the data showed unequal variances, the Box-Cox-Procedure was applied (Brown-Forsythe-test and Levene-test). If there were no differences in variances tested, the raw data were maintained and were not transformed.

Another important assumption of the analysis of variance is that the distribution is normal. This was tested by using the Shapiro-Wilk-W-test. In the analysis of variance the "F" statistic was used to assess the significance of the main effects, i.e. the way in which the species responded to the different carbon dioxide concentrations.

An a posteriori test for significance between means (response of each species to the different CO₂ concentrations) were made using CONTRAST statement in the JMP procedure. A p-value less than 0.05 was taken to show significant difference. Standard error bars displayed in the graphical presentation of this thesis show pooled standard errors of the means.

2.2.9. STOMATAL COUNTS (Stomatal frequencies, stomatal pore length and width)

Samples were taken from the midpoint of the leaf blade and stomatal frequency of the lower (abaxial) leaf surface was measured by using replicas prepared with cellulose acetate (nail varnish) mixed with 0.1% Aniline Blue (North, 1956). The replicas were mounted on slides with double sided tape. Counts were made with a grid reticule in one eyepiece of a microscope using a 20 x objective lens. Stomatal observations were confined to the abaxial surface. Treatment means were calculated from three leaves per treatment using counts from 20 areas per leaf from each environment. Stomatal pore length was measured by the same method mentioned using an additional grid scale.
2.3. RESULTS

2.3.1. GENERAL MORPHOLOGICAL OBSERVATIONS:

As shown in figures 1-6, all species of *Panicum* investigated show a clear qualitative response to elevated carbon dioxide concentrations. All plants grown under high CO$_2$ in the greenhouse showed a deeper blue-green leaf colour and an increase in height. The foliage showed a denser and more vigorous appearance. On average, these plants flowered about 4-5 days earlier than the controls grown at 350 ppm CO$_2$. Another feature of the grass plants grown at elevated carbon dioxide was stiffness of the leaves, which was accompanied by an increase in sharpness of the leaves when felt by hand.

*Panicum antidotale*, grown under 900 ppm carbon dioxide, flowered about 1 week earlier than the species grown under ambient conditions. They also displayed signs of early leaf senescence, showing a yellowish colour (see figures 3 and 6). Similar observations were made for *Panicum decipiens*, which produced an increased amount of flowerheads, which were big when grown under elevated carbon dioxide.
2.3.1.1. EFFECTS OF ELEVATED CARBON DIOXIDE CONCENTRATIONS ON THE MORPHOLOGY OF *PANICUM* GRASS SPECIES
**Figure 1:** Effects of high CO$_2$ on *Panicum trichanthum* (C$_3$) six weeks after seedling emergence. Note differences in height of plants grown under ambient CO$_2$ (left) compared to plants grown under high CO$_2$ (right). Scale bar = 30 cm

**Figure 2:** Effects of high CO$_2$ on *Panicum decipiens* (C$_3$/C$_4$) six weeks after seedling emergence. Note differences in height of plants grown under ambient CO$_2$ (left) compared to plants grown under high CO$_2$ (right). Scale bar = 30 cm
**Figure 3:** Effects of high CO$_2$ on *Panicum antidotale* (C$_4$) six weeks after seedling emergence. Note differences in height of plants grown under ambient CO$_2$ (left) compared to plants grown under high CO$_2$ (right). Scale bar = 30 cm

**Figure 4:** Effects of high CO$_2$ on *Panicum tricanthum* (C$_3$) ten weeks after seedling emergence. Note differences in height of plants grown under ambient CO$_2$ (left) compared to plants grown under high CO$_2$ (right). Scale bar = 30 cm
Figure 5: Effects of high CO$_2$ on *Panicum decipiens* (C$_3$/C$_4$) ten weeks after seedling emergence. Note differences in height of plants grown under ambient CO$_2$ (left) compared to plants grown under high CO$_2$ (right). Scale bar = 30 cm

Figure 6: Effects of high CO$_2$ on *Panicum antidotale* (C$_4$) ten weeks after seedling emergence. Note differences in height of plants grown under ambient CO$_2$ (left) compared to plants grown under high CO$_2$ (right). Scale bar = 30 cm
2.3.2. SPECIFIC MORPHOLOGICAL RESULTS

2.3.2.1. EFFECTS OF ELEVATED CO$_2$ ON LEAF AREA, LEAF WEIGHT AND PLANT DRY MATTER
Figure 7: Effect of high CO$_2$ on the leaf area of Panicum species: Panicum decipiens (C$_3$/C$_4$; Prob $> t = 0.05$), Panicum tricanthum (C$_3$; Prob $> t = 0.00$), Panic antidotale (C$_4$; Prob $> t = 0.08$) displayed an increase in leaf area when grown under 900ppm carbon dioxide.
Figure 8: Effect of high CO₂ on the leaf weight of Panicum species:

Panicum decipiens (C₃/C₄; Prob > t= 0.01), Panicum tricanthum (C₃; Prob > t = 0.001) and Panicum antidotale (C₄; Prob > t= 0.003) showed a significant increase in leaf weight when grown under 900ppm carbon dioxide.
Figure 9: Effect of high CO₂ on the plant dry matter of Panicum species:

Panicum decipiens (C₃/C₄; Prob > t = 0.001), Panicum tricanthum (C₃; Prob > t = 0.007) and Panicum antidotale (C₄; Prob > t = 0.000) displayed significant response to elevated carbon dioxide when grown under 900ppm carbon dioxide.
2.3.2.2. EFFECTS OF HIGH CO$_2$ ON STEM HEIGHT AND STEM WEIGHT, TILLERING, AND INTERNODE FORMATION
Figure 10: Effect of high CO$_2$ (900ppm) on the stem height of *Panicum* species. There was a high statistical significance for the three species (*Panicum antidotale* (C$_4$): Prob > t = 0.001; *Panicum tricanthum* (C$_3$) Prob > t = 0.003; *Panicum decipiens* (C$_3$/C$_4$) Prob > t = 0.002).
Figure 11: Effect of high CO$_2$ (900ppm) on the stem weight of *Panicum* species. There was a high statistical significance for the three species (*Panicum antidotale* (C$_4$): Prob > $t = 0.001$; *Panicum tricanthum* (C$_3$) Prob > $t = 0.002$; *Panicum decipiens* (C$_3$/C$_4$) Prob > $t = 0.2$).
Figure 12: Effect of high CO$_2$ (900ppm) on tiller frequencies of Panicum species. Panicum antidotale (C$_4$, Prob > t = 0.01), Panicum tricanthum (C$_3$; Prob > t = 0.00) and Panicum decipiens (C$_3$/C$_4$; Prob > t = 0.00) displayed statistical significance.
**Figure 13:** Effect of high CO₂ (900ppm) on the numbers of internodes of *Panicum* species. *Panicum antidotale* (C₄, Prob > t = 0.01), *Panicum tricanthum* (C₃, Prob > t = 0.00) and *Panicum decipiens* (C₃/C₄, Prob > t = 0.01) exhibited the same trend of a slight increase with statistical significance:
2.3.2.3. RESPONSE OF STOMATA OF *PANICUM* SPECIES TO INCREASED CO$_2$ CONCENTRATIONS
Figure 14: Stomatal frequencies (per mm$^2$) of Panicum species grown under elevated CO$_2$ (900 ppm). Panicum antidotale ($C_4$, Prob > t = 0.00) and Panicum decipiens ($C_3/C_4$; Prob > t = 0.02) exhibited a statistically significant increase. Note the decrease in Panicum tricanthum ($C_3$; Prob > t = 0.02)
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<td><em>Panicum tricanthum</em></td>
<td>350</td>
<td>44</td>
<td>52</td>
</tr>
<tr>
<td></td>
<td>900</td>
<td>44</td>
<td>52</td>
</tr>
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</table>

**Table 1**: Guard cell size (length and width) of *Panicum decipiens* (C₃/C₄), *Panicum tricanthum* (C₃) and *Panicum antidotale* (C₄) grown under ambient (350 ppm CO₂) and high carbon dioxide concentrations (900 ppm CO₂). Stomatal length and width did not alter noticeably under elevated carbon dioxide concentrations.
2.3.3. SCANNING ELECTRON MICROSCOPY (SEM)

2.3.3.1. RESPONSE OF THE EPICUTICULAR WAX LAYER OF *PANICUM* SPECIES TO INCREASED CO$_2$ CONCENTRATIONS: PRELIMINARY EXPERIMENTS
**Figures 15 - 20** show scanning electron micrographs of the last fully expanded leaf of the main tiller. Samples were prepared by different procedures: fixation, critical point dried and finally gold-coated; ambient (air-) dried and gold coated; freeze dried and gold-coated; cryo-fixed (cold stubs technique or LTSEM) and examination of the uncoated specimen.

**Figure 15 A, B; 16 C, D:** Scanning electron micrographs of the last fully expanded leaf of the main tiller of *Panicum decipiens* prepared using the freeze drying technique. (A) scalebar=45.4\(\mu\)m, specimen freeze-dried and broken transversely; (B) scalebar=66.6\(\mu\)m, specimen freeze-dried and epidermis viewed from above; (C) scalebar=83.3\(\mu\)m specimen freeze-dried and cut transversely; (D) scalebar=41.6\(\mu\)m, same as (C).

**Figure 17 E, F:** Scanning electron micrographs of the epidermal surface of the last fully expanded leaf of the main tiller of *Panicum antidotale* using the air-drying (ambient) technique. Scale bar (E)= 66.6\(\mu\)m; scale bar (F)= 50\(\mu\)m

**Figure 18 G, H:** Scanning electron micrographs of the epidermal surface of the last fully expanded leaf of the main tiller of *Panicum antidotale* using the replica technique. Note the strong presence of artefacts on the epidermal surface. Scale bar (G)= 42\(\mu\)m; scale bar (H)= 9.5\(\mu\)m

**Figure 19 I, J:** Scanning electron micrographs of the epidermal surface of the last fully expanded leaf of the main tiller of *Panicum decipiens* using the critical-point drying technique. Bald spots on the epidermal surface are caused by accidental physical abrasion. Scale bar (I)= 33.3\(\mu\)m; scale bar (J)= 7.6\(\mu\)m

**Figure 20 K, L:** Scanning electron micrographs of the epidermal surface of the last fully expanded leaf of the main tiller of *Panicum antidotale* using the cold-stubs technique. Scale bar (K)= 42\(\mu\)m; scale bar (L) = 6.6\(\mu\)m
2.3.3.2. RESPONSE OF THE EPICUTICULAR WAX LAYER OF *PANICUM ANTIDOTALE* (C₄) TO INCREASED CO₂ CONCENTRATIONS

Figure 21-24 A-H: Scanning electron micrographs of the epidermal surface of the last fully expanded leaf of the main tiller of *Panicum antidotale* (C₄). *(A/B)*: Abaxial epidermis of the last fully expanded leaf grown under 350 ppm CO₂, 21 A: scale bar = 23.2 µm; 21 B: scale bar = 5.5 µm; *(C,D)* Abaxial epidermis of the last fully expanded leaf grown under ambient 900 ppm CO₂. 22 C: scale bar = 23.2 µm; 22 D: scale bar = 5.5 µm; *(E,F)* Adaxial epidermis of the last fully expanded leaf grown under 350 ppm CO₂; 23 E: scale bar = 23.2 µm; 23 F: scale bar = 5.5 µm; *(G,H)* Adaxial epidermis of the last fully expanded leaf grown under ambient 900 ppm CO₂, 24 G: scale bar = 23.2 µm; 24 H: scale bar = 5.5 µm
2.3.3.3. RESPONSE OF THE EPICUTICULAR WAX LAYER OF *PANICUM TRICANTHUM* (C₃) TO INCREASED CO₂ CONCENTRATIONS

**Figure 25-28 A-H:** Scanning electron micrographs of the epidermal surface of the last fully expanded leaf of the main tiller of *Panicum tricantum* (C₃).

(25 A,B) Abaxial epidermis of the last fully expanded leaf grown under ambient 350 ppm CO₂, **25 A:** scale bar = 23.2μm; **25 B:** scale bar = 5.5μm;

(26 C,D) Abaxial epidermis of the last fully expanded leaf grown under 900 ppm CO₂, **26 C:** scale bar = 23.2μm; **26 D:** scale bar = 5.5μm; (27 E,F) Adaxial epidermis of the last fully expanded leaf grown under ambient 350 ppm CO₂; **207 E:** scale bar = 23.2μm; **27 F:** scale bar = 5.5μm; (28 G,H) Adaxial epidermis of the last fully expanded leaf grown under ambient 900 ppm CO₂, **28 G:** scale bar = 23.2μm; **28 H:** scale bar = 5.5μm
2.3.3.4. RESPONSE OF THE EPICUTICULAR WAX LAYER OF *PANICUM DECIPiens* (C₃/C₄) TO INCREASED CO₂ CONCENTRATIONS

**Figure 29-32:** Scanning electron micrographs of the epidermal surface of the last fully expanded leaf of the main tiller of *Panicum decipiens* (C₃/C₄). (29 A,B) Abaxial epidermis of the last fully expanded leaf grown under 350 ppm CO₂; 29 A: scale bar = 23.2 μm; 29 B: scale bar = 5.5 μm. (30 C,D) Abaxial epidermis of the last fully expanded leaf grown under 900 ppm CO₂; 30 C: scale bar = 23.2 μm; 30 D: scale bar = 5.5 μm. (31 E,F) Adaxial epidermis of the last fully expanded leaf grown under 350 ppm CO₂; 31 E: scale bar = 23.2 μm; 31 F: scale bar = 5.5 μm. (32 G,H) Adaxial epidermis of the last fully expanded leaf grown under ambient 900 ppm CO₂; 32 G: scale bar = 23.2 μm; 32 H: scale bar = 5.5 μm
2.3.4. QUANTITATIVE RESULTS: EXTRACTION AND QUANTIFICATION OF WAX COMPONENTS / THIN LAYER CHROMATOGRAPHY

The method used in this study successfully removed leaf waxes. This was revealed under the SEM: Leaf samples, which had been treated with the extracting solvent showed no signs of the glaucousness visible in the untreated leaves. However, the thin-layer chromatography method unfortunately appeared to be not sufficiently sensitive. The leaf sample size would have needed to be much larger to allow this method to work. This was not possible due to a restricted supply of material. Other methods such as electrophoresis and low-resolution magnetic resonance, which might permit success in quantification of the epicuticular waxes even in very small quantities, were unavailable to the researcher.
<table>
<thead>
<tr>
<th>species/concentration</th>
<th>TOTAL DRY WEIGHT (g)</th>
<th>LEAF WEIGHT (g)</th>
<th>LEAF AREA (cm²)</th>
<th>STEM WEIGHT (g)</th>
<th>STEM HEIGHT (cm)</th>
<th>TILLER FREQUENCIES</th>
<th>NUMBERS OF INTERNODES</th>
<th>STOMATAL FREQUENCIES (mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P.ant. 350 ppm CO₂</td>
<td>8.13</td>
<td>3.2</td>
<td>804.74</td>
<td>5.1</td>
<td>44.1</td>
<td>5.2</td>
<td>7.5</td>
<td>121.76</td>
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<tr>
<td>P.ant. 350 ppm CO₂</td>
<td>14.6</td>
<td>5.1</td>
<td>997.71</td>
<td>9.6</td>
<td>58.67</td>
<td>5.79</td>
<td>6.5</td>
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<tr>
<td>P.tric. 350 ppm CO₂</td>
<td>0.98</td>
<td>0.59</td>
<td>228.09</td>
<td>0.38</td>
<td>34.6</td>
<td>3.0</td>
<td>7.6</td>
<td>216</td>
</tr>
<tr>
<td>P.tric. 900 ppm CO₂</td>
<td>4.3</td>
<td>2.65</td>
<td>903.73</td>
<td>1.7</td>
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<td>8.7</td>
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<tr>
<td>P.dec. 350 ppm CO₂</td>
<td>1.61</td>
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<td>22.65</td>
<td>3.5</td>
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<td>124</td>
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<td>P.dec. 900 ppm CO₂</td>
<td>3.97</td>
<td>1.8</td>
<td>352.09</td>
<td>2.16</td>
<td>32.9</td>
<td>5</td>
<td>5.1</td>
<td>166.6</td>
</tr>
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</table>

**TABLE 2:** Data represent average values of morphological parameters of *Panicum* species grown under ambient (350 ppm) and elevated carbon dioxide (900 ppm).
<table>
<thead>
<tr>
<th>SPECIES</th>
<th>TOTAL DRY WEIGHT</th>
<th>LEAF WEIGHT</th>
<th>LEAF AREA</th>
<th>STEM WEIGHT</th>
<th>STEM HEIGHT</th>
<th>TILLER FREQUENCIES</th>
<th>NUMBERS OF INTERNODES</th>
<th>STOMATAL FREQUENCIES</th>
</tr>
</thead>
<tbody>
<tr>
<td>P.antidotale (C4)</td>
<td>1.79</td>
<td>1.59</td>
<td>1.24</td>
<td>1.88</td>
<td>1.33</td>
<td>1.09</td>
<td>0.86</td>
<td>1.28</td>
</tr>
<tr>
<td>P.tricanthum (C3)</td>
<td>4.3</td>
<td>4.49</td>
<td>3.96</td>
<td>4.47</td>
<td>1.29</td>
<td>1.40</td>
<td>1.14</td>
<td>0.78</td>
</tr>
<tr>
<td>P.decipiens (C3/C4)</td>
<td>2.46</td>
<td>2</td>
<td>1.27</td>
<td>3.18</td>
<td>1.45</td>
<td>1.42</td>
<td>1.27</td>
<td>1.34</td>
</tr>
</tbody>
</table>

**TABLE 3:** PROPORTION OF INCREASE OF MORPHOLOGICAL PARAMETERS OF THREE *PANICUM* SPECIES IN RESPONSE TO ELEVATED CARBON DIOXIDE (900 ppm : 350 ppm)
2.4. DESCRIPTION OF RESULTS AND DISCUSSION

2.4.1. GENERAL MORPHOLOGICAL OBSERVATIONS:

All three species of *Panicum* investigated show a significant response to elevated carbon dioxide concentrations in all morphological parameters used in this study (see figures 1-14). These results confirm the results obtained in other C₃ plants and also indicate that monocotyledonous perennial plants with the C₄ and C₃/ C₄ pathway may respond more positively with increases in biomass production to elevated carbon dioxide concentrations than generally considered (for review see Kimball, 1983; Cure and Acock, 1986 and Poorter, 1993). The C₃/ C₄ *Panicum decipiens* showed highly significant responses which correlated with the responses of the C₃ as well as the C₄ plants. This result for the first time gives evidence that intermediate monocotyledonous plants may not necessarily be intermediate either in a physiological or in an evolutionary sense under an environmental pressure such as high CO₂. Instead, they may be able to act according to the circumstances with responses similar to that of C₃ or the C₄ plants, as the results obtained from this thesis strongly suggest.

Increases in stiffness and sharpness of the grass leaves grown at high CO₂ were also qualitatively observed. These may have been caused by the increased deposition of silica, or alternatively by a production of calcium oxalate, which could be used as a form of carbon storage within the leaf cells. Calcium oxalate crystals are thought to play a role in plant protection, have a bitter taste and lower digestibility and attractiveness of the feeding plant.
2.4.2. SPECIFIC MORPHOLOGICAL OBSERVATIONS:

2.4.2.1. LEAF AREA:

Leaf area seemed is commonly increased under high carbon-dioxide concentrations, particularly in C₃ plants, because their photosynthetic pathway allows a greater accumulation of dry weight at elevated carbon dioxide concentrations since less carbon is dissipated via photorespiration (Tolley and Strain, 1984). Some C₄ grass species like maize, which do not lose carbon through photorespiration, also showed an increased leaf area when grown at high carbon dioxide concentrations (Gifford, 1988; Rogers and Dahlman, 1993). This was likewise found in the C₄ and C₃/C₄ Panicum species investigated in this study (see figure7; table 2). In line with other studies the C₃ plant Panicum tricanthum showed the highest increase in leaf area (see table 2; table 3).

All three Panicum grasses investigated displayed an increase of leaf area (see figure 7), responding highly significantly to the treatment ambient versus high (900ppm) CO₂. The response of the intermediate Panicum decipiens regarding leaf area was close to the C₄ plant (see table 3 and table 7).

Many researchers have found that leaf area is likely to increase in total and in rate of development in plants developing under high CO₂ concentrations (Ford and Thorne, 1967; Hofra and Hesketh, 1975; Imai and Murata, 1976; Wong, 1980; Sionit et al., 1981; Sionit, 1983; Rogers and Gifford, 1984; Morison and Gifford, 1984). This increase is often proportionally less than the increase in leaf dry weight, thus the leaf area to leaf dry weight ratio, the specific leaf area (SLA), decreases. Both Panicum decipiens and Panicum antidotale conformed to these previous findings and showed a higher increase in leaf weight in comparison to leaf area (see figures 7 and 8; table 2), whereas the C₃ Panicum tricanthum (see figures 7 and 8; table 2) gained a slightly higher proportion of leaf area than leaf weight. Exceptions to
general patterns have also been noted for wheat and maize (Sionit et al., 1981; Rogers et al., 1983).

Decreasing SLA is strongly associated with increasing starch and sugar accumulation in leaves (Hofstra and Hesketh, 1975). The change in SLA is generally caused by the increase in carbohydrates stored in the leaves in some plants, but not in others. All three Panicum species displayed a highly significant increase of starch, i.e. non-structural carbohydrate amount within their leaves (chapter four, figure 47). This result contributed to the increase in leaf weight (figure 8), which occurred with only minor adjustments in leaf thickness (figure 40).

The development of leaf area is dependent on cell division (important for the first steps of leaf development) and cell elongation, which is important for the further development of the leaf. Cell division seems to be especially sensitive to a change in carbohydrate-gain and use and the general ionic environment, and is also closely connected with water availability (Kriedemann, 1986). If a water deficit, as observed in Hordeum vulgare and Helianthus annuus (Delane et al., 1982; Munns Weir, 1981) causes an inhibition in the growth of leaf area, an increased water supply might be anticipated to increase the leaf area. The provision of an adequate nutrient supply for the Panicum grasses (chapter 2), combined with the thickened epicuticular wax layer (see figures 22 C/D; 24 G/H; 26 C/D; 28 G,H; 29 A/B; 32 G/H) and the closure of stomata occurring under high carbon dioxide could therefore have enhanced the water use efficiency and thus the development and increase of leaf area. This larger leaf area itself could be a factor enhancing the water use efficiency because of the correlated increase in transpirational area.

The results confirmed that even slight gains in the rate of leaf production will significantly increase the photosynthetic area (Baker and Enoch, 1983), because the larger leaf area allows increased net photosynthesis. This becomes compounded in turn into significantly more leaf area per plant in some species (Sionit, 1983; Tolley and Strain, 1984), but not in others (Tolley and Strain, 1984).
Higher photosynthetic rates, evidenced by gains in dry matter (figure 9), has been available to support leaf growth, as confirmed for all three *Panicum* species. The leaf area would have increased due to increased osmotic potential which, in turn is responsible for cell expansion in the elongation zone as stated by Munns and Weir (1981).

This study suggests that the increase in leaf area might be primarily due to young developing leaves initially being the most active sinks and benefitting from CO₂ enrichment of the older, source leaves. This was also found by Kramer (1981) and Porter and Grodzinski (1985), who found that during a period of several weeks of high CO₂ enrichment, leaves often accumulated more starch than they needed to export to sustain the rapid development of sinks, and therefore displayed an enhanced leaf area.

The enhanced leaf area might be a problem if the evaporative leaf area increases more than the absorptive root area. In the event of this problem, water stress would also occur earlier and become more extreme (Baker and Martin, 1967). However, the plants did not show any signs of water stress and I am therefore confident that the leaf area did not increase beyond the capacity of the root system, and the plants did not show any signs of water stress. Gifford (1979) and Sionit et al. (1981) found that the net effect of increased leaf area with decreased stomatal conductance improved the water status of wheat. Tolley and Strain (1984) likewise found that the net effect of high CO₂ concentrations on *Liquidambar styraciflua* and *Pinus taeda* was to improve the water status for both species.

In conclusion, the greater leaf area (figure 7) obtained by the *Panicum* grasses grown under high CO₂ has evidently led to increased light interception and higher water use efficiency. This was demonstrated by increased growth measured as the increments in plant dry matter (2.4.4., figure 9), tiller production (2.4.5, figure 12), internode production (2.4.5, figure 13), stem height (2.4.5, figure 10), stem weight (2.4.5, figure 11), and leaf weight (2.4.3, figure 8).
2.4.2.2. LEAF WEIGHT:

Increases in leaf weight in response to high carbon dioxide were thought to be an indicator for leaf thickness, which is often correlated with leaf starch content (Wulff and Strain, 1982). In this study this hypothesis was not supported for all species investigated. *Panicum tricanthum* shows an increase in leaf area (figure 7) as well as in leaf thickness (figure 40, chapter 3) and starch (figure 47, chapter 4). But in *Panicum decipiens* and *Panicum antidotale* (figure 8 and figure 40, chapter 3) the individual increases in leaf weight were accompanied by decreases in leaf thickness, even though they also display a large increment in the amount of starch (see figure 47, chapter 4). Therefore leaf thickness seems not to be a reliable parameter for the assessment of leaf starch content.

The leaf starch content increased significantly in all three species (see figure 47, chapter 4) parallel to the increase in leaf area (see figure 7), which results in increased dry matter production (see figure 9) and thus increased leaf weight (see figure 8). Increased leaf weight was found to increase leaf dry matter digestibility (Wilson et al. 1983), a factor not assessed in this study. However, the increase of leaf weight and plant dry matter observed in this study did suggest a general increase in DMD, mainly through the build up of starch, which contributes to the weight gain. This result makes the genus *Panicum* grown under high CO₂ concentrations a more attractive forage grass for future agronomic projects, i.e. global climates.
2.4.2.3. PLANT DRY MATTER (TOTAL DRY WEIGHT):

All three Panicum species showed a marked significant response in plant dry matter production under high CO₂ (see figure 9), the intermediate plant responding closely to the C₄ P. antidotale. The responses of all three species have involved a massive starch accumulation found within the leaves (see figure 47, chapter 4). This suggests again that many other monocotyledonous plants grown under high CO₂ might respond more strongly to elevated carbon dioxide concentrations, which can contribute to an increase of leaf starch content. This would confirm the findings of Madore and Grodzinski (1985) who investigated cucumbers, and of Servaites et al. (1989a; b;) and Geiger and Fondy (1991) who stated that source leaves accumulate large foliar carbohydrate pools to buffer variations in the rate of photosynthesis and to liberate a sufficient supply of sucrose at night. This would ensure a more or less continous supply of sucrose, which constitutes the major source of energy, for sink organs in many plant species.

Improvement in plant dry matter production is thought to be largely due to a permanent increase in photosynthetic efficiency, as a result of the reduced competitive effects on ribulose bisphosphate carboxylase-oxygenase under high carbon dioxide (Tolbert, 1980; Ingvardsen and Veierskov, 1994). This would explain the greatest response of the C₃ plant P. tricanthum to high carbon dioxide (see table 2). In the C₄ and C₃/C₄ plants the protective shield of the bundle sheath might not be as impermeable to external carbon dioxide as thought and might allow high CO₂ to leak through as suggested by Rathnam and Chollet (1978; 1980).

It is likely that the increase in biomass production is also due to greater leaf area as in Glycine max, where plant dry matter was increased between 24% - 74% (Patterson and Flint, 1980). Significant increases in leaf area were recorded for all Panicum grasses used in this study (see figure 7).
Another important carbon-budget component is respiration, which can release up to 50% of the carbon fixed in photosynthesis (Amthor, 1991). Because plants seem to grow larger and at higher rates in CO₂ enriched environments, one might anticipate higher dark respiration rates under such conditions (Amthor, 1991; Farrer and Williams, 1991), but reports of either no change or a reduction in dark respiration with high CO₂ are more prevalent (Reuveni and Gale, 1985; Bunce, 1990; Amthor, 1991). Sometimes the basis on which respiration rate is expressed can affect the interpretation of whether alterations have been imposed by elevated carbon dioxide.

2.4.2.4. STEM HEIGHT AND WEIGHT; INTERNODES AND TILLERS:

The Panicum species used in this study show a significant increase in the parameters of stem weight and height, numbers of tillers, and numbers of internodes under high CO₂ (see figures 10-13) compared with grasses grown under ambient CO₂ concentrations.

The increased stem growth under high CO₂ often results in a moderate increase in stem height (Sionit et al., 1985) or in case of the Tradescantia, internode length was twice as long in plants grown under elevated CO₂ (Boetsch et al., 1996). The increase in stem height is proportionally less than the increase in dry weight, thus stem dry weight per unit length increases (Ford and Thorne, 1967; Sionit et al., 1981; Rogers et al., 1983). This was confirmed for the three Panicum species (see table 2). The greater plant height under elevated CO₂ treatment was caused by enhanced stem elongation (internode length). The increased number of internodes then, in turn, caused the increase in stem weight.

Enhanced tillering (branching) in high CO₂ concentrations was observed in all Panicum species investigated (see figure 12) and has been reported for some monocots like barley, rice, wheat (Ford and Thorne, 1967; Gifford, 1977; Imai and Murata, 1978; 1980; Sionit et al., 1981; Sionit, 19820).
Branching has been stimulated in dicots such as soybean (Rogers et al., 1984). In addition, an increase in the rate of node formation was observed (Andersen, 1976; Paez et al., 1983). However, this effect varies with species. *L. styraciflua* produced more branches when grown under high CO₂, but *P. taeda* did not (Rogers et al., 1983).

As the leaf area (see figure 7) and the number of tillers (see figure 12) increase in response to elevated CO₂ the total number of leaves per plant increases, which may also affect light penetration of the plant. The distribution of radiation received at any given concentrations will change, i.e. lower plant parts could suffer from shading, whereas upper parts, for instance, would receive too much radiation. An increase in tillering can occur even when there is a small increase in photosynthetic rates and growth rates, and is apparently connected with an increased carbohydrate supply, which activates meristem production and development leading again to the appearance of tillers. All three *Panicum* grasses (see chapter 4, figure 47) displayed a highly significant increase in non-structural carbohydrate content (starch) within the leaf under high carbon dioxide in concert with increased tillering. The increase in the number of tillers did not have any apparent shading effect because the foliage showed a normal colour and development on all parts of the plants, even though the plants grown under high CO₂ appeared denser and leafier (see figures 1-6). A shading effect may apply to bigger plants than grasses like trees and shrubs with more dense branching and foliage. The increased tillering and node formation (see figures 12-13), together with the increased leaf area observed (see figure 7 and) could enable these *Panicum* grasses to exploit the space available to them more rapidly. They could then intercept more sunlight and grow even more rapidly.
2.4.2.5. RESPONSE OF STOMATA TO INCREASED CO₂ CONCENTRATIONS:

2.4.2.5.A STOMATAL FREQUENCIES (DENSITIES):

The waxy leaf cuticle and the layer of epidermal cells are formidable barriers to both water loss and CO₂ entry. The exchange of gases is facilitated by the stomata in the epidermis, which are a high conductance pathway for CO₂ influx.

Many previous studies have shown that leaf development and stomatal characteristics are sensitive to changes in environmental factors such as CO₂ concentration, irradiance, moisture content, temperature, humidity and soil moisture content (Ticha et al., 1985; Woodward, 1990; Morison, 1993). Several workers have shown that increases in atmospheric carbon dioxide from 280 - 1000 ppm have markedly reduced stomatal frequencies in various species (Madsen, 1973; Woodward, 1987; Woodward and Bazzaz, 1988; Miglietta and Raschi, 1993; Clifford et al., 1995), and North et al. (1995) explained this in terms of an increase in epidermal cells. Reduced stomatal frequencies also seemed to exhibit reduced stomatal diffusive conductance (DeLucia et al., 1985; Williams et al., 1986).

Doubt has been cast on these findings. Koerner (1988) suggested that experimental error rate might preclude meaningful interpretation of the data. Other species, such as the sensitive maple (Acer pseudoplatanus), leaves of herbaceous plants and Tradescantia leaves did not show any alteration in their stomatal frequencies when grown under carbon dioxide concentrations above 350 ppm CO₂ (Woodward, 1987; Woodward and Bazzaz 1988, Boetsch et al., 1996). A survey of stomatal frequencies in mountain and lowland plants sampled recently and at the beginning of the century revealed no difference, further supporting the view that downward photosynthetic adjustments to increased CO₂ may not as pronounced in the field (Koerner, 1988). But even within an ecological community, species respond differently
to increases in this atmospheric gas (Knapp et al. 1994). It is therefore impossible to find a rule for stomatal behaviour under high carbon dioxide concentrations and this dilemma has been well reviewed by Murray (1995).

For all three *Panicum* species the stomata were amphistomatomous. *P. antidotale* and *P. decipiens* exhibited a statistically significant increase of stomatal density on the abaxial surface under elevated CO$_2$ concentrations, whereas *P. tricanthum* showed a significant decrease in stomatal frequencies (see figure 14). The increase in stomatal frequencies may be attributed to an increase in the proportion of epidermal tissues (see chapter 3, figure 36) and therefore epidermal cell numbers, and has not been found in any other C$_4$ grasses. A decrease of stomatal frequencies is ascribed to overall larger leaves owing to formation of bigger cells under high carbon dioxide, as stated by several authors, and is mainly found in C$_3$ plants (Bristow, 1969; Madsen, 1973; Woodward, 1987). If this is the case, the results shown in figure 14 suggest that the C$_3$/C$_4$ *P. decipiens* anatomically responds like a C$_4$ plant when grown under high carbon dioxide concentrations.

The higher stomatal frequencies recorded in this study (see figure 14) could affect water usage and enhance transpiration/evaporation and would allow more gases, i.e. carbon dioxide, to enter the leaf. It could also be possible that through the stomatal narrowing which occurs under elevated carbon dioxide that growth could be enhanced. This again confirms the hypothesis that the bundle sheath in C$_4$ grasses allows a certain percentage of carbon dioxide to enter the Calvin cycle immediately.

A lower stomatal density as in the C$_3$ *P. tricanthum* would enhance water use efficiency, because fewer routes might exist for the escape of water vapor from the leaf. On the other hand, if high carbon dioxide forces the stomata to close, the transpiration rate would be lowered and the plant still would have an advantage over a plant grown at ambient conditions. Therefore the question arises as to whether an increase of stomatal density in response to
high carbon dioxide concentrations has an adverse effect on the improved water use efficiency.

2.4.2.5.B STOMATAL SIZE:

Stomatal pore size is regulated by guard cells that control the diameter of their aperture by changes in their turgor. Thus, CO₂ and water loss are regulated by the frequency of stomata (stomatal density) and their degree of openness. Stomata are known to close rapidly within a few minutes of exposure to high CO₂ (Raschke, 1975; Madore and Groszinki, 1985; Mott, 1990), but it is unknown if they close uniformly over the entire leaf surface (Downton, 1988).

A large reduction in stomatal conductance as shown by Morison (1987) was attributed to the direct effect of elevated CO₂ resulting in reduced stomatal aperture, or its indirect effects in reducing guard cell length (and hence again stomatal aperture), as it has been reported for Quercus pubescens (Miglietta and Raschi, 1993).

Very few authors have drawn attention to guard cell size. Thomas and Harvey (1983) found no alteration in the length/width ratios of stomatal pores of corn, sweetgum and soybean grown under 520, 718 and 910 ppm CO₂. The Panicum species used in this study show no significant change in stomatal size (see table1), although there are differences recorded for the stomatal length in P. decipiens (decrease) and the stomatal width in P. tricanthum (decrease), both grown under high carbon dioxide (see table1). The magnitudes of these changes are not large, and it is difficult to envisage their having a major effect on water use efficiency under elevated carbon dioxide.
2.5. SCANNING ELECTRON MICROSCOPY

2.5.1. CO₂ EFFECTS ON THE EPICUTICULAR WAX LAYER: QUALITATIVE INVESTIGATIONS:

The three Panicum species each exhibited different pattern of wax bloom or glaucousness (see figures 21-32). Thus rapid application of the LTSEM method applied in this study would make it possible to use such characteristic ultrastructural features of the leaf surface as part of a plant taxonomic key in botany.

P. antidotale showed qualitatively a high degree of glaucousness on both leaf surfaces. The epicuticular structure appeared with dense aggregate coatings of various combinations of minute crystalline deposits and thin plates, which varied in shape. The crystalline deposits were ubiquitous (see figures 21 and 22). They were present all over the interveinal groove and covered the guard cells (see figure 21 A,B) and bulliform cells (not shown). Abaxial leaf surfaces of plants grown under 900 ppm carbon dioxide exhibited thicker plates (see figure 22 C,D) than the abaxial leaf surfaces of plants grown in ambient concentrations (see figure 21). The adaxial side of the epidermis of leaves grown under high carbon dioxide showed a much denser aggregation of plates and crystalline deposits than the abaxial epidermal surfaces of plants grown under ambient conditions (see figures 23 E,F and 24 G,H).

Amorphous wax was present on the abaxial leaf surfaces of P. tricanthum grown under both, ambient and elevated carbon dioxide, but the amount of glaucousness appeared to be increased somewhat under high CO₂ (see figures 25 A,B and 26C,D). The adaxial leaf surface showed a dense aggregation of plates and crystalline deposits similar to those in P. antidotale and the deposition of the epicuticular wax increased perceptibly under high
CO₂ in comparison with the ambient concentration (see figures 27 E,F and 28 G,H).

The abaxial surface of *P. decipiens* exhibited a very low amount of amorphous epicuticular wax under ambient CO₂ concentrations, which increased particularly on the ridges under elevated carbon dioxide concentrations (see figure 29 A,B and 30 C,D). Like *P. antidotale*, the adaxial leaf surface showed a dense aggregate coating of various combinations of minute crystalline deposits and plates, which appeared to become thicker and more defined on leaves grown under elevated CO₂ (see figure 31G,H and 32 E,F).

The wax plates observed are quite common on the foliage of higher plants (Jeffree et al., 1976). The presence of plates creates a larger contact angle for water drops on leaves. Furthermore, it was shown that the presence of large wax plates was associated with drought tolerance in Lehmann lovegrass lines (Johnson et al., 1983). This mechanism allows the leaf surface to intercept, collect and channel water from diffusive sources such as fog. Drops are therefore less likely to roll across the leaf and stem, to the radicular area (Juniper and Jeffree, 1983). These three *Panicum* species were not grown under drought conditions, but the appearance of crystalline plates might be an adaptation to the tropical conditions, often connected with occasional water stress, in which they naturally occur.

The increase of amorphous waxes as shown for *P. tricanthum* and *P. decipiens* (see figure 25 A,B and 26 C,D and figure 29 A,B and 30 C,D) might be due to greater quantities of oily waxes governed by the proportion of unsaturated, short chain fatty acids (Juniper and Jeffree, 1983). These might be produced in higher quantities when *Panicum* grasses are grown in elevated carbon dioxide concentrations. The ‘stressor’ carbon dioxide can clearly produce a preferential accumulation of epicuticular waxes on the leaf surface.
The increase in the density and/or thickness of the platelike structures on the cuticle of leaves grown under high carbon dioxide in comparison to leaves developed under ambient concentrations (seen figures 23-24, 27-28 and 31-32), could be explained with a cuticular response of the grass plants to a general environmental stressor such as drought or, as in this study, elevated carbon dioxide. The increase in thickness enhances the protective function of the wax layer and would increase the water use efficiency by reducing non-stomatal transpiration.

An increased deposition of epicuticular waxes under high CO₂ probably causes improved water use efficiency by decreasing that component of transpiration generally attributed to cuticular water loss through transpiration/evaporation (van Volkenburgh and Davies, 1977; Thomas and Harvey, 1983). Soybean and sweetgum, grown under 910 ppm CO₂, showed a tendency for increased epicuticular wax deposition when observed by SEM (Thomas and Harvey, 1983), whereas Zea mays did not show any changes in the epicuticular wax deposition under high carbon dioxide. A more recent observation was a 30% thicker cuticle in Opuntia ficus-indica with more epicuticular wax under doubled carbon dioxide concentrations (North et al., 1995). To the best of my knowledge, the results obtained in this study showing an increase of epicuticular waxes in response to elevated carbon dioxide levels in all three Panicum species with different physiological pathways, are the first on monocotyledonous perennial plants.

The increase of epicuticular waxes as observed in all three Panicum species can influence the optical transparancy. The waxes scatter the light, by cellular inclusions that absorb and scatter light and by phenolic compounds such as flavonoids and anthocyanins which absorb and thus attenuate the light, which then does not reach the mesophyll and is more diffused (Vogelmann et al., 1996). This will be of protective nature in a time where ozone gaps increasingly become bigger and UV radiation has to be filtered to prevent damage and thus offering a adaptive property appropriate to a
changing climate. This could especially concern the stomatal guard cells, as recently investigated by Grammatikopoulos et al. (1994).

Another point connected with the influence of increased deposition of epicuticular waxes regarding changes in the optical transparency is of developmental interest and may lead to a decrease in photosynthetic activity observed in plants under long-term exposure to high carbon dioxide concentrations.

An important function of epicuticular waxes is to increase the plant's retention of water by reducing water loss after stomatal closure. Indeed, plant survival during severe water deficits depends, amongst other things, on the ability to restrict water loss through the leaf epidermis after the stomata close. Non-stomataly -controlled water loss through the epidermis (loss through cuticle plus loss due to incomplete stomatal closure) may comprise up to 50% of total transpiration in water stressed wheat plants during the day and 100% during the night (Rawson and Clarke, 1988). Decreased diffusive conductance of water vapour through the cuticle comes from decreased cuticular permeability and thickening of the boundary layer. Permeability of the cuticle to water is affected by the amount, composition and physical configuration of the cuticular wax deposits (Blum, 1982). Another important role of epicuticular waxes is in the enhancement of leaf reflectance in both the visible and near infrared wavelength (Blum, 1971a; 1975b; Johnson et al., 1983), which in turn may reduce transpiration.

The results obtained for the Panicum species investigated suggest in conclusion that some of the extra carbon gained is converted into waxes that will increase water use efficiency and leaf reflectance, enhancing protection against predators and ultraviolet radiation. The other hand, a waxy and thick cuticle lowers the dry matter digestibility by hindering the tissue digestion. The formation of epicuticular waxes seems an appropriate adaptation to a changing environment.
2.6. **SUMMARY:**

(i) C\textsubscript{3}, C\textsubscript{4} and C\textsubscript{3}/C\textsubscript{4} *Panicum* species investigated in this study show overall a significant morphological response to elevated carbon dioxide concentrations.

(ii) C\textsubscript{3}, C\textsubscript{4} and C\textsubscript{3}/C\textsubscript{4} *Panicum* species investigated in this study displayed a significant increase in leaf area when grown under high carbon dioxide. This was discussed to be caused by enhanced water use efficiency due to partial closure of stomata and possibly also to an increment in epicuticular waxes, that would have reduced non-stomatal transpiration.

(iii) significant increase of plant dry matter was recorded for the C\textsubscript{3}, C\textsubscript{4} and C\textsubscript{3}/C\textsubscript{4} *Panicum* species, an indicator that monocotyledonous plants can respond as strongly to elevated carbon dioxide as dicotyledonous plants. The increment was considered to being due to greater leaf area, which allows increased light interception and therefore increased photosynthetic efficiency. A significant component of the increase in dry matter is due to massive starch accumulation within the leaves as observed in this study, which suggests an improved dry matter digestibility for the grasses grown under high carbon dioxide.

(iv) The total leaf weight increased proportionally with the increase in plant dry matter and it is proposed that this increase is due to an accumulation of starch within the leaves as observed in all *Panicum* species investigated (see chapter 4); this result could be correlated with an increase in leaf thickness in *P. tricanthum*, but not in *P. antidotale* and *P. decipiens*. 
(v) The significant increase in stem height or length is due to an accompanying increase in internode length (stem height) and numbers of internodes. Both increments lead to significant increase of stem weight. The stem weight is also increased by the significant increase in the number of tillers. All these increments are related to an increase in the photosynthetic efficiency and increased carbon supply (see chapter 4).

(vi) Stomatal frequencies for the abaxial leaf surface were found to increase in *P. antidotale* and *P. decipiens*. This result was attributed to an increase in the proportion of epidermal tissues probably due to an increase in epidermal cell number. In *P. tricanthum* the stomatal frequencies decreased. This was explained by the formation of larger leaves and also increased epidermal tissue area, owing to the formation of bigger cells. The latter would eventually increase the water use efficiency by decreasing the transpirational area. Stomatal sizes did not change appreciably under elevated carbon dioxide concentrations.

(vii) All *Panicum* species show an increase in glaucousness (epicuticular wax bloom) when grown under elevated CO₂ and also an increase in epicuticular wax deposition when grown under 900 ppm carbon dioxide. Although none of the following points were investigated it is possible that the increase in epicuticular waxes could:

1) reduce non-stomatal transpiration
2) enhance the boundary layer for diffusion to/from stomata
3) render light penetrating the epidermis diffuse rather than focused
4) reduce UV-B penetration, according to content of UV-absorbing (phenolic) compounds
5) might reduce dry matter digestibility, thereby leading to appropriate adaptations for a more stressfull environment.
CHAPTER 3

ANATOMICAL:

EFFECTS OF HIGH CARBON DIOXIDE CONCENTRATIONS ON THE ANATOMICAL STRUCTURE OF PANICUM GRASS SPECIES WITH DIFFERENT PHOTOSYNTHETIC PATHWAYS

3.1. INTRODUCTION

One aim of this study was to obtain anatomical information on the Panicum species grown under elevated CO₂ (900ppm) and under ambient concentrations and to compare the outcome of these investigations with the findings from the morphological part of this study. A schedule for preparation of all species used for lightmicroscopical viewing had to be developed to allow the application of Image Graphics Analysis. Another important aim was the development of a fixation/dehydration and embedding system which could also be used for further transmission electron microscope studies.
3.2. MATERIAL AND METHODS

3.2.1. SAMPLING AND PREPARATION OF PANICUM LEAF SPECIMEN FOR THE LIGHT MICROSCOPE

The penultimate fully expanded leaf blade was harvested for analysis from fully developed main tillers of the Panicum material described in chapter 2 (see 2.2.1). As marker for the respective date of full expansion served ligule appearance, which has been found from past experience to coincide with the attainment of full expansion of the leaf blade.

For the purpose analysis of tissue anatomy a 3 mm wide piece was cut from the midpoint of two or three leaves per replicate and fixed in 3% (v/v) glutaraldehyde (0.025 M phosphate buffer), dehydrated in a graded ethanol or acetone series and embedded in Spurr’s low viscosity embedding medium (Spurr, 1969).

Sections (1μm) were cut using a glassknife on a UCUt microtome. The sections were then stained in 0.6% toluidine blue.

The sections were photographed using a Zeiss Orthomat light microscope onto a mirror and then onto the digitizer of a Dedicated Image Analyzer Noran TN/8502. Magnifications were calibrated using a stage micrometer.

The cross-sectional area of each type of tissue was obtained over a half leaf width (midpoint of midrib to leaf margin) and expressed as a percentage of the total cross sectional area of all tissues. Bundle sheath areas and vascular bundle areas were determined from a portion of the transverse section embracing three major vascular bundles on each side of the midrib. The midrib vascular bundle/bundle sheath was included. The epidermal cell area represents the average data from upper and lower epidermis. Mean sclerenchymatic tissue area was measured from the groups of fibres below each measured major vascular bundle. True mesophyll (spongy and palisade
mesophyll) was not distinguished and the tissue was thus classified as parenchyma. The bulliform cell and mesophyll cell areas were determined individually by selecting 5 cells within the field of view, measuring their area, repeating this 5 times in other areas within the selected tissue.

3.2.2. IMAGE GRAPHICS ANALYSIS:

The proportion of mesophyll, epidermis, sclerenchyma, bundle sheath, vascular tissue were expressed as a percentage of total tranverse sectional area (mm²) of the three different Panicum species. Two files were chosen (species/concentration), containing up to twelve frames (images) which contained six different phases (parameters chosen) each. For the bulliform and mesophyll cell area the files species/concentration were chosen, containing now five frames with two phases (parameters). The parameters used to calculate the cell areas were the ‘projected X’, which represents the tangential dimension or length (μm) of cells, and the ‘projected Y’, which represents the radial dimension or cell height. The leaf thickness was measured calculating the distance (mm) between upper and lower epidermis of the leaves of the three Panicum species at three different locations. Data were transferred to an Apple Macintosh II si Computer. Analysis of variance was obtained using the JMP procedure of the SAS program Version III (SAS Inst. Inc., 1994, USA).

Significance of the difference among means of all groups was reported by the ‘F’ statistic in the analysis of variance. No statistical comparison was made over different sampling dates and standard errors were calculated for each harvest.
3.3 RESULTS

3.3.1. LIGHT MICROSCOPY: ANATOMICAL FEATURES OF PANICUM

The general anatomical structure was not qualitatively changed in the Panicum species investigated in this study (see figures 33-35) at high CO₂.
Figure 33 A: Transverse section of the last fully expanded leaf of the main tiller of *Panicum triscanthum* (C3): Scale bar = 55.2 μm

Figure 33 B: Transverse section of the last fully expanded leaf of the main tiller of *Panicum triscanthum* (C3): m (mesophyll); bs (bundle sheath); ve (vascular elements). Scale bar = 28.4μm
Figure 34 A: Transverse sections of the last fully expanded leaf of the main tiller of *Panicum antidotale* (C₄). Scale bar = 55.2 μm

Figure 34 B: Transverse sections of the last fully expanded leaf of the main tiller of *Panicum antidotale* (C₄): m (mesophyll); bs (bundle sheath); ep (epidermis). Scale bar = 28.4 μm
Figure 35 A: Transverse sections of the last fully expanded leaf of the main tiller of *Panicum decipiens* (C₃/C₄). Scale bar = 55.2 μm

Figure 35 B: Transverse sections of the last fully expanded leaf of the main tiller of *Panicum decipiens* (C₃/C₄): ve (vascular elements); m (mesophyll); bs (bundle sheath). Scale bar = 28.4μm
3.3.2. GRAPHICS IMAGE ANALYSIS: EFFECTS OF HIGH CARBON DIOXIDE ON THE LEAF TISSUE AND CELL AREAS OF *PANICUM* SPECIES
**Figure 36**: Effect of high CO$_2$ (900 ppm) on the proportion of epidermal tissue area of *Panicum* leaves. *Panicum decipiens* (C$_3$/C$_4$; Prob > $t = 0.9$), *Panicum triscanthum* (C$_3$; Prob > $t = 0.9$) and *Panicum antidotale* (C$_4$; Prob > $t = 0$) showed an increase in the proportion of epidermal tissue.
Figure 37: Effect of high CO\textsubscript{2} (900 ppm) on the epidermal bulliform cell area of *Panicum decipiens* (*C\textsubscript{3}/*C\textsubscript{4}; Prob > t = 0.00), *Panicum tricantum* (*C\textsubscript{3}; Prob > t = 0.375). The bulliform cell area increased substantially in *P. decipiens*, but showed a trend of decrease in *P. tricantum*. 
Figure 38: Effect of high CO$_2$ (900 ppm) on the proportion of the mesophyll tissue area of *Panicum* leaves. *Panicum decipiens* (C$_3$/C$_4$; Prob > t = 0.5) and *Panicum antidotale* (C$_4$; Prob > t = 0.00) displayed a decrease in mesophyll tissue area, whereas *Panicum tricanthum* (C$_3$; Prob > t = 0.3) exhibited an increase.
Figure 39: Effect of high CO$_2$ (900 ppm) on the mesophyll cell area of

*Panicum* leaves. *P. decipiens* (C$_3$/C$_4$; Prob > t = 0.2), *Panicum tricanthum* (C$_3$; Prob > t = 0.02) and *Panicum antidotale* (C$_4$; Prob > t = 0.03).
LEAF THICKNESS


cO2
350 (ppm)
900 (ppm)

LEAF THICKNESS (mm)

P. antidotale P. decipiens P. tricanthum

PANICUM SPECIES

Figure 40: Effect of high CO2 (900 ppm) on the leaf thickness of Panicum decipiens (C3/C4), Panicum tricanthum (C3) and Panicum antidotale (C4). The leaf thickness showed a decrease in P. antidotale (Prob > t = 0.06) and P. decipiens (Prob > t = 0.2), whereas P. tricanthum (Prob > t = 0.5) displayed an increase.
Figure 41: Effect of high CO$_2$ (900 ppm) on the proportion of the vascular tissue area of Panicum leaves. Panicum decipiens (C$_3$/C$_4$; Prob > t = 0.3) displayed a decrease in vascular elements. Panicum tricanthum (C$_3$; Prob > t = 0.9) and Panicum antidotale (C$_4$; Prob > t = 0.00) showed an increase.
Figure 42: Effect of high CO₂ (900 ppm) on the proportion of sclerenchymatic tissue area of Panicum leaves. Panicum decipiens (C₃/C₄; Prob > t = 0.2), Panicum tricanthum (C₃; Prob > t = 0.02) and Panicum antidotale (C₄; Prob > t = 0.00).
Figure 43: Effect of high CO$_2$ (900 ppm) on the proportion of bundle sheath tissue area of Panicum leaves. Panicum decipiens (C$_3$/C$_4$; Prob > t = 0.2), Panicum tricanthum (C$_3$; Prob > t = 0.02) displayed a decrease. Panicum antidotale (C$_4$; Prob > t = 0.00) showed a significant increase.
3.4. DESCRIPTION OF RESULTS AND DISCUSSION:

3.4.1. GENERAL ANATOMY ON LEAF TISSUE PROPORTIONS AND CELL AREAS OF PANICUM TRICANTHUM, PANICUM DECIPIENS AND PANICUM ANTIDOTALE

The general anatomical structure was not qualitatively changed in the Panicum species investigated in this study (figures 33-35) at high CO₂. The C₃/C₄ intermediate P. decipiens displayed both Kranz and non-Kranz characteristics under both concentrations of CO₂ and did not show any dramatic alteration in form of either the C₃ or the C₄ anatomy.

Irrespective of their photosynthetic type, all species had well developed parenchyma bundle sheath cells, with the mesophyll cells arranged around the bundle in an orderly manner, each cell orientated with its long diameter at right angles to the bundle. Thus intransverse sections as in this study, the mesophyll appears to radiate from the bundles as described by Esau (1977). The varying arrangement of the mesophyll and the well developed bundle sheath are also a characteristic feature of Panicum, i.e. Panicoideae (Wilson et al., 1983; Prendergast et al., 1987) (see figures 33-35). The mesophyll cells in the C₃/C₄ and C₄ species were tightly packed, which is dictated by the necessity of contact between mesophyll cells and bundle sheath cells for their coordinate activities (Hatch and Osmond, 1976; Kemp et al., 1983). The mesophyll of P. antidotale did not contain as many chloroplasts when compared to the the C₃ and the C₃/C₄ plants. The mesophyll of the C₃ P. tricanthum displayed more and bigger intercellular spaces in plants grown under elevated carbon dioxide. The bundle sheath of the C₃ appeared to be devoid of organelles, in comparison with the densely packed bundle sheath of the C₄ type and the moderate packing of the C₃/C₄ plants. The bundle sheaths of all species extended to the end of bundles, so that the vascular tissue was rarely exposed directly to intercellular air spaces. The bundle
sheath chloroplasts had fewer grana than the mesophyll cells, but still contained an appreciable amount (see chapter 4, figure).

Grass leaves generally have strongly developed sclerenchyma. Commonly fibers appear in longitudinal plates extending from the larger vascular bundles to the epidermis (Esau, 1977). The *Panicum* species had strongly developed sclerenchyma in the form of longitudinal plates of fibres extending on both sides of the major vascular bundles to the epidermis, and the smallest bundles are only connected with one fiber plate (see figures 33-35).

The epidermis in *P. tricanthum* and *P. decipiens* displayed bulliform cells, which were absent from the epidermis of *P. antidotale* (see figures 33-35). The bulliform cells were visibly larger in *P. decipiens* grown under high carbon dioxide.

3.4.2. THE EPIDERMIS AND THE EPIDERMAL BULLIFORM CELLS:

The number and size of epidermal cells control leaf area, although leaf volume depends on the extent of intercellular spaces as well as cell volumes. In the mature leaf, with cell division completed, the ratio of cells in the epidermis to total cell number is constant (Dengler et al., 1985). Because cell division ceases first in the epidermis, the behaviour of the epidermal layers governs the leaf size. Extending the period over which the epidermal cells divide, or, perhaps increasing the development of epidermal cells through an agency such as high CO₂, would be likely to increase leaf cell number, but leaf area would only be increased if the size of the epidermal cells did not decrease to compensate for the greater number.

Thomas and Harvey (1983) found that epidermal cells remained constant in size over a range of elevated CO₂ concentrations in *Zea mays*, *Glycine max*
and *Liqidambar styraciflua*, whereas Madsen (1973) stated that epidermal cells per unit area decreased with increased carbon dioxide concentrations.

In contrast to the above findings the *Panicum* species investigated displayed an increase in the epidermal tissue area (see figure 36) when grown under high carbon dioxide. This might be due to an increased photosynthetic capacity leading to the formation of greater leaf area (see chapter 2, figure 7) involving an increase in the number of epidermal cells and/or an increase of the epidermal cell area through formation of bigger cells.

In *P. decipiens* the increase of epidermal tissue area was clearly due to an increase in modification of some of the epidermal cells, the bulliform cells (see figure 37). The bulliform cells in *P. tricanthum* in comparison showed a slight decrease under high CO₂ (see figure 37), whereas the epidermal tissue area in this species still increased (see figure 36).

The bulliform (meaning: bubble-like cells) appear as a feature in leaves of most monocotyledonous families and are a peculiar type of comparatively larger, highly vacuolated, thin, anticlinal walled cells in the epidermis (Esau, 1977). In transverse sections (see figure 33A; 34A) they appear as a fan-like band, because the median cell is usually largest in size. In *P. decipiens* and *P. tricanthum* they occur on the upper side of the leaves parallel to the veins, whereas in *P. antidotale* they are absent (see figure 35A). The bulliform cells remain restricted to the grooves, which are water-containing cells, thin walled with no chlorophyll, but with outer walls which are just as thick and cutinized as the other epidermal cells.

There are three generally accepted views regarding the functions of bulliform cells. Firstly, they are concerned with unrolling the developing leaves. Secondly, they are thought to play a role in the hygroscopic opening and closing movements of mature leaves, due to changes in turgor and are therefore described as cells participating in involution and folding movements of grass leaves. Thirdly, they might be simply concerned with water storage, and have no other function (Esau, 1977). Thus bulliform cells may be
multifunctional, so in *P. decipiens* and *P. tricanthum* all three aspects may apply.

*P. decipiens* showed a significant increase in size of the bulliform cell area under high carbon dioxide (see figure 34A and table ), and this could confirm that enhanced water use efficiency occurs in this species. Increased water use efficiency also causes changes in turgor, which could lead to folding or unfolding of leaves due to the specialized bulliform cells. Highly folded leaves were observed in *P. decipiens* when grown under high carbon dioxide. Anatomically an increase of in the size of bulliform cells occurred. In *P. tricanthum* there was a trend towards a decrease in bulliform cell area, but this was not statistically significant. This might be due to experimental error, and either the bulliform cell area does not change in this species under high carbon dioxide concentrations, or, due to enhanced water use efficiency due to lowered stomatal frequencies, stomatal closure and increased epicuticular waxes, there may be no need for the plants to produce larger water containing bulliform cells.

### 3.4.3. LEAF THICKNESS, MESOPHYLL TISSUE AREA AND MESOPHYLL CELL AREA

The main part of the ground tissue of a leaf blade is the mesophyll containing many chloroplasts and a large volume of intercellular spaces (Esau, 1977). The mesophyll of the *Panicum* grasses used in this study showed no distinct differentiation into palisade and spongy parenchyma as is the rule in the grass family.

In plants grown under high carbon dioxide concentrations in comparison to those grown under ambient conditions, an increase of mesophyll tissue area occurred in *Panicum tricanthum*, but *P. antidotale* and *P. decipiens* displayed a decrease (see figure 38). The C₄ species showed a high significance for this decrease, whereas the decrease in the C₃/C₄ species showed a trend,
which was not statistically significant. The C₃ species showed a trend to an increase of mesophyll.

An increase in leaf thickness in *P. tricanthum* when grown under high CO₂ correlated well with the observed changes in mesophyll, where both individual mesophyll cell area and the proportion of mesophyll in a leaf transverse section increased. These results confirm previous findings which showed that leaves of soybean, sweet potato, loblolly pine, poplars, and sweetgum (all C₃ species) became increasingly thicker as CO₂ concentrations were increased, although it should be noted that the leaf thickness of corn (a C₄ plant) remained unchanged (Hofstra and Hesketh, 1975; Rogers et al., 1983; Radoglou and Jarvis, 1990; Delgado et al., 1994). The increments obtained in soybean and sweetgum leaves were directly attributable to significant changes in the density and number of palisade mesophyll cells, and soybean in addition even showed a third palisade cell layer (Thomas and Harvey, 1983). In pine and sweetgum, however, the size of the entire mesophyll area increased with CO₂ enrichment, and significant changes could not be attributed to the development of any specific cell type in preference to another. The extra leaf thickness in the two species was considered by (Thomas and Harvey, 1983) to be the result of more rapid development of leaves in high CO₂ concentrations during the period of lamina differentiation and cell enlargement.

Increases in individual cell size and a slight increase in the number of cells increased both leaf thickness and leaf area in *P. tricanthum* (see chapter 2, figure 7). Increased leaf and palisade layer thickness, in this instance connected with reduced specific leaf area, was also observed in alpine plants by Koerner and Diemer (1994), who attributed this to an increased nitrogen content.

A greater cell expansion occurring at high CO₂ as observed in *P. tricanthum* can be attributed to the increased osmotic potential of leaf cells associated with their higher carbohydrate content, which causes cells to absorb water and thus enlarge (Madsen, 1973). This hypothesis was confirmed by the
results obtained showing a significant increase in the amount of starch (chapter 4, figure 47).

In contrast to *P. tricanthum* and other C₃ plants, *P. antidotale* and *P. decipiens* showed no increase in leaf thickness, but instead, a slight but significant decrease.

Since the area of individual mesophyll cells of these plants increased (figure 39, table 2), there must have been an increase in the area of individual leaves which led to the observed increase in leaf area in both species (figure 7, chapter 2), or fewer mesophyll cells per leaf, or both.

### 3.4.4. THICKENED TISSUES:

**VASCULAR ELEMENTS, SCLERENCHYMA AND BUNDLE SHEATH:**

The vascular system of the leaf, consisting of the principal water conducting tissues (xylem) and the principal food conducting tissues (phloem), is located in veins, distributed throughout the blade, and thus shows a close spatial relationship to the mesophyll. In this research, no marked change in the proportion of vascular tissues was observed (see figure 41).

*P. antidotale* showing a significant increase, but *P. tricanthum* only a slight trend towards increase, and *P. decipiens* responded with a trend towards a decrease of the vascular tissue area (see figure 41). The increase in vascular tissue in *P. antidotale* was not observed in maize by Thomas and Harvey (1983), the only C₄ plant whose anatomy has previously been studied, which is also in the same tribe (*Panicoideae*).

The results of the analysis of the sclerenchymatic tissue area showed significance in *P. antidotale* and *P. tricanthum* and a decreasing trend in *P. decipiens* (see figure 42). The heavily lignified bundle sheath again showed
a significant increase in tissue area in *P. antidotale*, but a decreasing trend in
the other two species (see figure 43).

The C₄ plant *P. antidotale* responded to the greatest extent (table 4 and table
5, figures 41-43) and in a highly significant manner to the carbon dioxide
treatment, whereas the other two species seemed to be slightly affected by
the high carbon dioxide concentration with regard to an increase in woody
tissues (table 4 and table 5, figures 41-43). The substantial increase in the
size of bundle sheath cells in the C₄ species *P. antidotale* (figure 43) may
have disposed these cells to accommodate the major accumulation of starch
observed in the bundle sheath chloroplasts (see figure 47, chapter 4).

Extra photosynthetic gain through the extra carbon dioxide in the C₃ and
the C₃/C₄ species may be allocated to starch rather than converted into cell
wall material, which is confirmed by the results of accumulation of structural
carbohydrates as shown in Chapter 4. This similarity is likely in the context
that the C₃/C₄ plant reacts physiologically like the C₃ plant (J. Wilson,
personal communication).

It is unlikely that the increase in vascular elements is due to a closer spacing
of bundles in C₄ compared to C₃ grasses as demonstrated by Takeda and
Fukuyama (1971) and Crookston and Moss (1974), because *P. antidotale*
clearly exhibited an individual response to high carbon dioxide for all woody
tissue parameters used.

The enhanced rate of xylem differentiation, resulting in numerous smaller
vessels and greatly thickened xylem fibers as found by Jitla (1995,
unpublished) in *Eucalyptus tereticornis* grown under high CO₂ could not be
detected in Panicum. Also an increase in the number of xylem vessels as
observed by Jitla, 1995 (unpublished) was not detected. Xylem vessels of
*Panicum* plants grown under elevated carbon dioxide appear not to have
changed appreciably.
<table>
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<th>species/concentration</th>
<th>EPIDERMIS</th>
<th>BULLIFORM CELL AREA</th>
<th>MESOPHYLL CELL AREA</th>
<th>LEAF THICKNESS</th>
<th>SCLERENCHYMA</th>
<th>VASCULAR ELEMENTS</th>
<th>BUNDLE SHEATH</th>
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</thead>
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<td>0.63</td>
<td>106.08</td>
<td>0.26</td>
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<tr>
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<td>nil</td>
<td>0.43</td>
<td>115.60</td>
<td>0.20</td>
<td>0.063</td>
<td>0.114</td>
</tr>
<tr>
<td>P.tric. 350 ppm CO2</td>
<td>0.042</td>
<td>2184.78</td>
<td>0.51</td>
<td>163</td>
<td>0.18</td>
<td>0.042</td>
<td>0.056</td>
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<tr>
<td>P.tric. 900 ppm CO2</td>
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<td>1972.04</td>
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<td>179.02</td>
<td>0.20</td>
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<td>1442.515</td>
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<td>63.36</td>
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<td>0.056</td>
<td>0.085</td>
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<tr>
<td>P.dec. 900 ppm CO2</td>
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<td>90.93</td>
<td>0.16</td>
<td>0.072</td>
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</tbody>
</table>

**TABLE 4:** DATA REPRESENT AVERAGE VALUES OF ANATOMICAL PARAMETERS OF *PANICUM* SPECIES GROWN UNDER AMBIENT (350 ppm) AND ELEVATED CARBON DIOXIDE (900 ppm).
<table>
<thead>
<tr>
<th>SPECIES</th>
<th>BULBIFORM</th>
<th>MESOXYLL</th>
<th>CELL AREA</th>
<th>LEAF THICKNESS</th>
<th>SCLERENCESHA MA</th>
<th>VASCULAR ELEMENTS</th>
<th>BUNDLE SHEATH</th>
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<tbody>
<tr>
<td>P. amandia</td>
<td>1.34</td>
<td>0.68</td>
<td>1.08</td>
<td>0.77</td>
<td>1.34</td>
<td>1.70</td>
<td>2.39</td>
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<tr>
<td>P. tricarinatum</td>
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<td>0.90</td>
<td>1.09</td>
<td>1.18</td>
<td>1.29</td>
<td>1.01</td>
<td>0.85</td>
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<tr>
<td>P. decipiens</td>
<td>1.28</td>
<td>3.28</td>
<td>1.43</td>
<td>0.89</td>
<td>1.28</td>
<td>1.08</td>
<td>0.98</td>
</tr>
</tbody>
</table>

**TABLE 5: PROPORTION OF INCREASE OF ANATOMICAL PARAMETERS OF THREE JAVICUM SPECIES IN RESPONSE TO ELEVATED CARBON DIOXIDE (000 ppm: 3.50 ppm)**

108
3.5. SUMMARY:

(i) All three *Panicum* species studied did not qualitatively change in their leaf anatomical structure when grown under high carbon dioxide. They displayed the typical anatomical features of the *Panicoideae*.

(ii) *P. tricanthum* showed an increase in mesophyll, mesophyll cell area and leaf thickness, whereas *P. antidotale* and *P. deciiens* showed a decrease in all three parameters when grown under high carbon dioxide concentrations. There is a clear positive or negative correlation between the three parameters mesophyll tissue area, mesophyll cell area, and leaf thickness.

(iii) All three *Panicum* species grown under high carbon dioxide responded with an increase in epidermal tissue area. An increase in modified epidermal cells, the bulliform cells, which could enhance water use efficiency in *P. deciiens*, was also observed. Epidermal cell number and size jointly govern the leaf size and therefore both parameters are in part responsible for the larger leaf area found (see chapter 2).

(iv) *P. antidotale* exhibited an increase in all thickened (woody) tissues, whereas *P. tricanthum* only showed slight increases in the vascular and sclerenchymatic tissues and decrease in the bundle sheath. By comparison, *P. deciiens* showed a decreasing trend in all woody tissue parameters applied in this work. The C₄ species showed an increased ratio of woody tissues and carbohydrates (starch, see chapter 4, figure) under high CO₂, whereas the C₃ and C₃/C₄ species allocate the extra carbon gained to starch rather than to cell wall material of strengthening or vascular tissues.
CHAPTER 4

HIGH CO₂ AND PHOTOSYNTHETIC CAPACITY IN
MONOCOTYLEDONOUS PERENNIAL PLANTS - AN
ANATOMICAL AND PHYSIOLOGICAL APPROACH

4.1. INTRODUCTION

A commonly observed phenomenon in studies of plants grown under elevated CO₂ is the accumulation of starch and soluble carbohydrates (Eamus and Jarvis, 1989; Arp, 1991; Stitt, 1991). This response seems to be accompanied by a gradual loss of photosynthetic capacity of the leaves (Eamus and Jarvis, 1989; Gunderson and Wullschleger, 1994).

Net photosynthesis has been found to be down-regulated (i.e. there is downward acclimation) during growth at high CO₂. Feedback inhibition by reduced sink demand due to a built-up of carbohydrates has been shown to cause this downward photosynthetic acclimation (DeLucia et al., 1985; Ehret and Joliffle, 1985; Eamus and Jarvis, 1989; Arp, 1991; Stitt, 1991). In extreme cases accumulated starch granules in plants grown under high CO₂ directly reduce leaf photosynthetic capacity by damaging chloroplast (thylakoid) structure (Cave et al., 1981; Wulff and Strain, 1982). Other investigations recorded a decline in leaf chlorophyll concentrations (DeLucia et al., 1985; Houpis et al., 1988; Mousseau and Enoch, 1989; Norby et al., 1992; Wullschleger et al., 1992) and the chlorophyll a/b ratio often became lower (Cave et al., 1981; De Lucia, 1985; Mousseau and Enoch, 1989; Drake,
1992). However, whether there exists a causal relationship between carbohydrate accumulation and photosynthetic inhibition remains controversial (Stitt, 1991).

In this study the possible effects of elevated carbon dioxide on photosynthetic capacity of three tropical perennial Panicum grass species with different photosynthetic pathways was assessed by determining four parameters: 1) leaf starch content, 2) leaf chlorophyll content, 3) chloroplast structure and the proportion of non appressed to appressed thylakoids of the Panicum grass plants. This was done for plants grown under ambient (350 ppm) and elevated (900 ppm) CO₂ concentrations. This study focussed on the following questions:

- Does the leaf starch content change in Panicum grasses grown under high CO₂ in comparison with the grasses grown under ambient conditions?
- Do chloroplasts with a constant chlorophyll a/b ratio, developed under ambient conditions, have a constant proportion of appressed to non-appressed thylakoids?
- Do the chlorophyll a/b ratio and proportion of appressed to non-appressed thylakoids change under high CO₂?
- Is there a connection between carbohydrate content within the cell and a change in thylakoid numbers and chlorophyll a/b ratios?
4.2. MATERIALS AND METHODS:

4.2.1. CHLOROPHYLL ANALYSIS:

4.2.1.1. PLANT CULTURE, HARVEST AND EXPERIMENTAL DESIGN

This investigation used samples from the plant culture described in chapter 2. The exception to the sampling procedure described in chapter 2 was that plants were harvested in the middle of the day and leaves were randomly taken to give 1g per sample from all parts of 10 plants per concentration per species at three different harvest times.

4.2.1.2. CHLOROPHYLL EXTRACTION:

Each 1g leaf sample was freshly cut into small pieces and then ground in 40ml of 80% acetone for about three minutes until the tissue appeared to be a fine pulp. The extract was then filtered and the final volume was adjusted by adding 80% acetone. The optical density of the chlorophyll extract was read and recorded in a 10nm cell with a spectrophotometer set at 645 and 663 nm. The 80% acetone was used as the solvent blank.
4.2.1.3. CALCULATION OF CHLOROPHYLL AMOUNTS

(mg chlorophyll/g tissue):

Calculations were carried out using the following formulas based on Witham et al., 1986:

\[
\text{mg chlorophyll a/g tissue} = (12.7D_{663} - 2.69D_{645}) \times \frac{\sqrt{V}}{1000 \times W}
\]

\[
\text{mg chlorophyll b/g tissue} = (22.9D_{645} - 4.68D_{663}) \times \frac{\sqrt{V}}{1000 \times W}
\]

\[
\text{mg total chlorophyll /g tissue} = (20.2D_{645} + 8.02D_{663}) \times \frac{\sqrt{V}}{1000 \times W}
\]
4.2.2. STARCH ANALYSIS:

4.2.2.1. SAMPLING PROCEDURE

The sampling procedure for the three *Panicum* species and the experimental design including the data analysis were as described in chapter 2. Exception here to the sampling described in chapter 2 was that leaf samples (one fully expanded leaf of the main tiller of each species per replicate) were taken at random just after the onset of the light period.

4.2.2.2. DETERMINATION OF STARCH

Starch analysis was carried out using the last fully expanded leaf of the main tiller of three different trials, drying the leaves for 2 days at 70°C. The dried leaves were then milled and accurately weighed to give 50 mg per sample. The starch content of the fully expanded leaves was determined enzymatically by allying the Megazyme AACC 76-11 method, assay format 2 (alpha-amylase/pullulanase/beta-amylase). The Megazyme format allows the measurement of total starch or "enzyme susceptible" in most cereal products and was therefore appropriate for the three *Panicum* forage grasses studied. For every three harvests up to 4 assays containing 6 samples each per species were run. The following formula was used to calculate starch % (dry weight basis):

\[
\text{STARCH} = \frac{\Delta E \times F \times 1000 \times \frac{1}{100} \times 162}{180}
\]
4.2.3. TRANSMISSION ELECTRON MICROSCOPE INVESTIGATIONS:

4.2.3.1. CHLOROPLAST STRUCTURE/NUMBER OF THYLAKOIDS:

The anatomical investigation to observe chloroplast structure and determine the proportion of appressed to non-appressed thylakoid was carried out using transmission electron microscopy. The last fully expanded leaf of the main tiller was harvested just after the onset of daylight from three different trials. A schedule for the fixation/dehydration and embedding of the specimen was developed which can be used for investigations using light and transmission electron microscopy. Sections (70 μm thickness) were viewed under the transmission electron microscope and up to 12 high quality micrographs per species per concentration were taken. Information concerning grana were obtained by inspection of electron micrographs of 5 mesophyll chloroplasts from five different leaves of each species. Only mesophyll chloroplasts were investigated to allow a comparison between all three species. 10 random lines of equal length were set into the TEM picture to determine the proportion of appressed to non-appressed thylakoids.

4.2.3.2. SPECIMEN PREPARATION FOR THE TRANSMISSION ELECTRON MICROSCOPY - PRELIMINARY EXPERIMENTS:

Several variations of the standard preparation technique (EM UNIT schedule, Biological Specimen Preparation, Sydney University, 1992) were used to guarantee optimal resolution of the specimen. This involved application of different incubation/fixation and embedding times, different dilutions of standard solutions, and use of different dehydrating solvents and resins. The main problems that occurred were due to the high amount of starch in the plants grown under elevated carbon dioxide which made fixation extremely difficult. The cutting of the specimen on the ultramicrotome was also made particularly difficult by the highly lignified cells in the bundlesheath and sclerenchyma in all three Panicum species.
4.2.3.3. SCHEDULE FOR FIXATION, DEHYDRATION AND EMBEDDING OF *PANICUM* SPECIES (modified after the EM Unit schedule, Biological specimen preparation, Sydney University, 1992)

Fix in 3% glutaraldehyde/ 0.025M phosphate buffer (Hayat, 1981; Bullock, 1984), pH 6.8-7.3
48 hours (4°C)

Wash in the same buffer
(3 changes, 3 mins. Each)

Postfix in 2% Osmium
(room temp.) 2 hours

Wash in distilled H₂O
(3 changes of 10 mins. Each)

Dehydrate

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<th>Time</th>
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<tr>
<td>30% acetone</td>
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</tr>
<tr>
<td>50% acetone</td>
<td>30 mins</td>
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<tr>
<td>100% acetone</td>
<td>30 mins</td>
</tr>
<tr>
<td>100% acetone</td>
<td>30 mins</td>
</tr>
</tbody>
</table>

Infiltrate

100% acetone : Spurr’s resin (1 : 1)
5 days (room temperature, on rotary wheel)

Transfer to

100% Spurr’s resin
2 days (room temperature, rotary wheel)

Embed (flat) in fresh Spurr’s resin and polymerize
(65°C) overnight

Contrast staining (1) of ultrathin sections with uranyl acetate
2% aqueous solution : 15 mins

Contrast staining (2) of ultrathin sections with lead citrate
15 mins
4.3. RESULTS:

4.3.1. EFFECTS OF HIGH CARBON DIOXIDE CONCENTRATIONS ON THE AMOUNT OF CHLOROPHYLL OF PANICUM SPECIES WITH DIFFERENT PHOTOSYNTHETIC PATHWAYS:

TOTAL CHLOROPHYLL (a/b);
CHLOROPHYLL a; CHLOROPHYLL b
Figure 44: Effect of high CO$_2$ on the total chlorophyll (a/b) concentration of Panicum on a fresh leaf basis. Panicum antidotale (C$_4$; Prob > t = 0.5) and Panicum tricanthum (C$_3$; Prob > t = 0.8) display a decreasing trend, whereas Panicum decipiens (C$_3$/C$_4$; Prob > t = 0.00) shows a significant increase in total chlorophyll.
Figure 45: Effect of high CO₂ on the chlorophyll a concentration of Panicum on a fresh leaf basis. Panicum antidotale (C₄; Prob > t = 0.2) and displays a decreasing trend, whereas Panicum tricanthum (C₃; Prob > t = 0.4) shows a slight trend of increase in chlorophyll a. Panicum decipiens (C₃/C₄; Prob > t = 0.00) shows a significant increase in chlorophyll a.
**Figure 46:** Effect of high CO$_2$ on the chlorophyll b concentration of *Panicum* on a fresh leaf basis. *Panicum antidotale* (C$_4$; Prob > t = 0.5) and *Panicum tricanthum* (C$_3$; Prob > t = 0.6) display a decreasing trend, whereas *Panicur decipiens* (C$_3$/C$_4$; Prob > t = 0.00) shows a significant increase in total chlorophyll.
4.3.2. QUANTITATIVE ASSESSMENT OF THE AMOUNT OF NON-STRUCTURAL CARBOHYDRATES: STARCH (% DRY WEIGHT):

The amount of starch increased in all three species when grown under high CO₂ with high statistical significance. The proportion of increase was found to be similar for all three Panicum species.
Figure 47: Percentage of starch on a dry weight basis in leaves of *Panicum* species grown under ambient (350 ppm CO₂) and high carbon dioxide concentrations (900 ppm CO₂). The starch amount increased with high significance in all three species when grown under high CO₂: *P. antidotale* (Prob > t = 0); *P. tricanthum* (Prob > t = 0); *P. decipiens* (Prob > t = 0).
4.3.3. TRANSMISSION ELECTRON MICROSCOPY:

EFFECT OF ELEVATED CARBON DIOXIDE CONCENTRATIONS ON THE CHLOROPLAST STRUCTURE:

- QUALITATIVE ASSESSMENT OF STARCH AND OBSERVATION OF GRANAL STRUCTURE THROUGH TEM INVESTIGATIONS

- RATIO OF APPRESSED TO NON—APPRESSED THYLAKOIDS
4.3.3.1: INVESTIGATIONS OF THE ACCUMULATION OF STARCH AND
THE GRANAL STRUCTURE WITHIN THE CHLOROPLAST IN PANICUM
GROWN UNDER ELEVATED CARBON DIOXIDE CONCENTRATIONS

The three Panicum species showed an enormous increase in starch grains
within the chloroplasts when plants were grown in 900 ppm carbon dioxide in
comparison to the control at 350 ppm CO₂. There is no disruption of the
granal structure in P. antidotale and P. decipiens neither in the mesophyll nor
in the bundlesheath chloroplasts. In P. trianthum (C₃) the starch grains
seem to interfere with the thylakoid structure and therefore cause
disintegration of the grana, but no visible disruption to the whole
chloroplast under high carbon dioxide occurred (49 B;C; 50 D,E).

The fixatives used in this study do not really fix starch grains. The starch
reacts with water and therefore folds over, which results in the dark bandings
seen on the starch grains in figures 49B/C; 50.

The C₄ plant P. antidotale exhibited an accumulation of starch in the
mesophyll cells only under high carbon dioxide concentrations (see figure 55
C), whereas the bundlesheath cells exhibited the normal accumulation of
starch in plants with Kranz anatomy under ambient concentrations. The
C₃/C₄ P. decipiens (see figures 51 A,B; 52 C,D,) shows an intermediate
behaviour, having starch stored in both the mesophyll and the bundlesheath
cells under ambient and high carbon dioxide concentrations.
EFFECTS OF HIGH CARBON DIOXIDE CONCENTRATIONS ON THE CHLOROPLAST STRUCTURE OF PANICUM TRICANTHUM (C₃)

Figure 48 A: A chloroplast showing clearly the appressed thylakoids (grana) and interconnecting (non-appressed) intergranal lamellae of Panicum tricantum grown at ambient (350 ppm) carbon dioxide concentrations. Starch grains are not visible. Scale bar = 1.3 μm

Figure 49 B: Transverse section of a mesophyll chloroplast adjacent to the empty bundlesheath and xylem vessels of Panicum tricantum (C₃) grown 900 ppm CO₂: m (mesophyll); bs (bundle sheath); xv (xylem vessels). Scale bar = 1.3 μm

Figure 49 C: A chloroplast showing clearly the appressed thylakoids (grana) and interconnecting (non-appressed) intergranal lamellae of Panicum tricantum grown at 900 ppm CO₂: mi (mitochondrion); sg (starch grain); la (lamellae). The lamellae appear disintegrated but intact despite of large starch grains. Scale bar = 0.58 μm

Figure 50 D;E: A chloroplast showing the high amount of starch grains within the chloroplasts of P. tricantum grown at 900 ppm CO₂: mc (mesophyll cell); sg (starch grain). Appressed thylakoids (grana) and interconnecting (non-appressed) intergranal lamellae appear disintegrated. Scale bar (D)= 1 μm; Scale bar (E)= 1.1 μm
EFFECTS OF HIGH CARBON DIOXIDE CONCENTRATIONS ON THE CHLOROPLAST STRUCTURE OF *PANICUM DECIPiens* (*C₃ /C₄*)

**Figure 51 A;B:** Micrographs show the accumulation of starch in the bundle sheath cells and mesophyll cells of *Panicum decipiens* grown at ambient (350 ppm) carbon dioxide concentrations: bsc (bundle sheath cell); sg (starch grain). Note the intact appressed thylakoids (grana) and interconnecting (non-appressed) intergranal lamellae. Scale bar (A) = 5.8 μm; Scale bar (B) = 1.8 μm.

**Figure 52 C;D:** Bundlesheath and mesophyll chloroplasts showing clearly the intact appressed thylakoids (grana) and interconnecting (non-appressed) intergranal lamellae of *Panicum decipiens* grown at (900 ppm) carbon dioxide concentrations: bsc (bundle sheath cell); mc (mesophyll cell); sg (starch grain). Starch grains occur in both mesophyll (C) and bundle sheath cells (C;D). Scale bar (C) = 5.8 μm; Scale bar (D) = 1.3 μm

**Figure 53 E:** Mesophyll chloroplast of *Panicum decipiens* grown at elevated (900 ppm) displaying large starch grains and clearly visible intact appressed and non-appressed thylakoids: mc (mesophyll chloroplast); sg (starch grain). Scalebar = 1.05 μm
EFFECTS OF HIGH CARBON DIOXIDE
CONCENTRATIONS ON THE CHLOROPLAST
STRUCTURE OF PANICUM ANTIDOTALE (C₄)

Figure 54 A;B: Mesophyll chloroplasts showing clearly the appressed thylakoids (grana) and interconnecting (non-appressed) intergranal lamellae of Panicum antidotale grown at ambient (350 ppm) carbon dioxide concentrations. Scale bar (A) = 2.1µm; Scale bar (B)=3µm

Figure 55 C;D: Chloroplasts of Panicum antidotale grown at (900 ppm) carbon dioxide concentrations. C: Mesophyll chloroplast of P. antidotale filled with starch grains (sg). Figure D: Bundle sheath chloroplasts showing clearly the appressed thylakoids (grana) and interconnecting (non-appressed) intergranal lamellae. Scale bar (C) = 2.1µm; Scale bar (D) =1.3µm
4.3.3.2 RATIO OF APPRESSED TO NON APPRESSED THYLAKOIDS OF PANICUM GROWN UNDER ELEVATED CARBON DIOXIDE CONCENTRATIONS

There was no appreciable change in the ratio of the number of appressed to non-appressed thylakoids in all three Panicum species investigated in this study (see table 6).

A physical disintegration of the thylakoid structure was only observed in P. tricanthum (figure 50 D;E). All mesophyll cells of the three Panicum species contained chloroplasts with well developed grana. Panicum tricanthum only exhibited smaller granal stacks, surrounding the structure disintegrating starch grains.
<table>
<thead>
<tr>
<th>SPECIES</th>
<th>ppm CO₂</th>
<th>thylakoid, non-appressed %</th>
<th>thylakoid, appressed %</th>
<th>total number of thylakoids</th>
<th>ratio of nonappressed/appressed thylakoids</th>
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</thead>
<tbody>
<tr>
<td><em>Panicum antidotale</em></td>
<td>350</td>
<td>38.9</td>
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</table>

**Table 6:** Ratio of appressed to non-appressed thylakoids (grana) of *Panicum* species with different photosynthetic pathways grown under ambient (350 ppm) and elevated (900 ppm) carbon dioxide concentrations. The data here are shown in full, because each value respresents an average of 10 determinations.
4.4. DISCUSSION

4.4.1. ANALYTICAL AND STRUCTURAL DETERMINATION OF STARCH ACCUMULATION IN PANICUM SPECIES GROWN UNDER HIGH CARBON DIOXIDE CONCENTRATIONS

4.4.1.1. QUANTITATIVE DETERMINATION OF NON-STRUCTURAL CARBOHYDRATES (STARCH)

The first stable products of carboxylation, the triose phosphates which are formed by RuBISCO within the chloroplast, have essentially three fates: first, they can be recycled within the chloroplast via the C₃ cycle; secondly, they can be stored as starch; thirdly, they can be transported from the chloroplast immediately, and converted to sucrose.

Carbon can be stored temporarily in the chloroplasts and is converted into starch for subsequent metabolism and export, a condition which is favoured by the high triose phosphate to inorganic phosphate ratio that occurs during carbon dioxide enrichment (Fondy and Geiger, 1980). This product starch forms most of the non-structural carbohydrate fraction in many plants. It is distinct from the structural carbohydrates which are components of cells walls and fibers, and is later available for utilization when soluble carbohydrate supplies are depleted, when it is mainly converted into sucrose for export via the phloem.

An altered concentration of non-structural carbohydrates in plants may be caused either by increased photosynthesis or decreased photorespiration. It is generally accepted that elevated concentrations of CO₂ cause an increase in photosynthesis and growth. However, exceptions are known such as in young ryegrass (Lolium perenne), where elevated CO₂ increases photosynthesis without affecting dry matter production (Ryle et al., 1992). Controlling the amount of CO₂ available to soybean plants was found to be
an effective means of altering leaf starch concentration, but not soluble sugar concentration according to Nafziger and Koller (1976). These results were similar to those of Madsen (1968), who found that the starch concentration of tomato leaves rose with increasing CO₂ concentrations. He noted that soluble sugar concentration did not increase as CO₂ concentration was raised above 400 ppm CO₂. The fact that raising the CO₂ concentration had no effect on the soluble sugar concentration, but increased starch content, indicates that soluble sugars were rapidly converted to starch. This has been confirmed by Yelle et al. (1989), who found an increase of 42% in non-structural carbohydrates of tomatoes grown at high carbon dioxide. Wong (1990) also found a substantial amount of carbon allocated to starch in the leaves of cotton grown under high carbon dioxide. There is considerable variation in the response of dicots as mentioned above in their response to elevated carbon dioxide, but comparative little is known about the response of monocots and their allocation of carbon. Barley (Hordeum vulgare), displayed a dry matter gain of 250 % under elevated carbon dioxide (675 ppm CO₂) which was not reflected in a significant concentrations of extractable carbohydrates (Ingvarsdson and Veierskov, 1994). Other monocots, such as wheat seem to allocate the extra photosynthate produced at high carbon dioxide concentrations to tiller production, and little starch accumulates in the leaves (Wong, 1990; Rogers, 1993).

The three Panicum species investigated in this study showed a significant increase in the amount of starch when grown under elevated carbon dioxide concentrations (see figure 47). This was confirmed by ultrastructural investigations (see figures 48A-55E). These results are consistent with to the increase in biomass production/plant dry matter gain (see chapter 2, figure 9). The amount of starch contents resported for Panicum are so far among the highest observed for leaves of plants grown under high carbon dioxide concentrations. Thomas et al.(1993) found 33% starch dry weight in Gossypium hirsutum under ambient conditions, which increased up to
33.4% when grown under elevated carbon dioxide concentrations. In soybean the amount of starch increased up to 20.5% (Allen et al., 1988)

An accumulation of structural carbohydrate may represent a temporary imbalance between production of assimilate in source leaves and its utilization by sinks for carbon, as suggested by Baxter et al. (1995). This evidently applies in Panicum, because the three species showed an obvious increase in size (figures 1-6, chapter 2), and these plants were investigated just before the transition from vegetative to floral growth, which provides an additional sink for assimilate late in plant development.

Nafziger and Koller (1976) found a decrease in photosynthetic rate as starch concentration increased. They suggested that starch accumulation may reduce net photosynthetic rate by impeding intracellular carbon dioxide transport, but there might be another mechanism: Cytoplasmic transfer could be a means of facilitating carbon dioxide transfer to the chloroplasts. The enlargement of chloroplasts due to starch granule growth may cause the chloroplasts to protrude further toward the center of the cell, thus reducing cytoplasmic streaming and resulting in less efficient transfer of carbon dioxide. Grub and Maechler (1990) also found a connection between starch accumulation and decreased photosynthesis. The formation of large starch granules in chloroplasts of red clover (Trifolium pratense) suggested a decrease in stromal space and an increase in stromal components, mainly inorganic and organic phosphates. However, elevated CO₂ seem to lower the inorganic phosphate concentration within the chloroplast leading to a decrease in the rate of the metabolism which circulates this inorganic phosphate, favouring starch formation over triose-phosphate export out of the chloroplast (Galtier et al., 1993; Galtier et al., 1995).

Inorganic and organic phosphates are also activators of RuBISCO. However, these compounds decrease the catalysis of CO₂ fixation per active enzyme site (Hatch and Jensen, 1980). The actual effect on RuBISCO seemed to be due to the binding and release of the enzyme to and from membranes and
changes in the degree of activation. High soluble carbohydrate concentrations are also thought to be involved in depressed transcription of the RuBISCO subunit gene (Krapp et al., 1993): it may be that decreased photosynthetic capacity in response to elevated CO₂ occurs only in circumstances where the extra carbohydrates generated by increased photosynthesis accumulate in the leaves. Accumulation of carbohydrates in the source leaves causes photosynthetic acclimation as observed in plants grown under elevated CO₂ and can directly or indirectly result in feedback inhibition of photosynthesis (Ascon-Bieto, 1983; Blehschmidt-Schneider et al.; Stitt, 1991; Bowes, 1994; Foyer et al., 1995). This can be discussed for the three Panicum species in which an increase of photosynthetic efficiency was needed in the first place to build up the large amount of non-structural carbohydrate found in their leaves when grown under high CO₂. It is also likely that due to the permanent formation of large starch granules an interference with the effective functioning of the membranes and transport mechanisms would occur sometime later after the active growth period. Such damage would reduce photosynthetic rates as hypothesized by Troughton (1975). Madsen (1975) demonstrated that tomato plants grown under high CO₂ concentrations accumulated starch and had deformed thylakoids. Carmi and Shomer (1979) and Wildman (1980) also reported that an accumulation of starch within the chloroplasts damaged the thylakoids and grana. The disintegration of the thylakoid structure caused by an enormous increase if starch grains in plants grown under elevated carbon dioxide (see figures 49 B/C; 50D/E; 52 C; D,E; 55C/D) combined with the decrease of the chlorophyll amount (see figures 44-46) in Panicum tricanthum might support this hypothesis.

Baxter et al. (1995) also found that an increase in starch was negatively correlated with photosynthetic capacity in the mature grasses Agrostis capillaris, Festuca vivipara and Poa alpina, after an initial increase in growth at elevated CO₂. Nafziger and Koller (1976) also observed a direct correlation between starch accumulation under high carbon dioxide and the
decline of photosynthesis. However, they found no significant decline in photosynthesis associated with starch concentration between 5% and 17%. Hence there are many different concentrations of carbohydrate accumulation in leaves reported in the literature which reflect differences in species as well as the range of environmental conditions used. Grasses of tropical origin, such as *Paspalum dilatatum* (Forde et al., 1975) accumulate starch up to 16-20% depending on temperature, whereas the temperate grasses only accumulate up to 4% of starch (Wilson and Ford, 1970). Because of this variation of species in response to elevated carbon dioxide concentrations it is difficult to find a means by which plants would respond in a negative manner. There also exists a genotypic control of starch (Galtier et al., 1993;1995), which is contrary of the opinion of Stitt (1991), who stated that starch would generally interfere with photosynthetic capacity. In conclusion, the implications of a higher starch content are:

a) better digestibility

b) faster production of a given quantity of forage and higher forage quality

c) if plants grow past the flowerstage, seed yield is postulated to increase

d) N- content might be reduced

e) eventually negative feedback on the photosynthetic capacity

The latter two points require further investigation
4.4.2. TRANSMISSION ELECTRON MICROSCOPY

4.4.2.1. ULTRASTRUCTURAL OBSERVATION OF THE CHLOROPLAST: ACCUMULATION OF STARCH AND ITS EFFECT ON THE RATIO OF APPRESSED TO NON-APPRESSED THYLAKOIDS IN PANICUM

Structural investigations, mainly carried out only on C₃ species, show that leaves grown under high carbon dioxide concentrations exhibit large starch grains in the chloroplasts of the mesophyll cells (e.g. Hofstra and Hesketh, 1975). When measured in the same CO₂ concentration, leaves grown under high CO₂ have often shown almost the same photosynthetic rate per unit leaf area as leaves grown under low and ambient CO₂ concentrations. In some instances the carbon dioxide concentration in which the leaves were grown had no significant effect on the photosynthetic rate (Ford and Thorne, 1967; Mauney et al., 1979). In other cases leaves grown in high carbon dioxide and containing large amounts of starch have often shown low photosynthetic rates (Hofstra and Hesketh, 1975; Mauney et al., 1979). Wulff and Strain (1982) also observed a depression of the photosynthetic rates in Desmodium paniculatum that was correlated with increased starch accumulation and reduced grana formation in the leaf chloroplasts. Obviously, complex and poorly understood adaptations are taking place.

Light and electron microscopy indicated that most, if not all, starch in bermuda grass, bahia grass and digit grass was present in the chloroplasts of the parenchyma bundle sheath cells (Akin and Burdick, 1977). This was also found by Downton and Tregunna (1968), who proposed that C₄ plants store starch (determined with IKI (tri-iodine)) preferentially in well-developed bundle sheath cells. However, Black and Mollenhauer, from TEM observations, observed that starch did not appear to be limited to sheath cells in all warm season grasses (mainly bermuda grasses), although the sheath cells always contained large amounts of starch. In contrast, C₃

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grasses appear to store starch throughout the mesophyll (Esau, 1965; 1977). Therefore it was expected that *P. tricanthum* would store starch, if present, only in the mesophyll cells, whereas the C₄ grass *P. antidotale* would store starch only in the bundle sheath cells. *P. decipiens* was thought to show intermediate behaviour.

These assumptions were not confirmed for all species. *P. antidotale*, grown under high carbon dioxide, showed an almost intermediate behaviour and stored large amounts of starch in both the mesophyll and the bundle sheath cells (figure 55C;D), a condition not observed at ambient carbon dioxide concentrations (figure 55A;B). The C₃/C₄ intermediate *Panicum decipiens* stored starch in both mesophyll and bundle sheath cells under ambient and high CO₂ concentrations (figure 51 A;B; 52 C;D, 53 E).

Byrd and Brown (1989) noticed a similar remarkable increase of starch in both mesophyll and bundle sheath cells in *Panicum milioides* (C₃/C₄) where the starch appeared to be responsible for a strong inhibition of apparent photosynthesis. In this case the carbohydrate increase was caused by a low O₂ concentration of 20 millimoles per mole. It was suggested that for these species O₂ is required to prevent detrimental effects on phloem loading of photosynthates and therefore provide sufficient translocation from the leaves.

The *P. tricanthum* leaves under ambient CO₂ showed no visible starch within their mesophyll chloroplasts (40A), but large starch granules appeared when the plants were grown under elevated carbon dioxide (figure 49B-50E). A physical disintegration of the thylakoid structure was observed in *P. tricanthum*, but there was no change in the ratio of the number of appressed to non-appressed thylakoids.

A disruption of the thylakoid structure may be caused by a change in turgor through an osmotic pressure caused by the accumulation of carbohydrates within cells but this is unlikely since starch is scarcely osmotically active. It is
more likely that the starch causes a physical pressure resulting in the disruption of the chloroplast structure.

Starch accumulation, leading to some feedback inhibition (negative feedback) or chloroplast disruption, has been most frequently postulated as responsible for the reduced photosynthetic rates in CO₂ enriched plants (Hofstra and Hesketh, 1975; Spencer and Bowes, 1986). Wulff and Strain (1982) attributed the depression of photosynthetic rates on a leaf area basis for *Desmodium paniculatum* plants grown under high CO₂ to reduced formation of grana in the chloroplasts correlated with an accumulation of starch. They suggested a possible detrimental effect on the thylakoid structure due to the high quantities of starch present which would account for a reduction in the photosynthetic rate. Yelle et al. (1989) found the distortion of thylakoids was caused by the build up of starch grains and was higher in *L. esculentum* then in *L. chmielewskii* when grown at 900 ppm carbon dioxide, but this did not improve the long-term photosynthetic efficiency of *L. chmielewskii*. Therefore it can be suggested that starch and sugar accumulation under high carbon dioxide cannot entirely explain the loss of photosynthetic efficiency of plants grown under elevated carbon dioxide. However, results obtained in this study show that there was also no gross disruption of the thylakoid structure, which could have caused a decrease in the ratio of appressed to non-appressed thylakoids in all three *Panicum* species (see table 6). The larger the starch grains, the less disruptive or disintegrating of the parent chloroplast structure they appear to be (figures 52 C,D and 53 E).

The starch content of a leaf results from synthesis and breakdown and is positively influenced by the CO₂ assimilation rate (Chatterton and Silvius, 1981; Sharkey et al., 1985). Neales and Incoll (1968) reviewed the research dealing with the relationship between leaf carbohydrate concentrations and photosynthetic rate. Most of the evidence supporting a product inhibition hypothesis has come from experiments in which the source/sink balance was altered in an attempt to change leaf carbohydrate concentrations. Such
alteration may also have changed the hormonal balance in the plant; certain hormones have been shown to affect photosynthesis (Treharne et al., 1970).

There are two hypotheses currently in the literature which might offer an explanation: Firstly, the sink activity is not adequate to utilize the additional carbohydrate produced as a consequence of increased photosynthesis at elevated CO₂, leading to an accumulation of carbohydrate in the source leaf and subsequent down regulation of photosynthesis. Thus the photosynthetic capacity at high CO₂ is reduced. Additionally, as down regulation of photosynthesis may result from a reduction in the amount of the photosynthetic machinery, leaf N content is expected to drop.

Secondly, photosynthetic rate and capacity are both functions of leaf N content; if N is reduced, for instance by nutrient dilution with carbon, then photosynthesis will be lower. Whether lower N content is a cause or consequence of reduced photosynthesis has yet to be established. The N content of plants were not taken into consideration in this study, and all Panicum grasses were treated with a generous nitrogen supply. More recent studies on mature source leaves of montane grasses like F. vivipara and P. alpina (Baxter et al., 1995) showed also a large increase in carbohydrates such as starch, coincident with lowered photosynthetic pigment concentrations and photosynthetic capacity. Therefore it is expected that the redistribution of carbon is paralleled by a significant reduction in photosynthetic capacity at some stage either during starch accumulation, or after the starch accumulation.

Starch accumulation did not seem to have a negative feedback on assimilation of the Panicum species under high carbon dioxide conditions; thus end product repression of photosynthesis seems unlikely. Temporary accumulation of starch by tropical grasses like Paspalum dilatatum study may reduce their sensitivity to this type of repression compared with other species, especially of temperate nature, where the temporary storage products are mainly fructosans and other soluble carbohydrates, as suggested by Forde et al.(1975).
4.4.2.2. LACK OF PERIPHERAL RETICULUM

The peripheral reticulum (PR) as observed by Carolin et al. (1973) in the C₄ grass Panicum pygmaeum was not present in the C₄ P.antidotale (figures 54 A;B, 55C;D), even though it is a consistent character of chloroplasts in both sheath and mesophyll of most C₄ plants. It was also not present in P. decipiens (figures 51A-53E) and P. tricanthum (figures 48A-50E).

The appearance of the peripheral reticulum is thought to be associated with a lack of, or low rate of, photorespiration (Hilliard and West, 1971). This was confirmed for some species with the C₄ pathway, which do not normally photorespire, but begin to do so, and also lose their peripheral reticulum when they become senescent. Thus it would have been interesting to observe the behaviour of a peripheral reticulum in plants with different photosynthetic pathways under high carbon dioxide, which suppresses or lowers photorespiration, especially in C₃ plants and eventually also in C₃/ C₄ plants. The questions would be whether there is a PR developed in a C₃ or a C₃ / C₄ plant under high CO₂, and what would happen to it in a C₄ plant, when grown under increased carbon dioxide?
4.4.3. CHLOROPHYLL ANALYSIS

The chlorophyll concentration varied within the *Panicum* species and also changed in response to elevated CO₂.

The chlorophyll concentration of many plants declines and the chlorophyll \( a/b \) ratio is often lower (Madsen, 1976; Cave et al., 1981; Wulff and Strain, 1982; DeLucia et al., 1985; Mousseau and Enoch, 1989; Drake, 1992; Norby et al., 1992.; Wullschleger et al., 1992). A constant trend of decrease in chlorophyll \( a,b \), and thus total chlorophyll was observed in *P. antidotale* when grown under high carbon dioxide. This decline in chlorophyll concentration is presumably a consequence of more rapid growth under elevated carbon dioxide (DeLucia et al.; Oberbauer et al., 1985) or may be an indicator of early leaf senescence (Houplis et al., 1988; Mousseau and Enoch, 1989). The latter was indeed observed in *P. antidotale* (figures 44-46). Baxter (1995) found a correlation of reduced leaf N content and chlorophyll in *F. vivipara*, whereas in *P. alpina* the nitrogen content in the leaf was not altered, but the chlorophyll concentration was significantly reduced and seemed to coincide with an increase in the amount structural carbohydrates (starch).

A lower chlorophyll \( a/b \) ratio might also reflect an adaptation to more shading through more vigorous growth of the plant (Anderson et al., 1988; Arp, 1991; Van Oosten et al., 1994), possibly caused by increased palisade mesophyll cells within the leaf (Rogers et al., 1983; Vu et al., 1989) or increased leaf area within the plant canopy at elevated CO₂. This coincides with the results achieved for *P. antidotale*, which displayed an increase in starch, leaf area and mesophyll cell area under high carbon dioxide concentrations. (see chapter 2, figure 7; chapter 3, figure 39; chapter 4, figure 47)

*P. tricanthum* showed a trend of decrease only in chlorophyll \( b \) (figure 46), whereas the amount of chlorophyll \( a \) (figure 45) compensated for this decrease by increasing. *P. decipiens* revealed a significant increase in chlorophyll \( a \) (figure 45), \( b \) (figure 46) and total chlorophyll (figure 44), which
confirmed a pattern of non-intermediate behaviour, neither at ambient, nor at elevated carbon dioxide concentrations. This is different from other intermediate species such as in the C$_3$C$_4$ *Flaveria angustifolia*, *Flaveria sonorensis*, *Flaveria ramossissima* and *Flaveria anomala*, which showed an intermediate chlorophyll a/b ratio under ambient conditions, indicating a development of the C$_4$ syndrome (Ku et al., 1996), here investigated only under ambient conditions. In case of *Panicum decipiens* there is a closer physiological correlation to the C$_3$ than to the C$_4$ (compare figures 44, 45 and 46), as stated by Wilson (1994, personal communication).

An increase in chlorophyll content in plants grown in high CO$_2$ has been reported by several authors (Vu et al., 1989; Drake, 1992), who suggested increased investment in light harvesting and electron transport constituents at elevated carbon dioxide concentrations, which explains the increased growth in this species here due to increased photosynthetic rates.

Others, like Delgado et al. (1994) found no change in total chlorophyll a and b amounts in the winter wheat *Triticum aestivum* grown at 700 ppm CO$_2$. Comparable results by Ingvarsdson and Veierskov (1994), who found no increase in chlorophyll in barley plants grown under 675 ppm CO$_2$. These results could not be confirmed in this study on *Panicum* grasses in response to high carbon dioxide concentrations, which shows again the enormous degree of variation in response to high CO$_2$ between species.
4.5. SUMMARY:

(i) An increase in the amount of starch in all three *Panicum* species was detected quantitatively and qualitatively (structurally) and was correlated with the increase in biomass and leaf area. The high starch content suggests better digestibility and eventually faster production of a given quantity of forage.

(ii) The chloroplast structure did not seem to be affected by an increase of starch in the C\(_4\) and C\(_3/C_4\) plant, but grana appeared to have disintegrated in the C\(_3\) species.

(iii) An increase in dry matter production was associated with changes in photosynthetic capacity in all three *Panicum* species (measured as chlorophyll content and number/proportion of appressed to non-appressed thylakoids). A decrease in chlorophyll content in *P. antidotale* suggests a negative feedback due to rapid growth, accumulation of starch and eventually shading effects, or early leaf senescence. In *P. decipiens* and *P. tricanthum* an increase of the chlorophyll amount was observed which was due to an increased investment in the photosynthetic machinery. A possible negative feedback was not observed.

(iv) The answer to the question concerning the relationship between membrane stacking and chlorophyll \(a/b\) ratio seems straightforward. It has been established that there is an inverse relationship between the extent of
membrane appression and the Chl a/b ratio of sun and shade plants due to the lateral heterogeneity in the distribution of thylakoid complexes between appressed and nonappressed domains (Anderson, 1986). Therefore I hypothesize that Panicum may respond to elevated CO₂ concentrations by maintaining a fixed ratio of appressed granal relative to nonappressed membranes. This is confirmed for all the three species investigated (see table 6) The amount of grana may be higher, but the ratio is clearly maintained.
CHAPTER 5

CONCLUSION AND GENERAL DISCUSSION:

5.1. MONOCOTYLEDONOUS PERENNIAL FORAGE SPECIES WITH DIFFERENT PHYSIOLOGICAL PATHWAYS GROWN UNDER HIGH CO₂: WHERE THIS CAME FROM

There have been very few anatomical studies of leaves from plants growing under elevated carbon dioxide concentrations, and most of these have concerned dicotyledons.

The results of this thesis support the view that growth of monocotyledonous plants at elevated carbon dioxide concentrations might result in changes in the morphogenesis of the individual leaves. A number of studies have noted modest increases in leaf size (Ford and Thorne, 1967), and changes in stomatal density (e.g. Woodward and Bazzaz, 1988).

Little work has been carried out to investigate the possible effects of CO₂ enrichment on altered internal cellular anatomy.

Madsen (1968; 1973). observed in tomato leaves an increase in lamina thickness under high carbon dioxide and attributed this entirely to cell enlargement. Thomas and Harvey (1983) reported that leaf thickness increased by the addition of an extra cell layer in some species at high CO₂. However, it has been recognized that more research is required on leaf area, dry matter and internal anatomy to enable generalizations to be made.

The present morphological and anatomical study investigated the influence of elevated carbon dioxide concentrations on monocotyledonous perennial plants with different photosynthetic pathways. The genus *Panicum* contains important tropical pasture grasses and was chosen because it includes
species with leaf blades of markedly diverse anatomical structure associated with variations in the photosynthetic pathway, i.e. C₄ (P. antidotale), C₃ (P. tricanthum) and C₃/C₄ (P. decipiens). This offered the opportunity to study the concordance of changes in morphology/anatomy with physiological change among related species in response to a single environmental change such as an altered CO₂ concentration.

From an agricultural point of view the investigations were of particular interest because the anatomical structure of forage grasses influences their digestibility, and as a result the daily intake of foraging animals may need to be lowered or raised at different CO₂ levels. This study also shed light on the question of the evolution of the C₄ species and the progression involved in plants with characteristics intermediate between those of C₃ and C₄ species. These intermediate species have been mainly characterized by CO₂ exchange and biochemical analysis, and there is little information either on the anatomical characteristics connected with their intermediate nature or their general response to elevated CO₂.

5.2. C₄ PLANTS CAN RESPOND TO ELEVATED CARBON DIOXIDE CONCENTRATIONS

Many papers and at least one textbook ("Plant Physiology" by Taiz and Zeiger, 1991) strongly suggest that elevated atmospheric carbon dioxide concentration has relatively little effect on the growth of C₄ plants. This opinion was confirmed in the 1980s through publication of two kinds of photosynthetic response curve, where photosynthesis is plotted against either light intensity or CO₂ concentration. Examples are those published by Downton, Bjorkman and Pike (1980, Fig.2.), where leaf responses indicated that C₄ plants will not show as great an increment in yields above a carbon dioxide concentration of approximately 400ppm, whereas C₃ plants will. However, C₄ plants such as Tidestromia oblongifolia do display improved
water use efficiency through the effect of high CO$_2$ on stomatal aperture (Downton et al., 1980).

Reinforcing this general view on C$_4$ plants, Carlsson and Bazzaz (1982) compared three C$_3$ annuals with three C$_4$ annuals, and observed that total plant growth increased under elevated carbon dioxide for the C$_3$ species, but varied only slightly with increased CO$_2$ for the C$_4$ species.

The results for another frequently investigated C$_4$ plant (Zea mays) have been variable. Wong (1980) found little growth response to elevated CO$_2$ concentrations, whereas recently Samarakoon and Gifford (1996) showed that there is little response to extra carbon dioxide under well-watered conditions, the gain becoming appreciable only under more "drying out" conditions, possibly reflecting authentic field situations. Thus earlier studies showing considerable gain for Zea mays (Rogers et al., 1980; Rogers and Dahlman, 1993), and after that, in four C$_4$ grasses (Sionit and Patterson, 1984) have been confirmed.

In conclusion, some C$_4$ species do appear to have the potential to respond to high CO$_2$ concentrations, perhaps because they can increase their internal CO$_2$ concentration further, which could explain the substantial dry matter gains under elevated carbon dioxide concentrations of the C$_4$ species P. antidotale as well as the C$_3$/C$_4$ intermediate P. decipiens. Alternatively the bundle sheath carbon dioxide concentration might not be increased still further in response to increased external carbon dioxide concentrations, and carbon gains might respond on faster throughput. Furbank and Hatch (1987), comparing the inorganic carbon pool sizes for one C$_3$ and six C$_4$ species, including P. maximum and P. miliaceum, found values for internal carbon dioxide and bicarbonate for the bundle sheath that approached 1mM on a whole cell basis (~0.35 and 0.83 respectively for these two species of Panicum).

Agostino et al. (1996) have suggested that the carboxylation potential of bundle sheath cells measured in vitro is not fully realized in vivo under conditions of ambient carbon dioxide.
5.3. THE C₃/C₄ INTERMEDIATE AS A PHYSIOLOGICAL CHIMERA

There is generally no reported effect on leaf morphology or anatomy of growth under elevated carbon dioxide concentrations of C₃/C₄ plants in the earlier literature.

In this study *Panicum antidotale* (C₄) and *Panicum decipiens* (C₃/C₄) have both been found to show increased dry matter gains under elevated carbon dioxide concentrations. The results also indicated increases in leaf area, but no increases in lamina thickness. There were in fact slight decreases in leaf thickness, together with changes in the distributions of tissue types within a leaf transverse section (see table 4).

The responses of this C₃/C₄ intermediate are superior to those of an intermediate *Atriplex* hybrid formed by crossing a C₃ species (*A. patula*) with a C₄ species (*A. rosea*) (Bjorkman et al., 1971). Brown et al. (1986) also obtained hybrids between C₃ and C₃/C₄ intermediate species of *Panicum*, but the C₃/C₄ parental type was never recovered even in the F₅ generation (Bouton et al., 1986).

The results also indicated increases in leaf area, but no increases in lamina thickness. There were in fact slight decreases in leaf thickness, together with changes in the distributions of tissue types within a leaf transverse section (see table 4).

Primarily, the term C₃/C₄ intermediate indicates that one or several features of the photosynthetic apparatus of these plants cannot be clearly classified as C₃ or C₄. It does not directly imply that these plants are evolutionary intermediate between C₃ and C₄.

Investigations on the C₃/C₄ intermediate taxa are generally based on the assumption that C₃/C₄ intermediate plants represent steps in the evolution of the C₄ plants and therefore an investigation of photosynthetic enzymes needs to be carried out in these. Until today it remains unclear whether the four known intermediate grasses *Panicum decipiens*, *Panicum milioides*,
*Panicum spathellosum*, and *Neurachne minor* represent steps towards the C\textsubscript{4}, if they are modifications of the photosynthetic pathway which evolved independently, if they are hydrids or even reversals. However, research on the phylogenetic relationship should prove extremely interesting and investigations are continuing (Sinha and Kellogg, 1996).

The C\textsubscript{3}/C\textsubscript{4} intermediate *Panicum decipiens* displayed clear responses to elevated carbon dioxide concentrations morphologically, anatomically and physiologically as shown in table 7. Being the second C\textsubscript{3}/C\textsubscript{4} intermediate in the genus after *Panicum milioides* to be investigated anatomically, it rarely revealed true intermediate behaviour. In fact, it was intermediate neither in a physiological nor in an evolutionary sense. Therefore the term "intermediate" might be a misnomer, and a term like 'physiological chimera' as suggested by Murray (1997, booktext, in preparation) might be introduced instead.
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Table 7: A comparison of the C₃/C₄ intermediate species (*P. decipiens*) with the C₃ (*P. tricanthum*) and the C₄ (*P. antidotale*)
5.4. CROP POTENTIAL

From the viewpoint of using tropical Panicum species as an energy crop, the reallocation of fixed carbon at elevated CO₂ concentrations to favour carbohydrates for cell wall formation, rather than protein, would be detrimental if the plant is to be used as animal feed. Conversely, a similar redistribution of carbon to favour starch formation, a common phenomenon among CO₂ enriched plants (Huber et al, 1984; Peet et al, 1986), would be beneficial. It seems reasonable to assume that there is a relationship between cell number and cell size and that this relationship varies according to the pattern of growth followed by different species. This may depend on nutrient availability and genetic predisposition of the particular plant which determines whether increasing cell size would be more economical than increasing cell number, which would have high unit costs of synthesizing nitrogen compounds, compared with polysaccharide.

Tropical grasses average about 13 percentage units lower in digestibility than temperate grasses (Minson and McLeod, 1970). A substantial part of this difference might be explained by the fact that tropical grasses grow under higher temperatures than temperate grasses. Another limitation of digestible energy or leaf dry matter digestibility is the anatomical structure of C₃/C₄ and C₄ species due to their high cell wall content associated with Kranz anatomy, which parallels the differences between the tropical C₃ and C₄ grasses in some Panicum species (Wilson et al., 1983). The most digestible C₃ Panicum species (among them P. tricanthum) were approximately 11 percentage units higher in leaf dry matter digestibility than the widespread cultivated C₄ species P. maximum and P. coloratum.

On the other hand, the C₃/C₄ intermediate P. decipiens exhibited a decrease in the proportion of thickened tissues and an increase of starch under elevated carbon dioxide levels, thus offering increased dry matter digestibility. This species, like other C₃/C₄ plants, could also have the advantage of
combining the high light intensity acceptance of the C₄ and the shade tolerance of the C₃, which, in combination with increased carbohydrate content and higher yield potential under a high CO₂ environment, could make it a future crop for more difficult environments.

5.5. **FOR FUTURE INVESTIGATION:**

The potential for C₄ and C₃/C₄ intermediate plants to utilize elevated carbon dioxide should now be systematically re-examined, with a watering regime much closer to field and/or natural conditions.

There are two avenues by which water use efficiency of a crop can be increased. Biomass may be increased or water use may decline. It has been frequently suggested that CO₂ enrichment should increase water use efficiency because elevated CO₂ increases biomass while at the same time causing partial stomatal closure, with a consequent reduction in transpiration (Kimball and Idso, 1983).

The *Panicum* grasses seem to offer this potential by providing an increased biomass production, connected with increased carbohydrate content. These grasses also show increased water use efficiency caused by increases in the deposition in epicuticular waxes.

However, it cannot be decided at this point if this will cause tropical species like *Panicum*, specially when grown under future high carbon dioxide concentrations, to be unsuited or suited for range grazing conditions. Further agronomic studies on these monocots are needed to take into account the effects of elevated carbon dioxide concentration identified here.

The higher the proportion of stem including the increase of internodes within the pasture, the lower will be the proportion of energy transferred to meat, milk or wool production. The long upper internodes of grasses have the lowest dry matter digestibility (DMD) due to greater amounts of lignification.
and degeneration of the central parenchyma; they also support leaves with a high proportion of sheath (Wilson and Minson, 1980, Wilson, 1991) which also has a low DMD. This might cause quality problems in tropical grasses like Panicum which respond with elongation of the stem and an increase of internodes to high CO₂ (see figure). Morphological observations made indicated a certain stiffness of the grasses, which also could be due to increased lignification or enhanced storage of calcium-oxalates, both plant products which would again lower the dry matter digestibility. This is contrary to the finding of an increment in non-structural carbohydrates (starch). Even though starch is not easily digestible, an increased amount of it within the grain or whole plant is thought to raise its DMD. On the other hand, it is possible that the monocotyledonous Panicum species use any carbon pathway available and therefore produce storage products as well as cell wall components. Therefore it would be interesting for future investigations to examine how the actual ratio of the digestible energy of plants grown under elevated carbon dioxide concentrations might be altered. The ratio might be increased, but the question is whether the balance between gain of storage products (like starch) and an increase in woody and therefore lignified tissues with low DMD is not expected to change, unless other genetic alterations may be involved?

From an agricultural point of view a thick cuticle hinders tissue digestion (Wilson, 1976, Akin et al., 1983), because it is indigestible and restricts the access of microorganism to the leaf and consequently affects the nutritional value of the herbage available to the grazing animal. Therefore the DMD in the Panicum species will be lowered through the increase in the density of the epicuticular wax, but the increase in non-structural carbohydrate may compensate for that. Since the wax thickness also decreases the optical transparancy, it is also useful for environments exhibiting high light intensities where this might filter the amount of ultraviolet light entering the tissues and thus offer a protective shield.
An important question for future investigation is whether the major genes controlling glaucousness are subject to considerable modification not only by the environment but also by the genotype as suggested by Johnson et al. (1983). For instance, glaucousness in wheat is controlled by a multiple allelic system of two dominant inhibitors on chromosomes 2B and 2D (Jensen and Driscoll, 1962) These major genes are subject to considerable modification by the environment and in particular the background genotype. In barley, two or more enzyme sites of different fatty acid chain length specificity could be involved in a given wax class. However, the influence of light and temperature on the composition of epicuticular waxes of barley leaves has been shown to depend on two enzymes of different sensitivity to different abiotic factors (Giese, 1975). Therefore the question arises as to whether the formation of epicuticular waxes in Panicum grass species grown under high carbon dioxide can be modified by genetic alterations.
5.6. OUTLOOK

5.6.1 UNDERSTANDING THE EFFECTS OF CO₂ ON THE ENVIRONMENT

The world wide efforts towards a better understanding of the consequences of increased carbon dioxide on plants and vegetation lack investigations of the ecological context and time frames which would allow plants to grow in an CO₂ enriched environment over evolutionary periods or periods which could enable them to achieve full acclimation.

Such situations may be found around geological CO₂ vents (Woodward et al., 1991), in CO₂ rich microsites on respiring substrate or by plants from contrasting altitudes (Koerner and Diemer, 1987 and 1994).

5.6.2. RESTRICTIONS ON MORPHOLOGICAL GROWTH RESPONSES

Whether or not total growth is increased by elevated CO₂ will depend on a number of factors including end product inhibition, size of available sinks, and nutrient availability.

Generally stresses seem to affect one type of organ more than another, and dry matter is preferentially partitioned to certain parts of the plant. Dry matter accumulation is thought to be fastest in those organs nearest the supply of material. For example, during water stress the roots grow faster than other organs. Once again, the mechanism seems to be one for balancing the supply and demand needs of the whole plant. At this point it is useful to discuss the maintenance of a functional balance between organs.

In the unmanaged biosphere, natural selection will determine which plants are best adapted or most fitted for the future high CO₂ world. The plants with
characteristics that ensure greater survival and reproductive advantages over all other plants will be selected. In the managed agricultural biosphere, on the other hand, growers and plant breeders will select plants whose characteristics will enable farmers to obtain the most profit. Those plants or crops will be grown that produce the greatest and most valuable yield in response to high carbon dioxide concentrations with least expense relative to other crops. Therefore, the plants that possess the largest yield response in response to high CO\textsubscript{2} will likely be selected, and such a yield response can be regarded as an adaptation to a higher CO\textsubscript{2} world.

An extrapolation of the extent to which a global increase in carbon dioxide concentrations will affect plant responses is, however, difficult to derive from the present study, largely because in "ideal" environmental conditions there are no other constraints on growth (Neales and Nichols, 1978; Kramer, 1981). Furthermore, as global CO\textsubscript{2} enrichment is a gradual process, it might lead to long term selection processes which cannot be considered here. The present study illustrates a variety of responses effected by an increase of carbon dioxide. For most plants, and especially for economically important plants such as forage grasses, even small effects on growth and photosynthetic responses or transient differences in dry matter partitioning may be ecologically significant because of their importance in competitive interaction during seedling establishment, distribution ranges, and reproductive capacity (Hurd, 1968, Neales and Nichols, 1978).
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7. APPENDIX:

ABBREVIATIONS:

bsc  bundle sheath chloroplast
mc  mesophyll chloroplast
bc  bulliform cell
bs  bundlesheath
ep  epidermis
m  mesophyll
mi  mitochondrion
la  lamellae
sc  sclerenchyma
sg  starch grain
ve  vascular elements
xv  xylem vessel

DMD dry matter digestibility
MORPHOLOGICAL AND STRUCTURAL INVESTIGATIONS INTO C₃, C₄ AND C₃/C₄ MEMBERS OF THE GENUS PANICUM GROWN UNDER ELEVATED CO₂ CONCENTRATIONS

by

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A thesis presented in fulfilment of the requirement for the degree of DOCTOR OF PHILOSOPHY

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ABSTRACT:

Three perennial tropical *Panicum* species were grown under ambient and elevated (900 ppm) carbon dioxide concentrations in especially designed microclimate chambers. The study aimed to investigate the influence of high carbon dioxide concentrations on morphology/anatomy with physiological change among three closely related species possessing distinctly different photosynthetic pathways.

The genus *Panicum*, which contains important tropical forage species, was chosen because it includes species with leaf blades of markedly diverse anatomical structure associated with variations in the photosynthetic pathway. *P. antidotale* was chosen as a representative of the C4 type, *P. tricanthum* of C3 species and *P. decipiens* as a representative of C3/C4 intermediate species. This provides an opportunity to study the changes in morphology/anatomy and their relationship to physiological change among related species responding to a single environmental 'stressor' such as elevated CO₂. Specifically, this investigation examined which photosynthetic type responded most to elevated (900ppm) carbon dioxide.

All species investigated showed significant morphological responses in the parameters of plant dry matter, leaf area, leaf weight, stem height, stem weight, tiller and internode numbers and stomatal frequencies when grown under 900 ppm CO₂. Stomatal sizes, detected using light microscopy, did not change appreciably. The results obtained suggest increases in biomass production and water use efficiency in *Panicum* plants grown under high CO₂.
The leaf surface was examined by scanning electron microscopy (SEM). Cold-stubs technique and critical point drying (CPD) technique were selected as giving the best resolution for viewing the epicuticular wax deposition on an ultrastructural level.

The three species exhibited different pattern in glaucousness, and also showed increases in the deposition of epicuticular waxes under high CO₂. It is proposed that this acts to protect the leaves by filtering the UV light, by enhancement of leaf reflectance and by reduction of transpiration.

The anatomy of the leaf was investigated using light microscopy (LM), transmission electron microscopy (TEM), and graphics image analysis. A suitable schedule for fixation, dehydration and embedding of leaf specimens for both forms of microscopy was developed.

The anatomy of the species investigated did not change qualitatively, but there were detectable changes in leaf thickness and tissue proportions of the epidermis, mesophyll and thickened tissues (sclerenchyma; bundle sheath; vascular elements) that differed with species.

All *Panicum* species showed a marked quantitative increase in starch grains within the chloroplasts as observed using transmission electron microscopy (TEM).

Significant disruption of the chloroplast structure occurred only in *P. tricanthum*. The thylakoid number was not affected by an increased carbon dioxide concentration, but three species responded by maintaining the ratio of appressed to non-appressed thylakoids.

This study is also relevant to the investigation of the evolution of C₄ species, and the progression involved in plants with characteristics intermediate between those of C₃ and C₄ species. These intermediate species have been
mainly characterized by CO₂ exchange and biochemical analysis, but they also display anatomical characteristics in between those of C₃ and C₄ plants. The evolutionary progression of the C₃ to C₄ species remains unsolved, although current studies indicate that the evolutionary step was from the C₃ plant to the C₄. Thus the intermediate C₃/C₄ plants may not be intermediate in an evolutionary sense and they could be seen as a simple hybridization between a C₃ plant and C₄ plant.

In most of the parameters measured the C₃/C₄ *P. decipiens* resembled either the C₃ *P. tricanthum* or the C₄ *P. antidotale*. It may therefore be likened to a physiological chimera rather than to a true intermediate form.
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CERTIFICATE OF ORIGINALITY

The text of this thesis contains no material which has been accepted as part of the requirements for any other degree of diploma in any University, or material previous published or written unless due reference to this material has been made.

Claudia Tipping (nee Decker)

Claudia Decker
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TO ROBERT BRUCE AND SONJA CHLOE TIPPING
INTRODUCTION:

There is no doubt that the increase in atmospheric carbon dioxide CO₂ from about 290 ppm mid 19th century to about 358 ppm at present results from accelerating use of fossil fuel, the harvesting of land for timber, and the conversion of forested land into agricultural production. This projected increase will have a major impact on the growth of plants, irrespective of any change of climate that may accompany this rise. It has been estimated that a valve of 660 ppm CO₂ may be attained sometime next century (Murray, 1995).

This investigation set out to study the influence of elevated CO₂ on monocotyledonous perennial plants with different photosynthetic pathways and which differed from a morphological and structural point of view. The genus *Panicum*, which contains important tropical forage species, was chosen because it includes species with leaf blades of markedly diverse anatomical structure associated with variations in the photosynthetic pathway. *P. antidotale* was chosen as a representative of the C₄ type, *P. tricarthum* of C₃ species and *P. decipiens* as representative of C₃/C₄ intermediate species. This provided an opportunity to study any changes in morphology/anatomy and their relationship to physiological change among related species responding to a single environmental ‘stressor’ such as elevated CO₂. This study is also relevant to the question of the evolution of C₄ species, and the progression involved in plants with characteristics intermediate between those of C₃ and C₄ species. These intermediate species have been mainly characterized by CO₂ exchange and biochemical analysis, but they also display anatomical characteristics in between those of C₃ and C₄ plants. The evolutionary progression of the C₃ to C₄ species is not resolved, and current studies indicate that the evolutionary step was from
the C₃ plant to the C₄. Thus the C₃/C₄ plants may not be intermediate in an evolutionary sense and they could be seen as a simple hybridization between a C₃ plant and C₄ plant.

It is possible that under higher CO₂ concentrations anatomical features within the species may respond with changes in their structure or ultrastructure. From an agricultural point of view the investigations are of particular interest because the leaf anatomical structure of forage grasses is related to their digestibility, and as a result the nutritional value can be lowered or raised. A greater carbon intake at the carboxylation sites within species grown under elevated carbon dioxide concentrations could have a dramatic effect and are expected to elicit higher cell wall content resulting in lower digestibility. On the other hand, it should be possible to improve forage quality if the extra carbon is assimilated into storage products and leads to increases in biomass and leaf area.

Within this context, this thesis has the following objectives:

1) **GENERAL OBJECTIVES:**

- To investigate the influence of high carbon dioxide concentrations on monocotyledonous perennial plants with different photosynthetic pathways
- To examine which photosynthetic pathway was most responsive to the elevated carbon dioxide concentration
2) **SPECIFIC OBJECTIVES:**

- To determine whether a change in morphological features occurs at elevated CO₂ and
- To determine how any observed morphological changes correspond to changes in the anatomical structure (micro- and/or ultramicroscopical structure)
- To ascertain whether the role of extra photosynthates obtained by extra carbon gain are allocated to cell wall production or to accumulation of storage products such as starch or both
- To determine via investigation of the chloroplast structure whether a change in photosynthetic capacity occurs under high concentrations of CO₂