Drought responses of C_3 and C_4 (NADP-ME) Panicoid grasses

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Abstract

The success of C_4 plants lies in their ability to concentrate CO_2 at the site of Rubisco thereby conferring greater efficiencies of light, water and nitrogen. Such characteristics should advantage C_4 plants in arid, hot environments. However, not all C_4 subtypes are drought tolerant. The relative abundance of NADP-ME species declines with increasing aridity. Furthermore, selected species have been demonstrated as being susceptible to severe drought showing metabolic limitations of photosynthesis. However there is a lack of phylogenetic control with many of these studies. The aims of this study were to determine whether the NADP-ME subtype was inherently susceptible to drought by comparing six closely related C_3 and C_4 (NADP-ME) Panicoid grasses. Gas exchange measurements were made during a natural rainless period and a controlled drought / re-watering event. Prior to water stress, the C_4 species had higher assimilation rates (A), and water use efficiencies (WUE_{leaf}) than the C_3 species, while transpiration rates (E) and stomatal conductances (g_s) were similar. At low soil water content, the C_3 species reduced g_s by a greater extent than the C_4 species, which maintained higher E during the driest periods. The C_4 species showed proportionally greater reductions in A than the C_3 species and hence lost their WUE_{leaf} and photosynthetic advantage. CO_2 response curves showed that metabolic limitation was responsible for a greater decrease in A in the C_4 type than the C_3 type during progressive drought. Upon re-watering, photosynthetic recovery was quicker in the C_3 species than the C_4 species. Results from whole plant measurements showed that the C_4 type had a significant whole plant water use efficiency advantage over the C_3 type under well-watered conditions that was lost during severe

drought due to a greater loss of leaf area through leaf mortality rather than reductions in plant level transpiration rates. The C_3 type had xylem characteristics that enhanced water-conducting efficiency, but made them vulnerable to drought. This is in contrast to the safer xylem qualities of the C_4 type, which permitted the endurance of more negative leaf water potentials than the C_3 type during low soil water content. Thus, the vulnerability of photosynthesis to severe drought in NADP-ME species potentially explains why NADP-ME species abundance around the world decreases with decreasing rainfall.

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Chapter 1: Introduction

Rationale

C_4 photosynthesis is a combination of anatomical, biochemical and physiological modifications that concentrate CO_2 at the site of Rubisco. Significant variations exist on this basic theme as characterized by the three-biochemical subtypes: NADP-ME, NAD-ME and PCK (described below). The C_4 mechanism confers potentially greater efficiencies of light, nitrogen and water use on C_4 species relative to C_3 species. Thus, it would appear that C_4 plants would have the greatest advantage over C_3 plants in arid, hot environments or under other environmental conditions that enhance photorespiration. However, not all C_4 subtypes are drought tolerant. The NADP-ME subtype shows a positive correlation to annual rainfall with numbers of species declining as aridity increases (Ellis *et al*. 1980 and Taub 2000). In addition, Ripley *et al.* (2007) demonstrated that the C_4 subspecies of *Alloteropsis semialata* was susceptible to severe drought, demonstrating that metabolic effects on photosynthesis reduced its photosynthetic capacity more than was observed for the C_3 subspecies. Furthermore, Ghannoum *et al*. (2002) found that under well-watered conditions, the water use efficiencies of nine NAD-ME and nine NADP-ME Australian C_4 grasses were similar. However, under drought conditions, the NAD-ME species had significantly higher water use efficiencies than the NADP-ME species.

It is the paradox between C_4 water use efficiency and the apparent inability of NADP-ME grasses to cope with severe drought that is the central theme of this thesis. This is considered in the context of both present-day conditions and for the role that it may have played in the evolution and expansion of C_4 grasslands.

This topic is pursued by asking the following questions: 1) is the photosynthetic and water use drought sensitivity of the C_4 subspecies of *Alloteropsis semialata* unique to just this species or can it be generalized to other C_4 species belonging to the NADP-ME

photosynthetic subtype? 2) Does the observed trend in the drought sensitivity of the NADP-ME subtype hold true when comparing species within the same subfamily? 3) And, what is the mechanism of this hypothesized drought sensitivity?

This study attempted to answer these questions by comparing six closely related C_3 and C_4 (NADP-ME) Panicoid grasses, monitoring both the field response of plants over a growing season that included rainless periods, and the response of potted material to a controlled drought and subsequent re-watering event.

Introduction

In order to explain the observed responses and their underlying mechanisms, it is necessary to review the biochemical and physiological differences between the photosynthetic types and the biochemical subtypes of C_4 photosynthesis. It is important to understand how and why C_4 photosynthesis evolved and the ecological implications of this evolution as seen today.

Approximately 8,000 of the 250,000 higher plant species use the C_4 photosynthetic pathway (Sage *et al*. 1999a). These plants are far more important than their numbers imply. They contribute about 25% of the world's primary productivity and comprise some of world's most important crops, including maize (*Zea mays*), sorghum (*Sorghum bicolor*), sugarcane (*Saccharum officinarum*), common millet (*Panicum miliaceum*) and teff (*Eragrostis teff*). They account for 70% of the grains grown in Africa and 30% worldwide (Brown 1999). C_4 plants dominate nearly all of the tropical, subtropical and warm temperate grasslands, but they are also well represented in disturbed and arid landscapes in the warm regions of the world (Sage *et al*. 1999b).

The importance of these plants to people around the world makes it crucial for us to accurately predict how tropical agriculture and grassland ecosystems will fare in the future. Anthropogenic increases of CO_2 concentrations should favor C_3 species, but the interaction of global warming, the timing and variation of precipitation and CO_2

enrichment will be important in determining the changes in the C_3 / C_4 dynamic (Sage and Kubien 2003). One approach to this problem is to first understand leaf level mechanisms and how they are impacted by environmental changes so we can scale up this knowledge to landscape processes.

The C_4 photosynthetic mechanisms

The success of C_4 plants lies in the ability to increase photosynthetic efficiency under conditions that promote photorespiration. C_4 plants are able to reduce the oxygenation activity of Rubisco by keeping it at near saturating CO_2 levels through specialized anatomical features and modifications of photosynthetic mechanisms. This results in a considerable advantage in terms of potential photosynthetic rates, and potentially greater efficiencies of light, nitrogen and water use. The underlying mechanisms of these characteristics are biochemical, physiological and anatomical.

The characteristic Kranz anatomy of most C_4 leaves is a wreathlike structure of cells comprising an outer layer derived from mesophyll cells that are in direct contact with the intercellular airspaces, and an inner layer, commonly referred to as the bundle sheath, which is positioned closer to the vascular tissue (Sage 2004). The bundle sheath cells are larger in C_4 plants relative to C_3 plants and they contain large, numerous chloroplasts. The mesophyll cells of C_4 plants are similar to C_3 plants, but they are enlarged radially such that contact with the bundle sheath cells is maximized. Mesophyll cells are rarely greater than 2 or 3 cells away from a bundle sheath cell (Sage 2004). An extensive network of plasmodesmata allows metabolites to diffuse freely between the two cell types. At the same time the Kranz anatomy structure of C_4 leaves divides the labor of these two cell types (Hatch and Osmond 1976). The mesophyll contains enzymes responsible for the initial fixation of CO_2, while the bundle sheath is modified to contain the photosynthetic carbon reduction cycle (PCR).

C_4 photosynthesis starts as CO_2 enters the mesophyll cells and is quickly converted to bicarbonate by carbonic anhydrase. Phosphoenolpyruvate (PEP) carboxylase uses

bicarbonate and PEP creating the four-carbon compound oxaloacetate (OAA) (O'Leary 1982). There are a few advantages of using PEP carboxylase to assist the initial assimilation reaction. Firstly, PEP carboxylase has a higher affinity for CO_2 than Rubisco. Secondly, the Michaelis-Menten constant (k_m) of Rubisco for CO_2 is 650 μbar, while the k_m of PEP carboxylase for CO_2 is only 80 μbar (von Caemmerer and Furbank 1999). Finally, PEP carboxylase has no oxygenase activity to offset CO_2 fixation unlike Rubisco. OAA is converted into either malate or aspartate and shuttled into the bundle sheath cells where it is decarboxylated to generate CO_2. The CO_2 is reduced to carbohydrate via the PCR cycle. Rubisco and other PCR enzymes responsible for carbon reduction are localized in the bundle sheath. The concentration of CO_2 in the bundle sheath is high enough to nearly saturate Rubisco, thus overcoming the oxygenation activity of the enzyme. The three-carbon acid pyruvate or alanine (the transamination of pyruvate), formed by decarboxylation of the C_4 acid is returned to the mesophyll and regenerated back to PEP.

Variations of C_4 photosynthesis

C_4 species can be divided into three distinct groups or subtypes: NADP-ME, NAD-ME and PCK based on anatomical and biochemical differences. They are named after the enzymes that catalyze their decarboxylation reaction. The NADP-ME subtype converts OAA into the C_4 acid malate in the mesophyll chloroplasts and transports it to the bundle sheath. Malate undergoes oxidative decarboxylation in the chloroplasts of the bundle sheath using the NADP dependent malic enzyme (Figure 1.1 *a*). Pyruvate is the three-carbon acid formed after decarboxylation that is returned to the mesophyll. The NAD-ME subtype transaminates OAA into aspartate in the cytosol and transports it to the bundle sheath (Figure 1.1 *b*). Aspartate is first reconverted to OAA in the mitochondria and then reduced and decarboxylated by the NAD dependent malic enzyme. Pyruvate (the product of decarboxylation) is converted into alanine and returned to the mesophyll. The PCK subtype also transaminates OAA into aspartate in the cytosol and shuttles it to the bundle sheath (Figure 1.1 *c*). Asparate is converted back to OAA in the cytosol and

decarboxylated by the enzyme, PEP carboxykinase. Pyruvate (the product of decarboxylation) is converted into alanine and returned to the mesophyll.

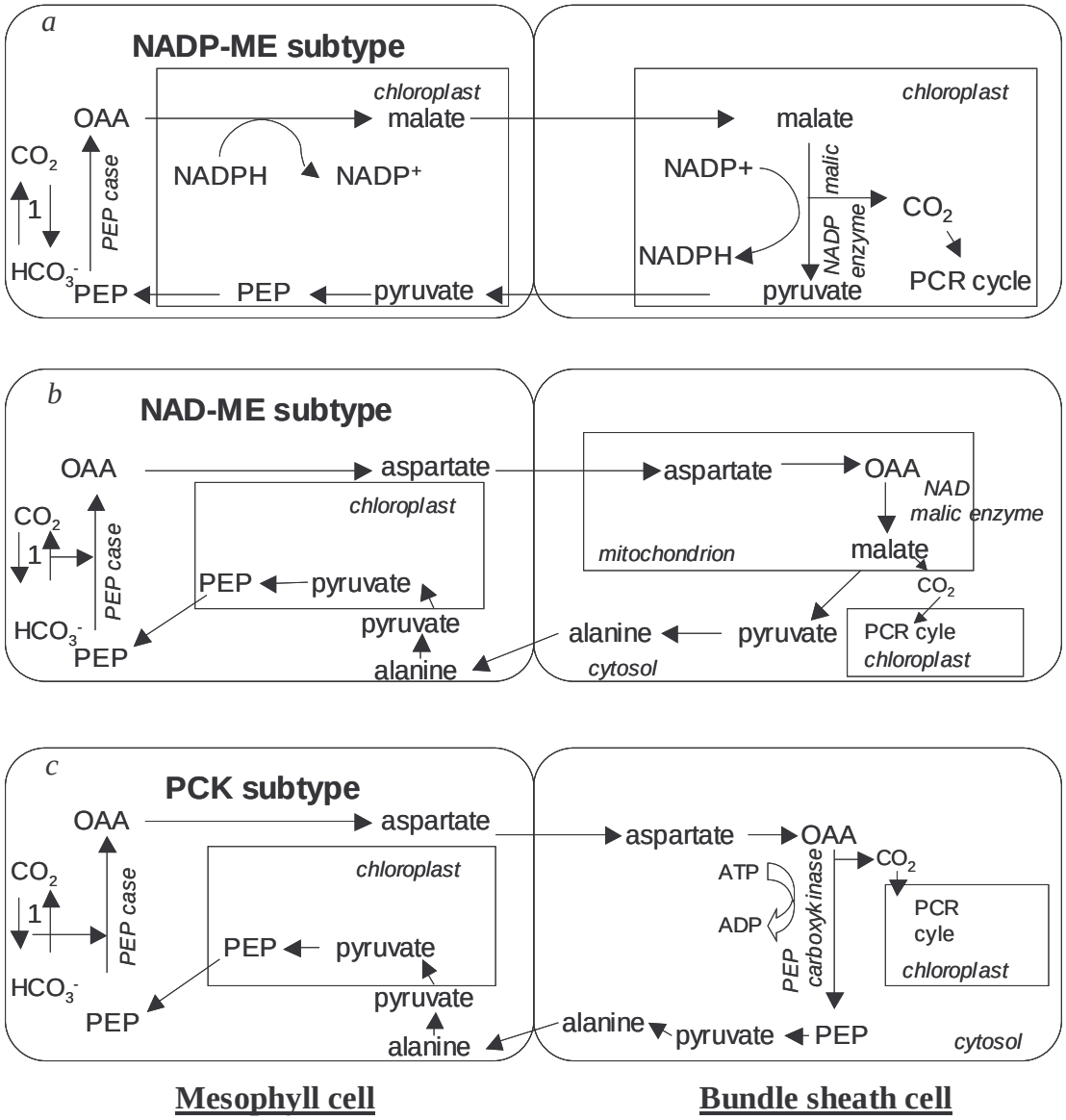

Figure 1.1: Comparison of the biochemistry of the three C_4 photosynthetic subtypes. The subtypes are named after the enzymes that catalyze their decarboxylation reaction. Other differences include the C_4 acid that is shuttled from the mesophyll to the bundle sheath, the organelles where decarboxylation occurs and the product returned to the mesophyll after decarboxylation. 1 = carbonic anhydrase (Modified, Lawlor 2001).

The structure of the bundle sheath, arrangement of the organelles inside the bundle sheath and the uptake and production of O_2 inside the bundle sheath are unique to each of the subtypes. The NADP-ME subtype has a single suberized bundle sheath around the vascular bundle that is uneven in outline. The bundle sheath chloroplasts are arranged centrifugally within the cell (Hatch *et al.* 1975). These chloroplasts have reduced grana reflecting low Photosystem II activity. They synthesize ATP by cyclic photophosphorylation using only Photosystem I (Lawlor 2001). In this process, electrons are cycled from Photosystem I back to the electron transport chain. Water does not need to be split; therefore no O_2 is evolved. The combination of a suberized bundle sheath wall and low Photosystem II activity keeps the ratio of CO_2/O_2 very high in the bundle sheath in this subtype.

The NAD-ME subtype has a double bundle sheath consisting of an outer sheath that lacks suberin, forms a smooth outline and is the site of PCR activity. The inner sheath is referred to as the mesotome sheath and is derived from vascular meristem tissue. The frequency of mitochondria to chloroplasts in the bundle sheath is the highest amongst the C_4 subtypes (Hatch *et al.* 1975). These mitochondria have well-developed internal membrane systems thought to deal with the large fluxes of metabolites between the mitochondria and the cytoplasm and because of the integral role they play in the decarboxylation reaction (Hatch *et al.* 1975). The NAD-ME subtype contains chloroplasts with well-developed grana that are centripetally arranged within the bundle sheath alongside the mitochondria (Hatch *et al.* 1975). The higher rate of O_2 uptake in the NAD-ME subtype as compared to the NADP-ME subtype may be due to pseudocyclic photophosphorylation that produces additional ATP needed for the C_4 cycle (Lawlor 2001). This process uses Photosystems I and II and passes electrons to O_2 as the terminal electron acceptor. The reduction of O_2 ultimately synthesizes water and evolves O_2.

The PCK subtype has a double bundle sheath both of which contain suberin. The outer sheath wall is much less regular in size and shape than the NAD-ME subtype and the mitochondria and chloroplasts are much more evenly distributed in the periphery of the bundle sheath (Hatch *et al.* 1975). The PCK subtype has bundle sheath Photosystem II

activities similar to C_3 plants (Kanai and Edwards 1999). Mitochondrial respiration inside the bundle sheath generates the additional ATP needed for the C_4 cycle, which contributes greatly to the higher rate of O_2 uptake in this subtype as compared to the NADP-ME subtype (Kanai and Edwards 1999).

Even though some oxygen production occurs in the bundle sheaths of these subtypes, the ratio of CO_2/O_2 remains high enough such that the oxygenation activity of Rubisco is much slower in these subtypes that it is in C_3 plants (Kanai and Edwards 1999).

C_4 attributes

The C_4 mechanism confers a range of attributes that have been ascribed as the reason for the past and present success of these species: higher photosynthetic rates and carboxylation efficiencies than C_3 plants, suppression of photorespiration at high temperatures, increased quantum yield relative to C_3 plants under low CO_2 concentrations and high temperatures, and efficient water use through lower stomatal conductances while fixing CO_2 at rates equal to or greater than C_3 plants. These are discussed in the following paragraphs.

Photorespiration

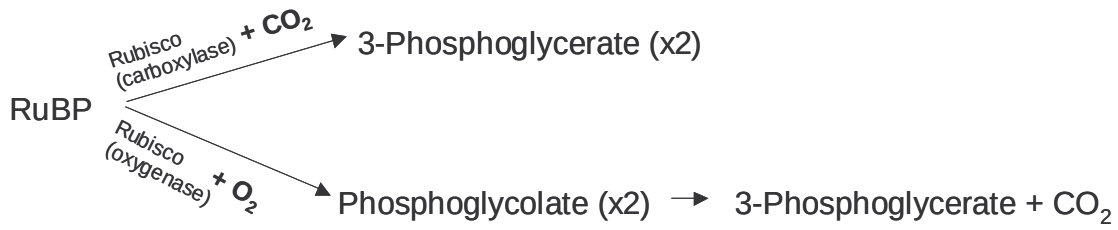

Figure 1.2: A simple representation of carboxylation and oxygenation reactions of Rubsico and RuBP**.** In the photorespiratory reaction, two molecules of phosphoglycolate (a total of four carbons) are needed to make one molecule of 3-phosphoglycerate (a three carbon compound) and one molecule of CO_2.

The enzyme Rubisco catalyzes the initial reaction of the PCR cycle of C_3 photosynthesis. CO_2 reacts with ribulose 1-5-bisphosphate (RuBP) to form two molecules of 3-phosphoglycerate, most of which are reduced to carbohydrate in the PCR cycle. However, Rubisco is also able to oxygenate RuBP (Figure 1.2). This is the primary reaction in a process known as photorespiration (it is called photorespiration because the process is light dependent). The initial chloroplastic product, phosphoglycolate is recycled and returned to the Calvin cycle as phosphoglycerate. This involves the metabolism in three organelles: chloroplasts, peroxisomes and mitochondria and results in the production and release of CO_2. Theoretically, for every two molecules of phosphoglycolate formed, one molecule of CO_2 or 25% of the carbon is lost in its conversion into 3-phosphogylcerate. This process diminishes net CO_2 uptake and leads to the consumption of NADPH (or NADH) and ATP from the light reactions, lowering the effective quantum yield of CO_2 fixation (Collatz *et al.* 1998).

The magnitude of the decrease in net CO_2 uptake depends on several factors: the kinetic properties of Rubisco, the concentrations of the substrates CO_2 and O_2 and temperature. As temperature increases, the solubility of CO_2 is reduced relative to O_2 and the availability of CO_2 as a substrate decreases. Compounding this effect the kinetic properties of Rubisco are influenced by temperature increases, which also increases the ratio of oxygenase activity to carboxylase activity of Rubisco (Ku and Edwards 1977). Photorespiration can inhibit photosynthesis by over 30% at warmer temperatures and current atmospheric conditions (Sage 2004). C_4 photosynthesis nearly suppresses photorespiration by concentrating CO_2 at the site of Rubisco in the bundle sheath cells, thus enabling C_4 plants to photosynthesize more efficiently than C_3 plants at higher temperatures. Thus this explains why C_4 grasses dominate in the semi-arid tropics and subtropics (Sage 2004).

Quantum yield

Quantum yield, also referred to as light efficiency, is the leaf level ratio of moles of CO_2 fixed per moles of photons absorbed (Ehleringer and Bjorkmann 1977). Changes in

quantum yield for CO_2 uptake are largely driven by changes in photorespiration, which is influenced by temperature and CO_2 concentrations. The quantum yield of C_3 plants decreases as temperatures increase reflecting a stimulation of photorespiration by temperature. Photorespiratory activity at higher temperatures can be suppressed by increasing CO_2 concentrations, thereby increasing quantum yield (Ehleringer *et al.* 1997). In contrast to C_3 plants, the quantum yield for CO_2 uptake in C_4 plants remains constant with temperature and CO_2 concentrations over the biologically relevant range of temperatures (Ehleringer *et al.* 1997). The maximum quantum yields measured in C_3 and C_4 plants are similar under current atmospheric CO_2 levels at around 25° C, with the C_3 having an additional investment in photorespiratory activity and C_4 plants having an additional investment of the C_4 cycle (Kanai and Edwards 1999). Higher temperatures at current atmospheric CO_2 levels or subatmospheric CO_2 levels at moderate temperatures will decrease the maximum quantum yield in C_3 plants relative to C_4 plants due to the increased oxygenase activity of Rubisco (Kanai and Edwards 1999).

Ehleringer and Pearcy (1983) showed that the quantum yield of fourteen different C_3 species showed little variation when measured under normal atmospheric conditions (330 μl l^{-1} CO_2, 21% O_2) at 30°C, but there was significant variation amongst the C_4 species surveyed. They speculated that the differences among the C_4 species might be due to two possibilities: the differential energy requirements and the differential rates of CO_2 leakage from the bundle sheath of the three biochemical subtypes of C_4 photosynthesis. Both of these possibilities would reduce quantum yield. The energy requirements of the C_4 subtypes are determined by decarboxylation enzymes and transport of metabolites between the mesophyll and bundle sheath. The NADP-ME and NAD-ME subtypes have similar energy requirements; 5 ATP and 2 NADPH are required per CO_2 assimilated, but the calculation for the PCK subtype is complicated by the coordination of PEP carboxykinase and NAD-malic enzyme in the decarboxylation step, thus making the relative stochiometries uncertain (Kanai and Edwards 1999).

The energy requirements of the C_4 subtypes are also complicated by O_2 uptake rates as determined by the processes used to produce the additional ATP needed for the C_4 cycle

(Kanai and Edwards 1999). As was explained earlier, the NADP-ME subtype uses cyclic photophosphorylation, which does not evolve O_2, keeping photorespiratory activity at a minimum. The higher rate of O_2 uptake in the NAD-ME subtype as compared to the NADP-ME subtype is due to the generation of ATP via pseudocyclic photophosphorylation and / or higher oxygenase activity of Rubisco in the bundle sheath (Kanai and Edwards 1999). The additional ATP needed by the PCK subtype is generated by mitochondrial respiration, which contributes greatly to O_2 uptake.

The differential CO_2 leakage rates observed in the C_4 subtypes is a result of the presence or absence of a suberized bundle sheath outer wall, which inhibits CO_2 leakage. Some of the CO_2 released in the decarboxylation of the C_4 acid in the bundle sheath may diffuse back into the mesophyll. Additional ATP is required to "refix" this CO_2 into a C_4 acid, thus lowering quantum yield (Ehleringer and Pearcy 1983). The NADP-ME and PCK subtypes have this feature, but the NAD-ME does not.

Ehleringer *et al*. (1997) discussed how interveinal distances in grass leaves should affect quantum yields since quantum yield reflects the ratio of photosynthetic CO_2 capture relative to photon capture. They state that by decreasing the number of mesophyll cells in the interveinal spaces across a leaf, quantum yield should increase because these cells contribute little to photon capture when activities are scaled to the leaf level and expressed on a projected area basis. Among C_4 grasses, interveinal distances in NADP-ME grasses are shorter than NAD-ME grasses, which correlate nicely with the reported higher quantum yield in NADP-ME versus NAD-ME grasses. Ehleringer *et al*. (1997) speculated that the differential distribution patterns of the C_4 subtypes within grasslands around the world is consistent with the higher quantum yield of the NADP-ME grasses providing a competitive edge over the NAD-ME grasses in ecosystems with higher productivities (discussed more later).

Water use efficiency

The stomata are pores that control the gas exchange between a plant and its environment. Through these stomata, CO_2 diffuses into the leaf for photosynthesis and water vapor exits through the transpiration stream facilitating the uptake and movement of important solutes and also the evaporative cooling of the leaf. This is a huge trade-off for a plant because several hundred molecules of water are lost from the leaf for each CO_2 molecule taken up (Raschke 1979). Water use efficiency (WUE_{leaf}) is a parameter used to describe the effectiveness of a plant in moderating the loss of water though transpiration while allowing sufficient CO_2 uptake for photosynthesis.

Fick's law of diffusion of gases in air governs the evaporative flux (transpiration) of water vapor from leaves. A plant is able to control the area available for vapor diffusion through the opening and closing of the stomata; therefore transpiration has units related to leaf area (mmol H_2O m^{-2} s^{-1}).

The value of transpiration rate (E) is given by:

$E = g_w (w_i\text{-}w_a)$

where g_w is the conductance for water vapor through the diffusional pathway that is largely controlled by stomatal conductance (g_s) and $w_i\text{-}w_a$ is the difference between the molar fraction of water vapor between the intercellular airspaces of the leaf and the atmosphere (Tyree 1999).

This equation can be applied to photosynthetic rate (A):

$A= g_c (c_a\text{-}c_i)$

where g_c is the conductance for CO_2 through the diffusional pathway and $c_a\text{-}c_i$ is the difference between the molar fraction of CO_2 between the intercellular airspaces of the leaf and the atmosphere.

$g_c = g_s/1.6$ to correct for the slower diffusion of CO_2 than water vapor

Now, WUE_{leaf} can be written as: photosynthetic rate / transpiration rate (A/E).

$$A/E = \frac{(c_a - c_i)}{(w_i - w_a)} 1.6$$

g_s varies with irradiance, leaf temperature, vapor pressure deficit and CO_2 concentrations (Cowan 1977). Any changes in g_s impact photosynthesis and transpiration directly. Decreased g_s induces a strong negative feedback on photosynthesis. Stomatal closure causes c_i levels to drop; the drop in c_i increases the CO_2 limitation on photosynthesis and photosynthetic rates decrease. Transpiration is affected in a similar way. Water vapor loss causes evaporative cooling that lowers the vapor pressure of the intercellular airspaces of the leaf, decreasing the vapor gradient between the inside of the leaf and the air causing transpiration to decrease, which results in the subsequent overheating of the leaf (Raschke 1979).

The relationship between transpiration rate and g_s is proportional if boundary layer conductance of the leaf is infinite and the water vapor gradient between the intercellular airspaces of the leaf and the atmosphere is constant. Therefore, the graphs of E vs. g_s for C_3 and C_4 plants will be the same under these conditions (Figure 1.3 *right*). On the other hand, photosynthesis will increase linearly (initially) to an increase in g_s as the inhibition of low c_i levels is overcome, and will eventually saturate under high light intensities because of other factors, namely changes in the rate of RuBP (C_3) or PEP (C_4) regeneration, changes in the rate at which triose phosphates are utilized (C_3) or an electron transport limitation in both types (von Caemmerer and Furbank 1999 and von Caemmerer 2000; Figure 1.3 *left*). At low g_s, the C_4 plant is not as inhibited by low c_i levels as the C_3 plant because PEP is has a higher affinity for CO_2 than Rubisco and is therefore, very efficient at assimilating CO_2 from very low concentrations. The suppression of photorespiration allows C_4 plants to achieve higher photosynthetic rates relative to C_3 plants. As g_s increases so does the photosynthetic advantage of C_4 up to a point.

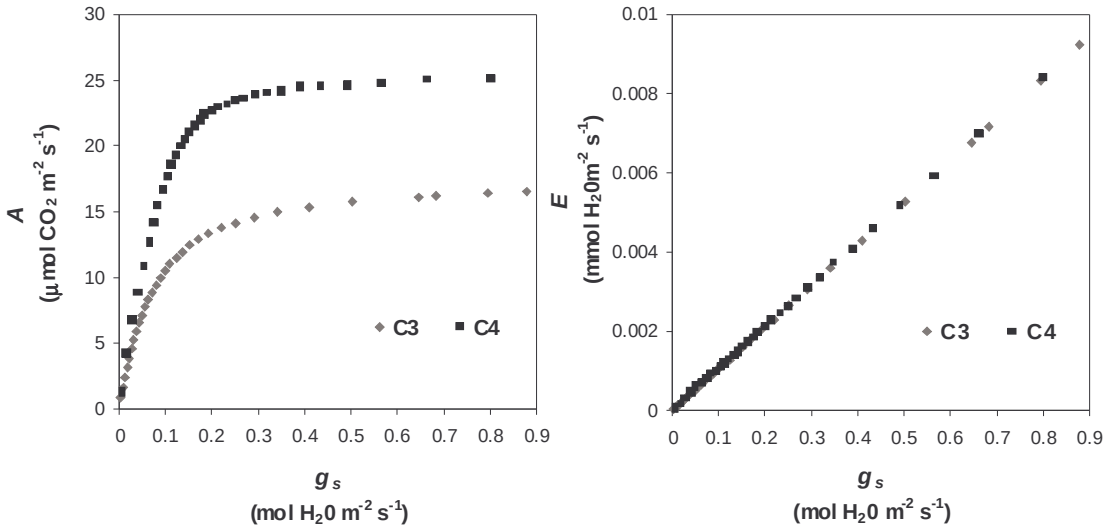

Figure 1.3: Theoretical model of how stomatal conductance (g_s) affects photosynthetic (*left*) and transpiration (*right*) rates of C_3 and C_4 grasses**.** The photosynthetic advantage of C_4 plants increases as g_s increases. Transpiration rates of both types are largely dependent on the water vapor gradient (assuming the leaf boundary layer conductance is infinite). In this case, 1 kPa was used to generate the graph. Photosynthetic rates were calculated using the equations from von Caemmerer (2000). Refer to chapter 3 'methods and materials' for these equations. Transpiration = g_s x VPD.

WUE_{leaf} of the C_4 plant will be consistently higher than the C_3 plant over a range of g_s assuming infinite boundary layer conductance and constant leaf-to-air vapor pressure deficit (Figure 1.4). As g_s increases, the WUE_{leaf} of the C_3 plant decreases more steeply because photosynthesis saturates more quickly than the C_4 plant, while transpiration continually increases.

Theoretically the WUE_{leaf} advantage of a C_4 plant is due to a higher photosynthetic rate than a C_3 plant if the environmental conditions are such that both types are transpiring at the same rate. This is clearly demonstrated by comparing the photosynthetic rates of the two types at a g_s of 0.2 mol H_2O m^{-2} s^{-1} (Figure 1.3). The C_4 plant has twice the photosynthetic rate of the C_3 plant thus enabling it to improve its $WUE_{leaf.}$ However, under the same atmospheric conditions, g_s is on average about 40% lower in C_4 plants than C_3 plants (Long 1999). The affinity of PEP carboxylase for HCO_3^- is so great that it is effectively saturated at ambient CO_2 concentrations, thus enabling C_4 plants to reduce stomatal aperture while fixing CO_2 at rates equal to or greater than C_3 plants and thereby

conserving water and improving WUE_{leaf} (Taiz and Zeiger 1991). Both of these examples convey how C_4 plants are better able to exploit more arid environments than C_3 plants.

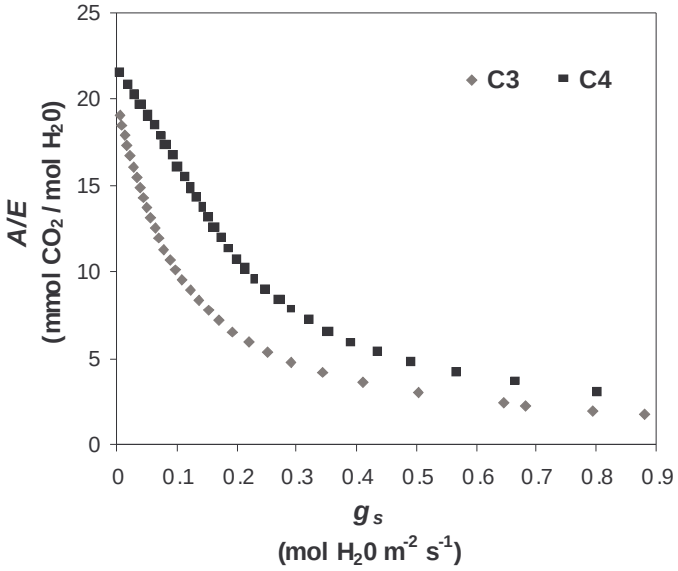

Figure 1.4: Theoretical model of how g_s affects WUE_{leaf} in C_3 and C_4 grasses (assuming infinite leaf boundary layer conductance and constant leaf-to-air vapor pressure differential). At low g_s, WUE_{leaf} of both types is the highest and this value decreases as g_s increases. The C_4 type maintains its WUE_{leaf} advantage over the C_3 type over a range of g_s.

The Link between Hydraulic and Stomatal Conductances

The soil-plant-atmosphere-continuum is the pathway of water from the soil, through the plant and into the atmosphere. Ohm's Law has been applied to whole plant hydraulics because a continuous "current" of water (or water vapor) flows through a plant across a series of potential differences in the root, xylem, leaf and stomatal cells, everywhere meeting a corresponding resistance (Tyree 1999). For example, the potential difference in the xylem vessels is a pressure gradient and the resistance is dependent on the width and the length of the vessels. Xylem with wider and longer conduits will have less resistance than xylem with narrower and shorter conduits. According to van den Honert (1948), the successive transport of water through the components of a plant may be considered as a catenary process, where the slowest partial process governs the velocity of the whole. The resistance of the stomata to water vapor diffusion is greater than any resistance to the movement of liquid water encountered in the roots, stems or leaves. Hydraulic conductance is the inverse of resistance; it is the flow rate of liquid water through the plant divided by the change in hydraulic pressure, which is driving the flow.

Water deficits develop in plants when the water lost from the leaves through transpiration is greater than the absorption of water from the roots. Plants preserve the hydraulic soil-leaf continuum by regulating gas exchange. Stomata function to regulate leaf water status by balancing transpirational flow to the supply of water through the xylem. Without this regulation, damaging decreases in plant water potential develop and result in the formation of embolisms (gas bubbles) in the xylem vessels, which ultimately lead to cavitations (the breaking of the water column), rendering the these vessels temporarily or permanently dysfunctional (Sperry *et al*. 2002). Consequently, hydraulic conductance is severely compromised. Changes in hydraulic conductance do not directly affect stomatal conductance, rather they induce changes in the water status within the leaf. This effect, coupled with high evaporative demand and low soil moisture indirectly drives stomata to close which affects both transpiration and carbon assimilation (Sperry *et al.* 2002).

C_4 plants are less sensitive to stomatal closure than C_3 plants and since C_4 plants require less water than C_3 plants for a given photosynthetic rate, one might predict that they need less conductive tissue and have lower plant hydraulic conductances than C_3 plants. Kocacinar and Sage (2003) suggested that the secondary consequence of C_4 eudicots having higher WUE_{leaf} than C_3 eudicots is to allow the modification of xylem structure and function in improving hydraulic safety and / or enhancing photosynthetic potential depending on the environment in which they are growing. In an arid environment, a C_4 plant may have safer xylem that has less flow capacity, but is less vulnerable to cavitations. In a mesic environment, a C_4 plant's photosynthetic potential may be enhanced by having a larger leaf area per unit of xylem.

The evolution of C_4 photosynthesis

Throughout most of the Earth's history, the atmospheric CO_2 levels were high enough to saturate Rubisco and limit the oxygenation of RuBP (Cerling *et al.* 1997). However, uplift of the Tibetan plateau and increased chemical weathering of the late Cenozoic may have triggered global climate change including the decrease of atmospheric CO_2 (Raymo and Ruddiman 1992). Some researchers have speculated that this decline in CO_2 attributed to the global expansion of C_4 plants in the late Miocene (Cerling *et al.* 1997, Ehleringer *et al.* 1991). However, new data collected has suggested that falling CO_2 was an insufficient driver of C_4 expansion (Pagani *et al.* 1999, Huang *et al.*2001, Osborne and Beerling 2006). Low latitude aridity and changes in seasonal precipitation and temperature exerted stronger controls over the expansion of C_4 plants (Pagani *et al.* 1999)

A suite of traits: biochemical, anatomical and genetic had to be acquired or modified for the evolution of C_4 photosynthesis, yet it has evolved independently and in unrelated families many times. C_4 photosynthesis occurs in fifteen eudicot families and three monocot families (Sage *et al.* 1999a). Extensive studies of anatomy, histology, biochemistry and gene expression have demonstrated that C_4 photosynthesis did not evolve the same way each time it originated in the grass family (Sinha and Kellogg 1996). This point is clearly featured in the differences discussed earlier between the

three-biochemical subtypes of C_4 photosynthesis. Sinha and Kellogg (1996) discovered that the only commonality amongst the origins was the up regulation of PEP carboxylase and the down regulation of Rubisco in the mesophyll. Histologically, the only common element was reduced spacing between the veins. C_4 photosynthesis also arose independently several times in the subfamily Panicoideae (Poaceae) (Giussani *et al.* 2001). Within this subfamily, NAD-ME subtype evolved once, as did the PCK subtype, while all other origins are NADP-ME (Giussani *et al.* 2001)

Past and present distribution of C_4 grasses

The ability of C_4 photosynthesis to nearly suppress photorespiration at high temperatures and low CO_2 concentrations and the associated benefits of this: high photosynthetic efficiency, high water use efficiency and high quantum yields, have been the foundation for explaining past and present distributions of C_4 grasses.

The C_4 savannahs of the present day tropics, subtropics and the warm temperate zones comprise one-eighth of the Earth's surface area (Long 1999). The abundance of C_4 grass species seems to be dependent upon latitude with most occurring in regions of low latitude (Long 1999). Many studies have been conducted around the world to determine the major factors that influence present-day C_3 and C_4 grass distributions (Teeri and Stowe 1976, Vogel *et al.* 1978, Tieszen *et al.* 1979, Boutton *et al.* 1980, Ellis *et al.* 1980, Rundel 1980, Hattersley 1983, Paruelo and Lauenroth 1996, Taub 2000, Murphy and Bowman 2007). Teeri and Stowe (1976) performed one of the earliest surveys of C_4 grass distributions in North America and showed that the higher the minimum temperature during the growing season the greater the proportion of C_4 grasses. Subsequent studies have also demonstrated similar correlations (Ellis *et al.* 1980, Vogel *et al.* 1978). While others have showed that temperature and rainfall are equally reliable predictors (Boutton *et al.* 1980, Tieszen *et al.* 1979, Rundel 1980). Most recent studies (Paruelo and Lauenroth 1996, Murphy and Bowman 2007) have included seasonal water availability as yet another criterion.

Vogel *et al.* (1978) surveyed the distribution of C_3 and C_4 grasses in South Africa. C_3 and C_4 grasses co-occupied areas that had as little as 100 mm of annual rainfall and as much as 1000 mm. C_4 species were excluded from very particular locations, such as the winter rainfall region of the Western Cape and along the summits of the of the Drakensberg and other Eastern Cape mountain ranges. They hypothesized that low temperatures (below a mean daily maximum of 25˚C) during the growth period (rainy season) gave C_3 grasses an advantage over C_4 grasses.

Ellis *et al.* (1980) expanded upon the previous study when conducting a survey of grasses in Namibia. Namibia has a warm and uniform average maximum summer temperature (30˚C) except for a narrow region along the Atlantic coast, whose average maximum summer temperature is 20˚C. The south-west regions of the country receive less than 50 mm of winter rainfall per year and the extreme northeast receives over 500 mm of summer rainfall a year. More than 95% of the grass species occurring at any particular location were C_4. Even though C_3 grasses were found in both regions, they occupied very specific and specialized niches. In the arid areas, C_3 grasses were restricted to moist microenvironments, like deeply shaded areas. In the hot and moist environments, the C_3 grasses became hydrophytes or obligate sciophytes (shade plants).

Surveys of C_3 and C_4 grasses along altitudinal gradients (Tieszen *et al.* 1979, Rundel 1980, Boutton *et al*. 1980) showed clearly that C_4 grasses dominate low altitudes and that the high altitudes are mainly or only C_3 grasses. In some cases, the low elevations are characterized by increasing water stress and high light intensities, which favor C_4 grass growth (Tieszen *et al*. 1979, Boutton *et al.* 1980), but in Hawaii the C_4 grasses predominated in the mesic rainforest communities at intermediate elevations. Rundel's (1980) study of Hawaii also demonstrated that the transition zone between C_3 and C_4 grasses corresponded to a mean maximum temperature between 19-21˚C and mean minimum temperature range of 9-11˚C, which was lower than previously recorded. He concluded that distributions of C_3 and C_4 grasses along temperature gradients in the tropical latitudes differed from those reported in the temperate regions (Teerie and Stowe 1976 and Ehleringer 1978).

Grass distributions have also been described by theoretical models. Collatz *et al*. (1998) classified climate as favoring the occurrence of only C_3 grasses, only C_4 grasses or both. The most consistent criterion for occurrence of C_4 grasses was a mean temperature greater than 22˚C and a mean precipitation above 25 mm for any given month. A mean temperature of 22˚C and precipitation that was never greater than 25 mm for the same month favored C_3 growth. Mixed C_3/C_4 grasslands have months with greater than 25 mm of rainfall and temperatures at or below 22˚C. Ehleringer (1978) applied his quantum yield model to the observed geographical distributions of C_3 and C_4 grasses. He concluded that the lower quantum yield seen in C_3 species relative to C_4 species at high temperatures is a significant factor in limiting C_3 grass distribution.

The most interesting result uncovered by these studies and models is that the distribution of C_4 grasses occurs over a range of rainfall gradients. In an effort to untangle the effects of precipitation on C_4 grass distributions, Ellis *et al.'s* (1980) survey of Namibia investigated the distributions of the three C_4 biochemical subtypes. The results showed that NADP-ME subtypes occurred primarily in regions with high rainfall, NAD-ME subtypes dominated the most arid part of the precipitation regime and PCK subtypes attained maximum abundance in areas of intermediate precipitation (Figure 1.5).

Taub's (2000) study of the C_4 grass flora in 32 sites in the United States was consistent with the previous findings. NADP-ME grasses greatly increased in abundance with increasing annual precipitation, while the abundance of NAD-ME and PCK decreased. However, the correlations may have been due solely to the tight association of the C_4 subtypes and the taxa from which they evolved. The Chloridoideae subfamily has no NADP-ME species, while the Arundinoideae and Panicoideae subfamilies are virtually all NADP-ME. The graphs show clearly that either subfamily or subtype could explain the trends observed in C_4 grass distributions along rainfall gradients (Figure 1.6).

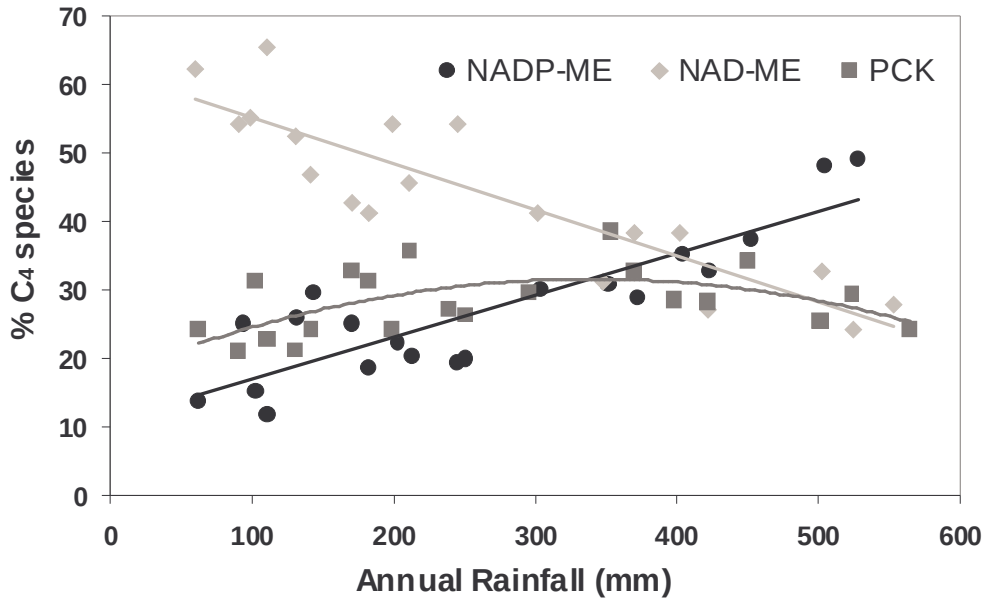

Figure 1.5: Percentage of C_4 grasses with the NADP-ME, NAD-ME and PCK subtypes in Namibia related to rainfall (re-drawn from Ellis *et al.*1980).

C_3 response to water stress

The effects of water stress on photosynthesis have been researched primarily on C_3 plants (Jones 1973, Lawlor and Cornic 2002, Parry *et al.* 2002, Bota *et al*. 2004, Noctor *et al.* 2002, Cornic and Fresneau 2002, Flexas *et al.* 2006a, Flexas *et al*. 2006b, Galmés *et al.* 2007). There has been some considerable debate amongst these studies as to whether the stomata or metabolic impairment is the primary limitation to photosynthesis. There is some general agreement that in the early stages of water stress, reduced CO_2 diffusion from the atmosphere to the site of carboxylation, manifested as reduced stomatal conductance is the dominant limitation. More recently, it has been shown that reduced mesophyll conductance also plays an important role (Flexas *et al*. 2006a). Stomatal limitation can be reversed by increasing atmospheric CO_2 concentrations so that intercellular CO_2 concentrations rise thereby restoring photosynthetic rates (Lawlor and Cornic 2002).

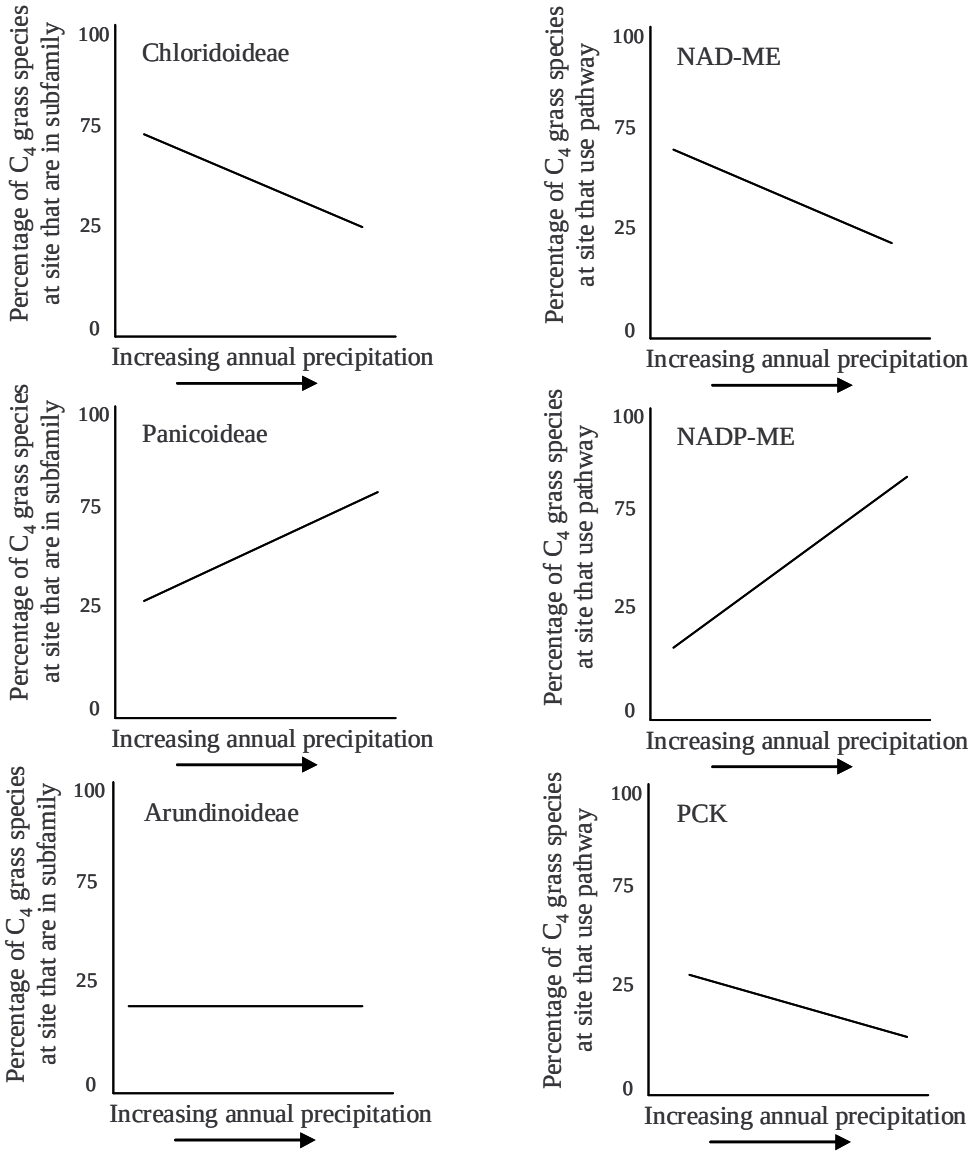

Figure 1.6: Taub's (2000) relationship between the proportions of C_4 grasses and rainfall at 32 sites in USA**.** Grass species are grouped according to subfamily: Chloridoideae, Panicoideae, and Arundinoideae and biochemical subtype: NAD-ME, NADP-ME and PCK.

In the more advanced stages of water stress, metabolic limitations progressively increase, although Flexas *et al*. (2006a) claims that these limitations may occur indirectly as a result of oxidative stresses that develop under high light rather than as a direct response to water stress. This phase of drought is characterized by the fact that increasing CO_2 levels does not restore photosynthesis to its unstressed rate. There is no consensus on the actual cause/s of metabolic inhibition. Lawlor (2002) listed some of the possibilities: reduced Rubisco activity through the non-activation of its active sites or through inhibition, decreased rate of the PCR cycle as a result of low enzyme activity, which in turn inhibits the regeneration of RuBP, a decreased supply of ATP and NADPH to the PCR cycle, a change in the rate of the electron transport and the regeneration of the proton gradient across the thylakoid membrane, damage to the photosystems and accumulation of phosphorylated assimilated intermediates which may lead to P_i deficiency resulting in impaired synthesis of ATP and RuBP.

The reduction of the photosynthetic reduction cycle generates excess photochemical energy. It has been argued that photorespiration and the Mehler ascorbate peroxidase reaction act as alternative electron sinks in C_3 species. These prevent chronic photoinhibition, stimulate photon utilization through non-assimilatory electron transport and help to preserve photosynthetic competence in bright light (Osmond and Grace 1995). However, others have argued that if the electron flow to alternate sinks is limited, which is likely to occur when a leaf is exposed to saturating or near-saturating light, the whole electron transport chain is down regulated (Cornic and Fresneau 2002).

C_4 response to water stress

How does water stress affect carbon assimilation in C_4 plants and how does this differ from the responses mentioned earlier for C_3 plants? Once again it is debated as to whether stomatal or non-stomatal factors prevail in the decrease of photosynthesis in C_4 species during drought. C_4 photosynthesis operates at near saturation under current ambient CO_2 levels (Ghannoum *et al.* 2000). Therefore, small decreases in stomatal conductance during moderate water stress, may not affect photosynthesis initially in C_4 plants as it would in C_3 plants (Lal *et al.* 1996). Still, as drought progresses and stomatal conductance is greatly reduced; the availability of CO_2 to Rubisco may limit photosynthesis (Lal *et al.* 1996). However, it has also been shown that decreases in photosynthesis are independent of ambient CO_2 levels, indicating metabolic limitations are involved (Ghannoum *et al.* 2003).

The causes of decreased photosynthetic rates may also be dependent on whether the drought was rapidly or slowly induced (Marques da Silva and Arrabaça 2004, Du *et al.* 1996, Saccardy *et al.* 1996). Decreased enzyme activity and lower mesophyll conductance have been proposed as possible non-stomatal factors responsible for decreased CO_2 assimilation rates in C_4 species (Du *et al.* 1996, Carmo-Silva *et al.* 2007). The dissipation of excess photochemical energy through alternative electron sinks under water stress, namely photorespiration, has been shown in some C_4 plants (Lal and Edwards 1996) while others have demonstrated this not to be the case (Ripley *et al.* 2007). Rather electron transport rate reduction and decreased photochemical energy dissipation are the major responses to drought (Ripley *et al.* 2007).

Phylogenetically controlled experiments

As Taub (2000) pointed out, the correlations between the distributions of the three biochemical subtypes of C_4 photosynthesis: NADP-ME, NAD-ME and PCK and annual rainfall in the United States may have been due solely to the tight association of the subtypes and the subfamilies to which they belong. Therefore, any results from studies comparing C_3 and C_4 grass species may just be a result of a species belonging in a particular subfamily and not actually an inherent C_3/C_4 effect.

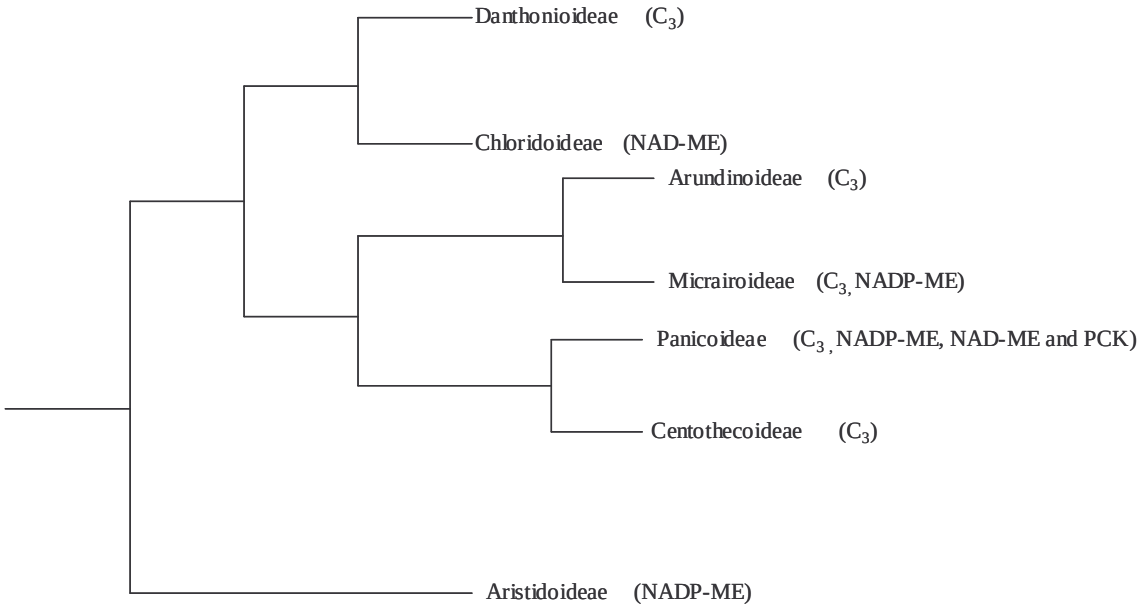

Figure 1.7: The new PACCMAD phylogeny with Micrairoideae reinstated as a subfamily within the larger PACCAD clade (sensu Grass Phylogeny Working Group 2001). The subfamily Panicoideae has two tribes: Andropogoneae that is only represented by species with the NADP-ME subtype of C_4 photosynthesis and Paniceae that is represented by C_3, NADP-ME and NAD-ME species. The diagram is based on Sanchez-Ken *et al.* 2007.

Ripley *et al.* (2007) addressed the phylogenetic issue by conducting a series of experiments on a native South African species of grass called *Alloteropsis semialata*, unique in having both C_3 and C_4 subspecies. *Alloteropsis semialata* belongs to the subfamily Panicoideae, which contains C_4 species within the NADP-ME subtype. Induced drought treatments were performed on the two subspecies grown in a common garden and in pots. During non-drought periods, photosynthetic rates were greater in the C_4 subspecies than in the C_3 subspecies. As drought progressed, the C_4 subspecies lost it photosynthetic advantage. At this time there was no significant difference between the photosynthetic rates of the subspecies. The decline in photosynthesis was three times greater in the C_4 subspecies than the C_3 subspecies. The mechanisms for the loss of photosynthetic advantage in the C_4 subspecies were investigated in a pot experiment. CO_2 response curves were generated for both subspecies under well-watered and water-stressed conditions. It was shown that the C_4 subspecies had greater metabolic (as opposed to stomatal) limitations to photosynthesis than the C_3 subspecies. These results indicate that C_4 photosynthesis may have an inherent sensitivity to drought independent of phylogeny and may explain why NADP-ME grasses decrease in abundance with decreasing rainfall. In order to assess if these results are unique to just the C_4 subspecies of *Alloteropsis semialata* or whether they can be generalized for all NADP-ME grasses, this study compared the drought responses of C_3 and NADP-ME grass species belonging to the subfamily Panicoideae.

The study species are restricted to the Panicoideae subfamily due to the instability of the grass phylogeny. The previous phylogeny had the subfamily Panicoideae and its sister group Centothecoideae basal to the other subfamilies whereas the most recent phylogeny has the subfamily Aristidoideae basal to the other subfamilies with 100% bootstrap support (Figure 1.7).

Three Panicoid C_3 species: *Alloteropsis semialata, Panicum aequinerve* and *Panicum ecklonii* and three Panicoid NADP-ME species: *Heteropogon contortus, Themeda triandra* and *Tristachya leucothrix* were selected because of their natural abundance around Rhodes University in Grahamstown, South Africa.

Field site and species descriptions

These study species are widely distributed in Southern Africa and co-occur on Faraway farm about 8 km outside of Grahamstown, South Africa (33º S 27º E). This area consists of 31 ha of land, of which 26 ha are composed of Suurberg quartzite fynbos. Suurberg quartzite fynbos is described as grassy fynbos with localized patches of dense proteoid and ericaceous fynbos (Mucina and Rutherford 2006). The soils of Faraway farm are sandy, and the aspect is predominately south facing. Grahamstown is in a semi-arid region in the Eastern Cape of South Africa with a bimodal distribution of rainfall occurring in the spring and autumn (681mm). Mean daily maximum and minimum temperatures are 27.7˚C and 4.7˚C for February and July respectively. Frost occurs 2-10 days of the year (Mucina and Rutherford 2006).

C_3 species

Alloteropsis semialata (R.Br.) Hitchc. subsp. *eckloniana* is a densely tufted perennial grass that forms the largest tufts among the C_3 grasses in this study (up to 1000 mm tall). Leaves are hairy and relatively thick and are about 50-480 mm long, 3-12 mm wide. This species is found in rocky places and forest margins. *A. semialata* occurs in Southern Africa as far north as Tanzania in the higher-lying regions (van Oudtshoorn 1992).

Panicum aequinerve Nees is a short-lived perennial or annual grass that grows along the ground and roots at the nodes. Leaves are about 40 mm long, 4 mm wide and are generally smooth. This species grows on shallow soils of forest margins or open grasslands, mainly in damp places and around boulders (Gibbs Russell *et al.*1991). It is one of the most difficult species to locate at Faraway farm because of its small size and small leaves. This species tends to lose its leaves during winter and sometimes during drought (personal observation). *P. aequinerve* is distributed in Southern Africa northwards to Uganda, Ethiopia and in Madagascar (Gibbs Russell *et al.*1991).

Panicum ecklonii Nees. is a relatively short perennial tufted grass. Leaves are about 60-200 mm long and 3-8 mm wide with dense velvety hairs. Healthy leaves look similar to *A. semialata,* but are greener in color. Plants tend to be small in size with just a few leaves on each plant, and are usually positioned close to other grass tufts. This species grows on sandy soils often in moist areas in mountainous regions that are subjected to burning (Gibbs Russell *et al.*1991). *P. ecklonii* occurs in Southern Africa as far north as Tanzania and the Democratic Republic of the Congo, and also in West Africa (van Oudtshoorn 1992).

C_4 species

Heteropogon contortus (L.) Roem. & Schult is perennial grass that grows up to 500 mm in height. Leaves are 30-300 mm long, 3-8 mm wide with rounded tips that are often folded. This species can show considerable variation in height, branches and color from one region to the next (van Oudtshoorn 1992). This species grows well on hillsides and rocky places on well-drained soils. *H. contortus* occurs in all tropical and subtropical parts of the world (van Oudtshoorn 1992).

Themeda triandra Forssk. is a perennial tufted grass that grows up to 500 mm in height. Its physical characteristics are extremely variable. Leaves are 150-300 mm long, 1-8 mm wide with tapered tips and are often folded. *Themeda triandra* and *Heteropogon contortus* can be confused for one another as both are approximately the same height and have similarly shaped leaves. This species is referred to as rooigras because of its reddish color late in the season. *T. triandra* is distributed in the tropical and subtropical parts of the Old World (van Oudtshoorn 1992).

Tristachya leucothrix Nees is a densely tufted perennial grass that grows up to 900 mm tall. It forms the largest tufts amongst the C_4 grasses in this study. Leaves are about 50-400 mm long and 2-7 mm wide. This species grows on marshy grasslands, mountain sourveld and on hillsides, and is found in the fynbos, savanna and grassland biomes (Gibbs Russell *et al.*1991). T. leucothrix occurs in Southern Africa and tropical Africa (van Oudtshoorn 1992).

Aims

The aim of this study was to determine if the NADP-ME subtype is more sensitive to water stress than the C_3 type on both leaf and whole plant levels. This was executed by comparing the responses of photosynthesis and water usage of closely related species during drought.

Do NADP-ME grasses:

- have higher instantaneous water use efficiency (WUE_{leaf}) than C_3 grasses? Is this advantage lost during drought?
- have higher WUE_{leaf} because they have higher assimilation rates or alternatively have lower transpiration rates?
- sustain their photosynthetic advantage during water stress?
- have greater stomatal or metabolic limitations to photosynthesis and how does this compare to C_3 grasses?
- have higher whole plant water use efficiency? Can it be maintained during slow dehydration?
- have a lower plant hydraulic conductance than C_3 grasses because of their lower water requirement?
- have less vulnerable xylem than C_3 grasses?
- recover their photosynthetic rates from drought more quickly than C_3 grasses?

Chapter 2: Leaf gas exchange in response to drought

Introduction

A trade-off exists between the efficient CO_2 uptake for photosynthesis and moderating water loss through transpiration. The carbon concentrating mechanism of C_4 photosynthesis has relaxed this constraint. It allows greater photosynthetic efficiency than C_3 plants under conditions that enhance photorespiration while allowing efficient water use through lower stomatal conductances. Leaf gas exchange was measured on three C_3 and three C_4 species of Panicoid grasses under well-watered and drought conditions to test this assumption. The effect of water stress on photosynthesis, transpiration, stomatal conductance and instantaneous water use efficiency were compared between the two photosynthetic types. Measurements were initially carried out on naturally co-occurring field plants during periods of differing vapor pressure deficits and soil water contents. The observed responses were further investigated by subjecting pot-cultivated plants to a controlled drought in an attempt to simulate the field observations, but allowing for better experimental control. Plants were drought stressed by gradually withholding water over a period of forty-eight days and were subsequently re-watered to the soil water content of the control pots to monitor their recovery (Figure 2.1). Gas exchange parameters were assessed on the pot-cultivated plants during both the dry down and recovery periods. The measurements from both the field and pot experiments are presented in this chapter.

In addition to gas exchange, further experiments were performed on the pot-cultivated plants to 1) explain the mechanisms for the loss of photosynthetic advantage in the C_4 type during drought, 2) to determine whether whole plant water use efficiency would exhibit similar trends to those observed in leaf level water use efficiency and 3) to correlate xylem anatomical characteristics with water use. The experimental time-course showing pot dehydration, re-watering and the timing of these supplementary experiments is illustrated in Figure 2.1.

In order to determine the stomatal and non-stomatal contributions to reductions in photosynthesis, CO_2 response curves (A:c_i) curves were constructed for control and drought stressed plants on two selected occasions that represented progressive and severe drought stress (Figure 2.1 and Table 2.1), and these results are presented in Chapter 3. Whole plant water use efficiency (WUE_{plant}), whole plant relative leaf expansion (RGR_{area}) and whole plant water loss per leaf area (E_{plant}) were measured during periods of no water stress, moderate drought and severe drought (Figure 2.1 and Table 2.1), and these results form the basis for Chapter 4. Whole plant hydraulic conductance, leaf water potentials and pre-dawn water potentials were determined on field and pot-cultivated plants at the same time gas exchange measurements were made. However, these parameters were only considered during the dry down period of pot experiment. In addition, anatomical analysis of characteristics relating to whole plant hydraulic conductance: average length of longest xylem vessel, total xylem lumen area, theoretical leaf hydraulic conductance, vascular bundle size class frequency and average maximum xylem diameter were measured on leaves that were slowly water stressed for forty-two days. These results were compared to values obtained from leaves that were collected in the field under well-watered conditions and are presented in Chapter 5. The discussions for all of the chapters have been compiled and presented in Chapter 6.

Table 2.1: The average percent soil water contents (% mass) associated with the watering treatments imposed on the pot-cultivated plants during CO_2 response curves and whole plant water use efficiency measurements**.** Drought terms are based on the relationship between pre-dawn $\Psi_{leaf,}$ and SWC (see Methods and Materials for further explanation).

Measurements	Well-watered	Moderate drought	Progressive drought	Severe drought
CO_2 response curves (μmol CO_2 m^{-2} s^{-1})	18.8 ± 0.1		3.8 ± 0.1	2.7 ± 0.1
WUE_{plant} (mmol CO_2 / mol H_20)	19.9 ± 0.2	9.7 ± 0.3		2.9 ± 0.1
RGR_{area} (cm^2 cm^{-2} d^{-1})	19.9 ± 0.2	9.7 ± 0.3		2.9 ± 0.1
E_{plant} (g H_20 d^{-1} cm^{-2})	19.9 ± 0.2	9.7 ± 0.3		2.9 ± 0.1

Values are means ± s.e. (For CO_2 response curve data: n = 36 for well-watered, n = 23 for progressive drought, and n = 27 for severe drought. For whole plant measurements: n = 210 for well-watered and severe drought, and n = 420 for the progressive drought.)

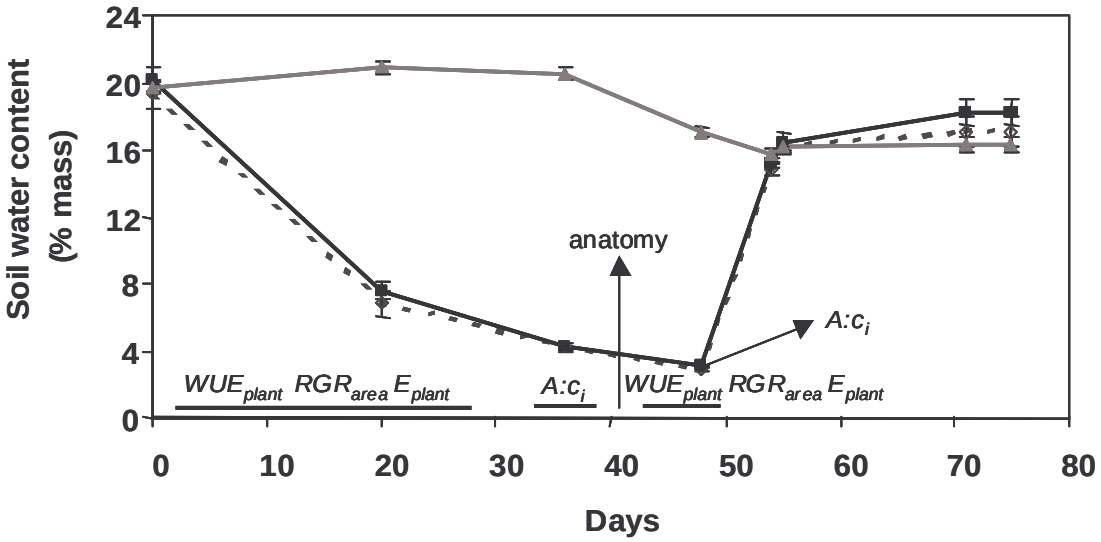

Figure 2.1: The experimental time-course of changes in percent soil water content (SWC) during the dry down / recovery experiment of pot-cultivated Panicoid grasses. SWC's were averaged for the three C_3 (hashed black line) and the three C_4 species (solid black line). The solid gray line represents the average SWC for the combined C_3 and C_4 control pots. Vertical bars represent standard errors. The periods when whole plant water use efficiency (WUE_{plant}), whole plant relative leaf expansion (RGR_{area}), whole plant water loss per leaf area (E_{plant}), CO_2 response curves ($A:c_i$) and anatomy were sampled or measured is indicated on the figure. Days 3-29 are referred to as moderate drought, days 33-38 are referred to as progressive drought and days 44-50 are referred to as severe drought. Well-watered whole plant measurements were made a week before the dry down experiment and are therefore not shown on the figure.

Methods and Materials

Field Experiment

Soil water content

A soil moisture probe (ECH20, Decagon Devices Inc. Pullman, Washington, USA) was buried at the Faraway farm field site and left to record hourly soil water contents for a five month period. On nine occasions during the period August 2006 through January 2007 the water content (% volume) of the soil at ten randomly selected locations around the field site were measured using a dielectric probe to a depth of 6 cm (ThetaProbe, type ML2x, Delta-T Devices). On three of those occasions (17th August 2006, 20th October 2006, 29th January 2007), three soil samples were collected from the field site to determine gravimetric SWC. Each sample was weighed to determine fresh mass and then oven dried at 60º C for two weeks to determine dry mass. The measurements made with the ECH2O and ThetaProbe were converted to gravimetric soil water content via relationships established for the soils present at the site.

Soil water potential was determined from pre-dawn Ψ_{leaf} measurements (see Chapter 5) and plotted against soil water content to generate a soil suction curve. This curve was used to define the drought terms used in the pot experiment. Moderate drought corresponded to a soil water potential that was approximately –0.8 MPa, the progressive drought treatment corresponded to a soil water potential that was less than –2 MPa and the severe drought corresponded to a soil water potential that was less than –4 MPa.

Leaf gas exchange

Measurements of net CO_2 assimilation rate (A), and transpiration rate (E), stomatal conductance (g_s) were carried out at midday (11am - 3pm) at the field site on 6th October 2006, 14th November 2006 and 24th January 2007 using a Li- 6400 photosynthesis system (Li-Cor Inc., Lincoln, NE, USA). Measurements were made on an attached, fully expanded leaf of each plant (first non apical leaf). Leaf area was measured before the leaf was clamped into the 2 x 3 cm chamber of the gas analyzer for about 30-60 seconds or until g_s values displayed by the instrument were stable. In order to maximize the surface

area of particularly narrow leaves, two leaves were placed inside the chamber. Ten leaves of each species, each leaf from a different plant was measured. A photosynthetic photon flux density of 2000 $\mu molm^{-2}s^{-1}$ was supplied by the red-blue LED internal light source. The vapor pressure deficit (VPD) and leaf temperature were allowed to track ambient conditions and varied on the particular days; however the gas exchange system was shaded to prevent excessive temperatures. The gas chamber gasket of the photosynthetic system was held open for a few seconds three times during the day to get an average measurement of ambient air temperature. Gas exchange parameters were calculated according to von Caemmerer and Farquhar (1981) and instantaneous water use efficiency (WUE_{leaf}) was calculated as A/E.

Pot experiment

Plant collection, growth conditions, and experimental set up

Six plants of each species were collected from the Faraway farm field site on 25th June 2006. Each plant was divided into six smaller plants, potted and tagged. *Themeda triandra* and *Panicum ecklonii* did not survive. These species were recollected on 5th February 2007. The surviving plants from the first collection were again subdivided into smaller plants and repotted along with *T. triandra* and *P. ecklonii* into 10L pots with 6.7 kg of soil. The soil used for potting was a natural topsoil of similar type to that of Faraway farm, collected from the Waainek study site near Grahamstown and left to air dry prior to use. The number of tillers planted in each pot was selected so that about 50 grams of water was transpired daily and was based on the results of a previous experiment (data not shown). A reasonably uniform rate of transpiration between species was desirable as it simplified maintaining uniform rates of soil drying during the drought experiment (see below). The pot plants were transferred to a naturally lit, clear polyethylene tunnel. The average day / night temperatures in the tunnel were 6.7ºC ± 0.2 s.e / 34.4ºC ± 0.6 s.e. The maximum light intensity of growth tunnel was 1400 $\mu mol\ m^{-2}\ s^{-1}$. The plants were kept well-watered for the month leading up to the experiment. Each week, hydroponics fertilizer was added to the pot plants (Chemicult, approximately 1 teaspoon per 5 liters). On 12th March 2007, the pot plants were arranged inside the clear

polythene tunnel according to either a well-watered or drought treatment. Each treatment contained seven plants of each species.

Determination of soil water contents

One kg of fine (<1 cm in diameter) stone was placed on the surface of the soil of each pot to prevent soil evaporation. The pots were soaked in water to saturate the soil and were weighed the following day after the pots had drained to determine the field capacity of the soil of each pot. The associated soil water content (% volume) of each pot was measured using a dielectric probe (ThetaProbe, type ML2x, Delta-T Devices). The field capacity weights and probe measurements were used to estimate the gravimetric SWC of the pots. Well-watered control pots were maintained at 20% SWC by replacing water on a mass basis. Similarly, drought-treated pots were initially maintained at 20% SWC for four weeks after which water was slowly withheld from pots. The drought treatment was imposed such that the SWC decreased by approximately 1% every two days. This was complicated by the fact that the different species transpired at different rates and hence dried soil at different rates. Therefore the average SWC of the slowest transpiring pots (*Alloteropsis semialata* and *Tristachya triandra*) were used as a reference to which the other pots SWC were manipulated. Pots were weighed every second day and water was added such that SWC declined at the same rate as that of the reference pots over time. The estimated values of SWC were corrected for at the end of the experiment when the actual SWC of each pot was determined. Each pot was weighed and a sub-sample of wet soil was weighed to determine fresh mass and then oven dried at 60º C for two weeks to determine dry mass.

Leaf gas exchange

Gas exchange measurements were made on the pots on 10th April 2007, representing well-watered conditions. Gas exchange was monitored during a gradually imposed drought on the days 30th April, 16th May and 28th May. After re-watering on 1st June, measurements on 3rd, 4th, 20th and 24th June were used to assess the recovery after drought (Figure 1). Measurements of A, E, g_s and WUE_{leaf} were made between 11am-3pm in a similar manner as those descried for the field experiment using a Li-6400

photosynthesis system (Li-Cor Inc., Lincoln, NE, USA). A photosynthetic photon flux density of 2000 $\mu molm^{-2}s^{-1}$ was supplied by the red-blue LED internal light source, air temperature was set at 25°C and relative humidity ranged from 35-65%.

Data analysis

A nested general linear model was used to detect the effects of photosynthetic type, species, treatment and their interactions. Species were treated as nested within photosynthetic type to account for each species belonging only to one photosynthetic type, hence making a factorial design unsuitable. Levene's test was used to determine homogeneity of variance. Transformations of the data were performed when needed. Statistical differences between means were determined by Tukey HSD post-hoc tests if the general linear model effect was significant.

Results

Field experiment

Abiotic conditions

Three separate days of gas exchange measurements were used to define three natural environmental treatments: 1) a day of low VPD and high SWC, referred to as the low VPD treatment 2) a day of high VPD and high SWC, referred to as the high VPD treatment 3) a day of low SWC and high VPD, referred to as the low SWC treatment. The relevant environmental parameters and the soil water contents of the field site during the course of the experiment are shown in Table 2.2 and Figure 2.2 respectively.

Table 2.2: Vapor pressure deficits, soil water contents and ambient temperatures measured on the indicated dates at the Faraway farm field site.

	October 6, 2006 **High VPD**	November 14, 2006 **Low VPD**	January 24, 2006 **Low SWC**
VPD (kPa)	2.4 ± 0.1*b*	1.1 ± 0.1*c*	2.8 ± 0.1*a*
SWC (% mass)	24.8 ± 0.7*a*	14.5 ± 0.5*b*	5.2 ± 0.3*c*
Ambient temperature (°C)	29.9 ± 0.4*a*	29.5 ± 0.5*a*	33.90± 1.6*a*

In each row, different letters indicate significant differences between means on the different days at $P < 0.05$ (Tukey HSD test). Values are means ± s.e. (n =60 for VPD, n = 40 for SWC, n = 60 for soil water potential and n = 3 for ambient temperature)

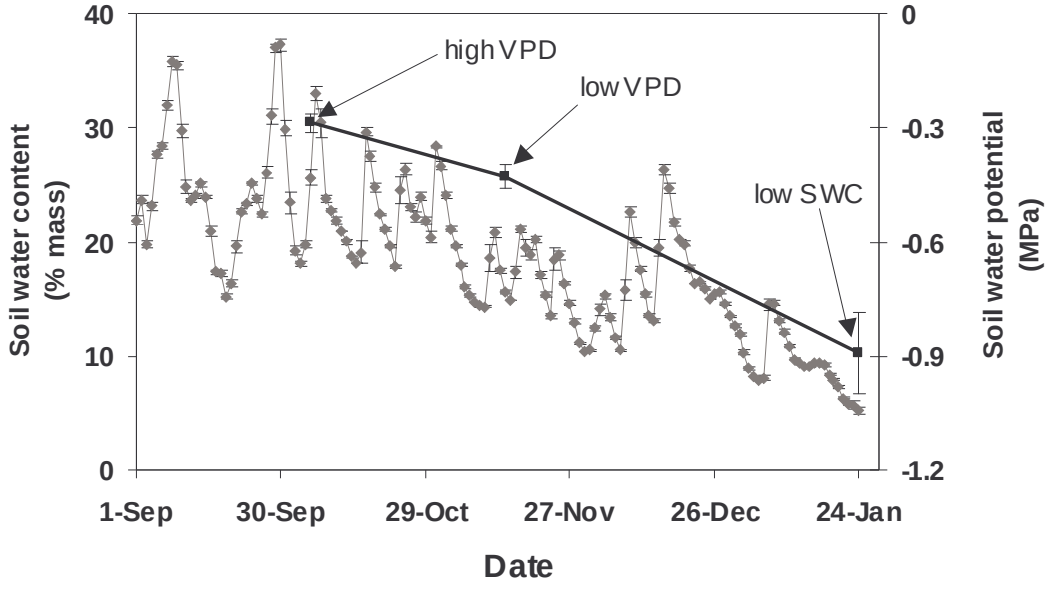

Figure 2.2: The time course soil water content and soil water potential (based on pre-dawn Ψ_{leaf} values) of Faraway farm field site. Arrows indicate the specific days and associated environmental treatments when gas exchange measurements were made on field-grown plants.

Instantaneous water use efficiency

The photosynthetic types showed similar responses to high VPD and low SWC relative to the low VPD treatment (n.s. type and treatment interaction Table 2.3). However, the C_4 type had significantly higher WUE_{leaf} than the C_3 type under all treatments (Figure 2.3 *a*). The individual species did not respond in the same way to drought and high VPD (significant species and treatment interaction Table 2.3). The low SWC treatment induced the lowest WUE_{leaf} values amongst all of the species, except for *P. aequinerve* (Figure 2.3 *b*).

Photosynthetic rate

In comparison to the controls (low VPD), both types significantly decreased photosynthesis under low SWC, but not when exposed to high VPD (n.s. type and treatment interaction Table 2.3). The C_4 type had significantly higher photosynthetic rates than the C_3 type across the treatments (Figure 2.3 *c*). The species responded differently to the three treatments (significant species and treatment interaction Table 2.3). *P. aequinerve* had a similar photosynthetic rate to the three C_4 species under the high VPD treatment and *T. leucothrix* and *H. contortus* had the highest photosynthetic rates under the low SWC treatment (Figure 2.3 *d*).

Transpiration rate

Both types increased transpiration under the high VPD treatment relative to the controls, but when subjected to low SWC, the C_3 type showed significant reductions whereas the C_4 type did not (Figure 2.3 *e*). The transpiration rate of the C_4 type was only significantly lower than the C_3 type under the control treatment. Transpiration was significantly higher in the high VPD treatment relative to the control in all species except *A. semialata* and *P.ecklonii* (Figure 2.3 *f*). *T. leucothrix* had the lowest transpiration rate under the control treatment.

Stomatal conductance

The photosynthetic types showed distinct responses to the three treatments (significant type and treatment interaction Table 2.3), but in such a way that overall the C_3 type was

similar to the C_4 type (n.s. type effect Table 2.3). The C_4 type increased g_s under the high VPD treatment (relative to the control) but the C_3 type had similar responses during the two treatments (Figure 2.3 *g*). The low SWC treatment induced significantly lower g_s in both types relative to the control. The species responded differentially to the three treatments (significant species and treatment interaction Table 2.3). *T. leucothrix* had similar g_s under both control and low SWC treatments whereas the other species decreased g_s (relative to the control) under the low SWC treatment (Figure 2.3 *h*).

Table 2.3: Summary of statistical significance of photosynthetic type, species (represented as species nested in type) and three naturally occurring environmental treatments (typified by three days in the field: low VPD, high VPD, low SWC) on instantaneous water use efficiency, photosynthetic rate, transpiration rate, and stomatal conductance of six species of Panicoid grasses. n.s., not significant; P>0.05; *, P<0.05; **, P<0.01; ***, P<0.001.

	Type	Species (type)	Treatment	Type x treatment	Species (type) x treatment
WUE_{leaf}	*** $F_{1,163}$= 95	*** $F_{4,163}$= 5.9	*** $F_{2,163}$= 130	**n.s.** $F_{2,163}$= 1.6	*** $F_{8,163}$= 4.6
A	*** $F_{1,163}$= 84	*** $F_{4,163}$= 5.5	*** $F_{2,163}$= 190	**n.s.** $F_{2,163}$= 0.31	*** $F_{8,163}$= 4.8
E	* $F_{1,163}$= 4.1	**n.s.** $F_{4,163}$= 1.8	*** $F_{2,163}$= 104	* $F_{2,163}$= 4.5	** $F_{8,163}$= 3.4
g_s	**n.s.** $F_{1,161}$= 3.9	* $F_{4,161}$= 3.3	*** $F_{2,161}$= 130	*** $F_{2,161}$= 7.6	*** $F_{8,161}$= 3.9

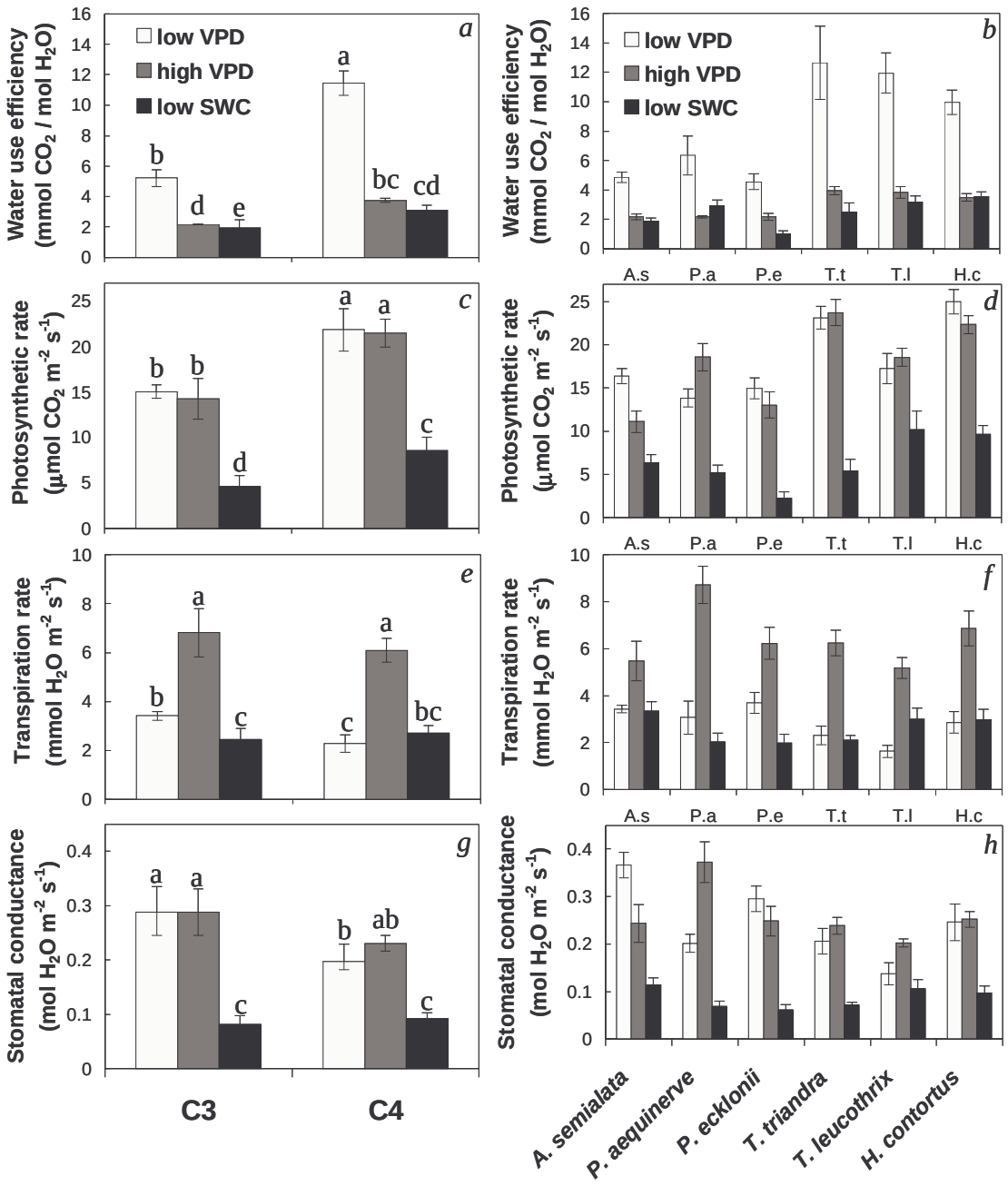

Figure 2.3: Instantaneous water use efficiency (*a*,*b*), net photosynthetic rate (*c*,*d*), transpiration rate (*e*,*f*) and stomatal conductance (*g*,*h*) of field-grown Panicoid grasses presented as individual species (*right*) or grouped by type (*left*) for three days in the field. Each day represents a different natural environmental treatment: low VPD, high VPD and low SWC. Values are means and vertical bars are standard errors (n = 9-11). Different letters indicate significant differences between means on the different days (environmental treatments) at $P < 0.05$ (Tukey HSD test).

Pot experiment

Table 2.4: Summary of statistical significance of photosynthetic type, species (represented as species nested in type) and days of drought or recovery on instantaneous water use efficiency, photosynthetic rate, transpiration rate and stomatal conductance of pot-cultivated Panicoid grasses**.** n.s., not significant; P > 0.05; *, P < 0.05; **, P < 0.01; ***, P < 0.001.

	Type	Species (type)	Treatment	Type x treatment	Species (type) x treatment
WUE_{leaf}	*** $F_{1,281}$= 58	*** $F_{4,281}$= 8.2	*** $F_{6,281}$= 110	*** $F_{6,281}$= 23	*** $F_{24,281}$= 3.7
A	*** $F_{1,315}$= 120	*** $F_{4,315}$= 11	*** $F_{7,315}$= 110	*** $F_{7,315}$= 3.8	*** $F_{28,315}$= 2.9
E	*** $F_{1,308}$= 15	*** $F_{4,308}$= 9.5	*** $F_{7,308}$= 77	*** $F_{7,308}$= 9.0	*** $F_{28,308}$= 3.2
g_s	* $F_{1,310}$= 6.0	*** $F_{4,310}$= 8.3	*** $F_{7,310}$= 50	*** $F_{7,310}$= 7.7	*** $F_{28,310}$= 3.0

Instantaneous water use efficiency

The C_4 type had significantly higher WUE_{leaf} than the C_3 type at the start of the experiment. This difference was maintained through the early stages of drought (days 0-20), but was eventually lost by day 36 due to a decrease in WUE_{leaf} of the C_4 type between days 20-36 while the WUE_{leaf} of the C_3 type increased slightly (significant type and treatment interaction Table 2.4 and Figure 2.4 *a*). Severe water deficit (by day 48) decreased the WUE_{leaf} of both types and resulted in the C_4 type having significantly lower WUE_{leaf} than the C_3 type. The C_4 type regained a significantly higher WUE_{leaf} than the C_3 type within four days of re-watering.

A. semialata had a similar WUE_{leaf} to the three C_4 species under moderate water stress (day 20), but as the drought progressed (day 36) all species had similar values, except for *T. leucothrix*, which had highest WUE_{leaf} (significant species and treatment interaction Table 2.4 and Figure 2.5 *a*). Severe water stress (day 48) caused *T. leucothrix* to lose its earlier advantage and have a WUE_{leaf} that was significantly lower than all C_3 species.

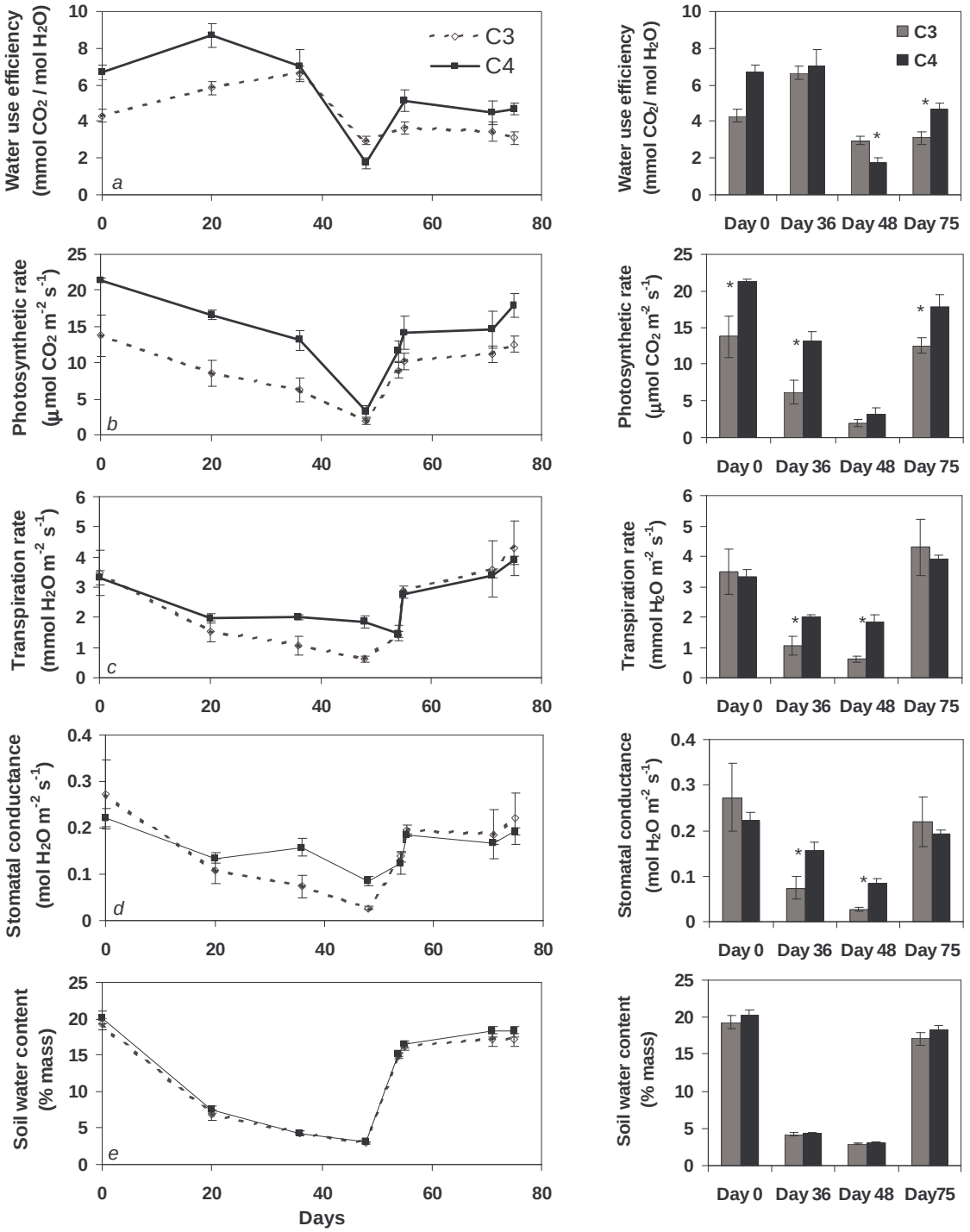

Figure 2.4: Instantaneous water use efficiency, photosynthetic rate, transpiration rate, stomatal conductance and soil water content for the two photosynthetic types: C_3 and C_4 during a controlled dry down and recovery experiment. ($n \geq 52$ for day 0 and n = 15-21 for the other days). Bar graphs show WUE_{leaf}, A, E, g_s and SWC at selected days of the experiment: 0, 36, 48 and 75. Vertical bars are standard errors. * indicates significant differences between means on the different days at $P < 0.05$ (Tukey HSD test).

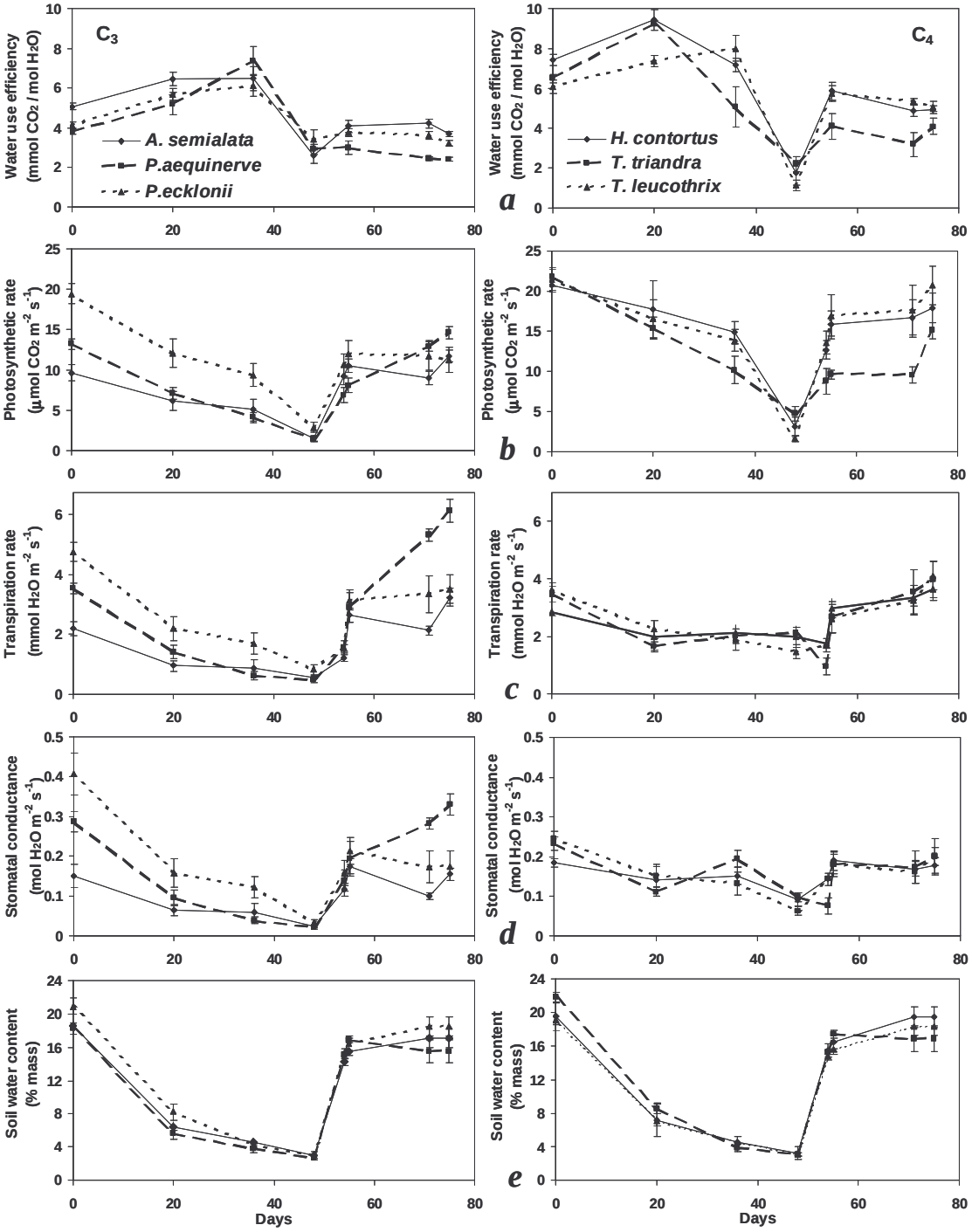

Figure 2.5: Instantaneous water use efficiency, photosynthetic rate, transpiration rate, stomatal conductance and soil water content of pot-cultivated Panicoid grasses during the dry-down / recovery pot experiment. Watering gradually decreased for 48 days and then pots were re-watered on day 51 and maintained at the SWC of the control pots for two weeks. The C_3 species are shown on the left and the C_4 species are shown on the right. Each point represents a species average ($n \geq 14$ for day 0 and n = 5-7 for the other days. Vertical bars represent standard errors).

Photosynthetic rate

The C_4 type had a significantly higher photosynthetic rate than the C_3 type under well-watered conditions (day 0) and throughout most of the gradually induced drought (Figure 2.4 *b*). On the day of severest water stress (day 48) the C_4 type lost photosynthetic advantage. This advantage was not regained until fourteen days after watering (day 75) although WUE_{leaf} recovered earlier.

The C_4 species maintained higher photosynthetic rates than the C_3 species until day 36 at which time *P. ecklonii* had similar photosynthetic rate to the three C_4 species (significant species and treatment interaction Table 2.4 and Figure 2.5 *b*). The photosynthetic rates of all the species were similar on the day of severest water stress (day 48) and throughout most of the recovery period (until day 75).

Transpiration rate

The C_3 and C_4 types had similar transpiration rates at the start of the experiment and during the initial stages of drought (up to day 20) even though both types decreased their rates relative to the start of the experiment (day 0) (Figure 2.4 *c*). The intensifying drought (days 36-48) resulted in the C_4 type having a significantly higher transpiration rate than the C_3 type (significant type and treatment interaction Table 2.4). Both types maintained similar rates upon re-watering which continued throughout the recovery period.

P. aequinerve had the lowest transpiration rate as drought intensified (days 36 and 48), however its rate quickly recovered upon re-watering. By days 71-75, *P. aequinerve* had the highest transpiration rates of all the species (Figure 2.5 *c*).

Stomatal conductance

The two types had similar g_s under well-watered conditions. The early stages of drought (up to day 20) caused both types to decrease conductance. However in the more advanced stages of drought (days 36-48), the C_4 type maintained significantly higher g_s than the C_3 type (Figure 2.4 *d*). The g_s of the two types increased similarly during the recovery

period. *P. ecklonii* maintained similar g_s as the C_4 species during early stages of the dry down experiment (days 20-36) (Figure 2.5 *d*).

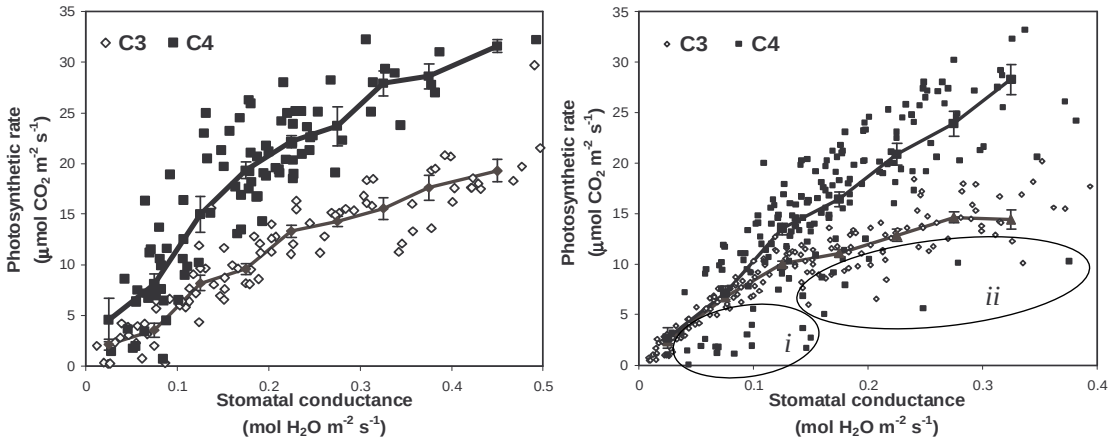

Figure 2.6: The relationship between photosynthetic rates and stomatal conductances for six species of Panicoid grasses grouped according to photosynthetic type. Each point signifies a single gas exchange measurement made in either the field (*left)* or during the pot experiment (*right*). The average photosynthetic rate of each type for a given stomatal conductance interval of 0.05 mol H_2O m^{-2} s^{-1} is shown. Vertical bars represent standard errors. Circle "*i*" indicates data from all three C_4 species on day 48. Circle "*ii*" indicates data for *T. triandra* on other days of the experiment.

As g_s increases so does the magnitude of the photosynthetic advantage of the C_4 type over the C_3 type under both field and greenhouse conditions (Figure 2.6). The C_4 type lost photosynthetic advantage at g_s of 0.1 mol H_2O m^{-2} s^{-1} during the greenhouse experiment (Figure 2.6 *right*), but this loss was not evident in the field data. Severe water stress (day 48) caused all three C_4 species to have more depressed photosynthetic rates for a given g_s relative to the C_3 species (Figure 2.6 circle *i*). *Themeda triandra* also had lower photosynthetic rates for a given g_s than the C_4 average on days other than day 48 (Figure 2.6 circle *ii*).

Refer to Chapter 6 and the section titled "Leaf level water use efficiency" on page (**84**) for the discussion of this chapter's results.

Chapter 3: Mechanisms of drought limitation of photosynthesis

Introduction

The previous chapter revealed that the photosynthetic advantage of the C_4 type was lost under severe water stress. However at the same time, g_s and transpiration rates were significantly higher in the C_4 type than the C_3 type. Hence, photosynthesis of the C_4 type does not appear to be limited by decreased CO_2 supply as a result of stomatal closure. This chapter aims to determine whether the susceptibility of C_4 (NADP-ME) Panicoid grasses to severe drought is a result of greater metabolic limitations on photosynthesis than co-occurring and closely related C_3 Panicoid grasses.

A CO_2 response curve is a useful tool in quantifying biochemical and stomatal limitations on photosynthesis (Figure 3.1). It is a demand function that shows the dependence of photosynthetic rate on the partial pressure of CO_2 at the sites of carboxylation (it is assumed that the partial pressure of the sites of carboxylation equals the partial pressure of the intercellular airspaces, Manter and Kerrigan 2004). The C_3 curve can be divided into two sections: the “linear region” defined as the Rubisco-limiting section and the “saturating region” defined as the RuBP regeneration limited section (and occasionally limited by triose phosphate availability, Harley and Sharkey 1991). Rubisco activity is limited at low CO_2 levels and as CO_2 increases, the activity of Rubisco increases. The slope of this linear section is referred to as carboxylation efficiency. It is a measure of Rubisco’s ability to assimilate CO_2 at maximum efficiency.

At low CO_2 levels, C_4 photosynthesis is limited by PEP carboxylase activity since Rubisco is kept at near saturating CO_2 levels. C_4 plants tend to have greater initial slopes (carboxylation efficiencies) than C_3 plants and their photosynthetic rates saturate at lower c_i values than C_3 plants because PEP carboxylase has a higher specificity for CO_2 than Rubisco and photorespiration is nearly suppressed in C_4 plants. The saturating section of

the curve develops because as photosynthetic rates increase, the pool of RuBP is depleted, as consumption is faster than synthesis. This regeneration step is limited by electron chain transport at high light intensities. At high CO_2 concentrations, PEP carboxylase activity is limited by PEP regeneration in C_4 plants (von Caemmerer 2000). Low PEP carboxylase activity also causes a reduction in the saturated portion of the curve due to Rubisco not being completely saturated with CO_2 in the bundle sheath (von Caemmerer 2000).

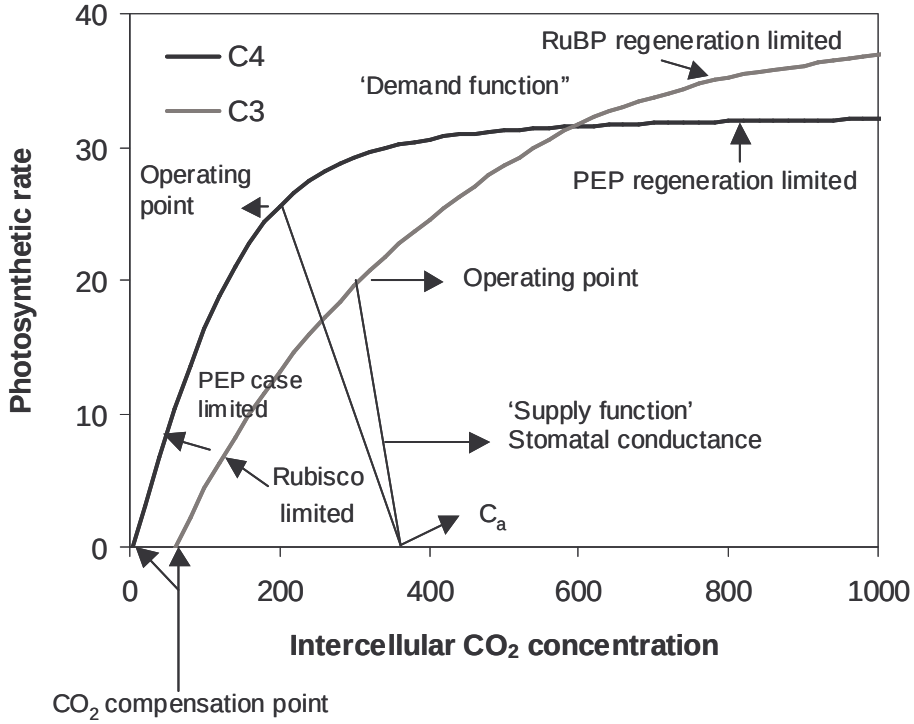

Figure 3.1: Changes in photosynthetic rates in response to intercellular CO_2 concentrations of representative C_3 and C_4 grasses. These responses are referred to as demand functions (bold gray curve for C_3 and bold black curve for C_4). The supply functions (lines drawn from the demand functions to the x-axis) represent the reduction in CO_2 concentrations from the atmosphere to the intercellular airspaces and the slopes of these lines are determined by stomatal conductance**.**

The supply function describes how CO_2 is "supplied" to the intercellular airspaces from the atmosphere via diffusion through the stomata (Figure 3.1). The slope of this line is stomatal conductance to CO_2. C_4 plants tend to have lower stomatal conductances than C_3 plants because PEP carboxylase activity is less inhibited by low intercellular CO_2 concentrations than Rubisco. The operating point of a leaf is defined by its particular

photosynthetic rate and c_i value at a particular point in time and is dependent on prevailing conditions. Changes in environmental conditions such as VPD, temperature, light intensity, ambient CO_2 concentration will induce changes in the operating point of a leaf. C_4 plants have an operating point at lower c_i values than C_3 plants (Figure 3.1). The point where CO_2 fixation by photosynthesis balances the CO_2 lost through respiration is called the CO_2 compensation point. Photorespiration occurs under low c_i increasing the compensation point of C_3 plants relative to C_4 plants.

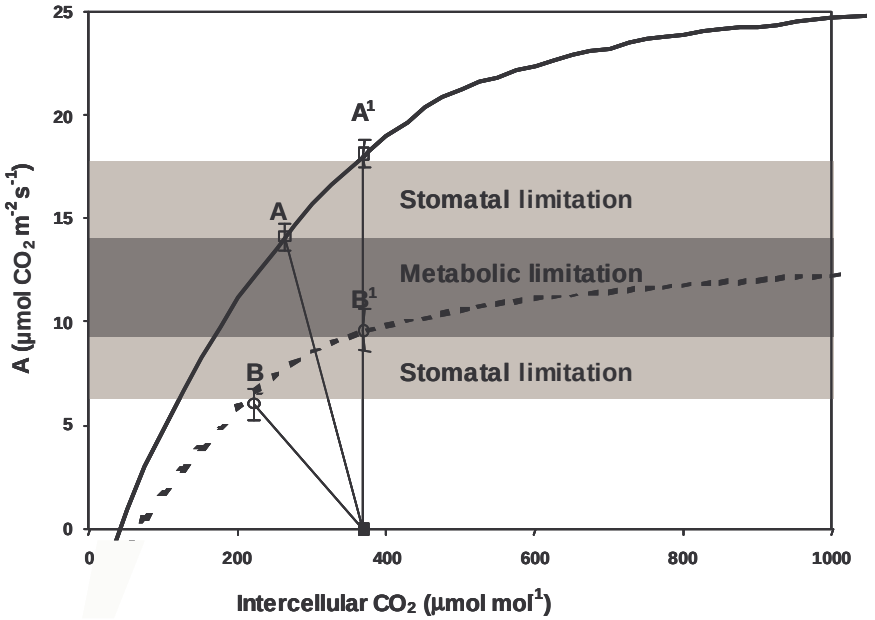

Figure 3.2: Stomatal (L_s), relative stomatal (R_{sl}) and metabolic (R_{ml}) limitations to photosynthesis calculated under well-watered (solid line) and drought (dashed line) conditions. For the control leaf, $L_s = (A^1 – A) / A^1$ and for the drought stressed leaf, $L_s = (B^1 – B) / B^1$. $R_{sl} = (B^1 - B) / A$ for the drought treated leaf (see Farquhar and Sharkey 1982). By definition, R_{ml} of the control leaf is equal to 0. For the drought stressed leaf, $R_{ml} = (A - B) / A - R_{sl}$ (see Ripley *et al.* 2007)

Limitations of photosynthesis caused by changes in stomatal conductance and mesophyll metabolism can be calculated from CO_2 response curves. In Figure 3.2, A and B represent the photosynthetic rates of the control and water-stressed leaves, respectively. The lines that connect each one to the x-axis are the supply functions representing the limitation imposed by the stomata on the diffusion of CO_2 from the air to the intercellular airspaces. The slopes of these lines are g_s and A^1 and B^1 represent the photosynthetic rates

of the control and drought treated leaves respectively, if there were no limitation imposed by the stomata (g_s = infinite). Metabolic limitations on photosynthesis during drought are characterized by changes to CO_2 uptake that cannot be explained by reduced CO_2 diffusion. These include biochemical and metabolic factors such as reduced Rubisco activity and mesophyll conductance, decreased ATP synthesis, photoinhibition and the regeneration limitation of RuBP.

Methods and Materials

CO_2 response curves

The responses of photosynthesis to internal concentrations of CO_2 (c_i) were measured on well-watered pot-cultivated plants with a SWC of 18% and drought stressed plants with a SWC of 3.8% using the Li-6400 photosynthesis system (Li-Cor Inc., Lincoln, NE, USA). Plants were transferred to the lab and acclimated to high intensity sodium lamps (PPFD of 1400 µmol m^{-2} s^{-1}) for thirty minutes before measurements were made. Photosynthetic rates were made on a fully expanded, first non-apical leaf after it adjusted to the environment of the cuvette (leaf temperature = 25º C, light intensity = 2000 µmol m^{-2} s^{-1}, VPD = 1.3 kPa, CO_2 concentration = 370µmol mol^{-1}) The external concentrations of CO_2 (c_a) were supplied in the following sequence: 370, 250, 150, 100, 50, 35, 370, 500, 750,1000, 1300 and 1600 µmol mol^{-1} and photosynthetic parameters were calculated according to von Caemmerer and Farquhar (1981).

A severe drought was induced by withholding water from the plants for an extra week. Photosynthetic rates were measured at c_a of 370 µmol mol^{-1} representing ambient conditions and photosynthetic rates at infinite stomatal conductance were simulated by adjusting the external supply of CO_2 so that c_i was 370 µmol mol^{-1}. These two points allowed for the calculation of stomatal and metabolic limitations during this treatment.

C_3 curves

According to von Caemmerer (2000), the equation for Rubisco-limited rate of CO_2 assimilation:

$$A_c = \frac{(c_i - \Gamma_*)V_{c\max}}{c_i + K_c(1 + O/K_o)} - R_d$$

where A_c is the Rubisco-limited rate of CO_2 assimilation, c_i is the intercellular CO_2 concentration, Γ_* is the C_3 compensation point in the absence of mitochondrial respiration, V_{cmax} is the maximal Rubisco carboxylation rate, K_c is the Michaelis-Menten constant of carboxylation, O the atmospheric oxygen partial pressure and K_o is the Michaelis-Menten constant of oxygenation.

The equation for the RuBP-limited rate of CO_2 assimilation:

$$A_j = \frac{(c_i - \Gamma_*)J}{4c_i + 8\Gamma_*} - R_d$$

where A_j is the RuBP-regeneration limited rate of CO_2 assimilation, c_i is the intercellular CO_2 concentration, Γ_* is the C_3 compensation point in the absence of mitochondrial respiration and J is the electron transport rate at a given irradiance.

Temperature correction equations

K_o, K_c and Γ_* were converted from values at 25˚C to the temperatures at which the CO_2 response curves were measured using the following equation from Bernacchi *et al.* (2001):

$$parameter = \exp(c - \Delta H_\alpha / RT_k)$$

where R is the molar gas constant and T_k is the leaf temperature, c represents a scaling constant and ΔH_α represents activation energy.

These values were used to fit the data to the Rubisco-limited and RuBP-limited equations to estimate V_{cmax}, R_d and J. These estimates for ambient conditions were corrected to a standard 25˚C using the following equation from Bernacchi *et al.* (2003):

$$Parameter = Parameter_{25} \times \exp(c - \Delta H_a / (R(T_l + 273)))$$

where $Parameter_{25}$ is the absolute value of the parameter at 25˚C, c represents a scaling constant, ΔH_α represents activation energy, R is the molar gas constant and T_l is the leaf temperature.

C_4 curves

C_4 curves were fitted according to Collatz *et al*. (1992). R_d was obtained by determining the y-intercept of the initial slope of the CO_2 response curve. c_i was converted to P_i. R_d, α, θ and $A:P_i$ data were used in the following equation to fit the parameters β, k *and* V_{max}:

$A = (W)\text{-}(SQRT(W)^2)\text{-}(4*\beta*(W))/(2*\beta)\text{-}R_d$ where

$W = (V_{max}+ (\alpha*Q))\text{-}(SQRT\ (V_{max}+(\alpha*\ Q))^2)\text{-}(4*\theta*(V_{max}*\alpha*\ Q))/(2*\theta)+(k)*(P_i/10/101600)$

and Q = light intensity (2000 μmol m^{-2} s^{-1}), A = assimilation rate (μmol CO_2 m^{-2} s^{-1}), P_i intercellular partial pressure of CO_2 (Pa), R_d is leaf respiration (μmol m^{-2} s^{-1}), k is initial slope of the photosynthetic CO_2 response curve (mol m^{-2} s^{-1}), V_{max} represents the CO_2 saturated region of the curve as determined by the maximum Rubisco capacity (μmol m^{-2} s^{-1}), β is the curvature factor for the CO_2 response curve, α is the initial slope of the photosynthetic light response = 0.04 mol m^{-1}, θ is a curvature parameter for light response curve = 0.83.

Stomatal limitation of photosynthesis (L_s) was calculated following Farquhar and Sharkey (1982). Relative stomatal limitation (R_{sl}) and relative metabolic limitation (R_{ml}) were calculated following Ripley *et al.* (2007). R_{sl} depicts the effect of the stomata on photosynthesis during a particular treatment in relation to the control, whereas L_s is the actual stomatal limitation of photosynthesis.

Data analysis

The same statistical design used in the previous chapter was applied to L_s, R_{sl} and R_{ml} data. A nested general linear model was used to detect the effects of photosynthetic type, species, treatment and their interactions. The three treatments in this case refer to three watering treatments imposed on the plants: well-watered, progressive drought and severe drought conditions. Species were treated as nested within photosynthetic type to account for each species belonging only to one type, hence making a factorial design unsuitable. Levene's test was used to determine homogeneity of variance. Transformations of the data were performed when needed. Statistical differences between means were determined by Tukey HSD post-hoc tests if the general linear model effect was significant.

Results

CO_2 response curves

Soil water contents

CO_2 responses were made on pot-cultivated plants growing in soils with three differing water contents (Table 3.1). Well-watered pots had an average SWC of 18.8% (termed controls) and this was reduced to 3.8% after thirty-five days (termed progressive drought) and to 2.7% after forty-eight days of withholding water (termed severe drought). There are no significant differences between the SWC of the species during the three treatments ($F_{5,128}$ = 0.25, P = 0.940, one-way ANOVA).

Table 3.1: The soil water contents of pot-cultivated Panicoid grasses during CO_2 response curve measurements**.**

Species	Soil water content for control pots (% mass)	Soil water content during progressive drought (% mass)	Soil water content during severe drought (% mass)
A.semialata	17.6 ± 0.3	3.8 ± 0.2	2.9 ± 0.3
P. aequinerve	18.7 ± 0.3	3.9 ± 0.3	2.9 ± 0.2
P.ecklonii	20.0 ± 0.2	3.7 ± 0.4	2.8 ± 0.4
T.triandra	17.9 ± 0.5	3.8 ± 0.2	2.9 ± 0.3
T.leucothrix	19.9 ± 0.4	3.8 ± 0.5	3.0 ± 0.1
H.contortus	18.5 ± 0.3	3.8 ± 0.5	3.0 ± 0.8
C_3	18.7 ± 0.7	3.8 ± 0.1	2.9 ± 0.1
C_4	18.4 ± 0.6	3.8 ± 0.1	3.0 ± 0.1

Values are averages ± s.e. (n = 6 for control pots, n = 4 for the progressive drought, except *T. triandra* n = 3, and n = 4 - 5 for the severe drought).

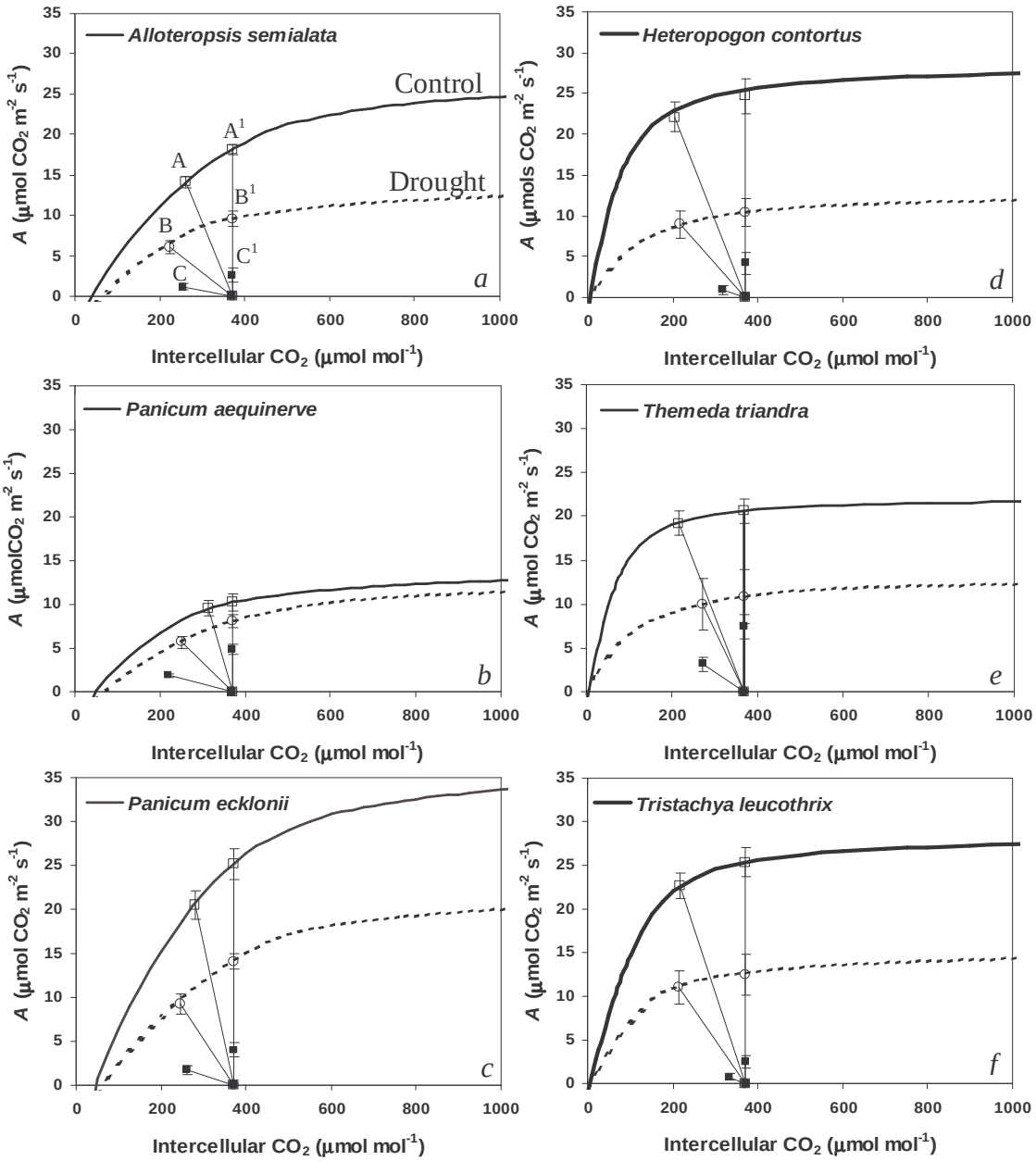

Figure 3.3: CO_2 response curves for pot-cultivated Panicoid grasses during three watering treatments: well-watered (▬), progressive drought (----) and severe drought (■) at PPFD of 2000 µmol mol^{-1}, 25°C and a VPD of 1.3kPa. During the severe drought treatment, photosynthetic rates were only measured at c_a=370 µmol mol^{-1} and c_i of 370 µmol mol^{-1}. Average photosynthetic rates at c_a = 370 µmol mol^{-1} of each treatment are shown as: A = well-watered, B= progressive drought, C = severe drought. Photosynthetic rates at infinite stomatal conductance are shown as: A^1 = well-watered, B^1 = progressive drought, C^1 = severe drought. Supply functions representing the limitation on photosynthesis by the diffusion of CO_2 through the stomata are also included. Each function has a slope equal to stomatal conductance and intercepts the c_i axis at c_a. (n = 6 for the control curves, n = 4 for the progressive drought curve, except for *T. triandra* which had n = 3 and n = 4 - 5 for the severe drought data. Vertical bars represent standard errors).

The CO_2 response curves of the well-watered pots demonstrated typical C_3 and C_4 variation (Figure 3.3 *a-f*). The C_4 species had consistently lower CO_2 compensation points, higher carboxylation efficiencies and saturated photosynthesis at lower c_i's than the C_3 species. The curves of the C_4 species saturated at c_i around 400µmol mol^{-1} with an operating c_i between 205-215 µmol mol^{-1} (Figure 3.3 *d-f*). *A. semialata* and *P. ecklonii* had representative C_3 curves with photosynthesis saturating at c_i greater than 1000 µmol mol^{-1} and operating c_i of 263 and 279 µmol mol^{-1} respectively (Figure 3.3 *a*, *c*). The curve of *P. aequinerve* saturated at a c_i similar to the C_4 species, but had an operating point of 314 µmol mol^{-1} (Figure 3.3 *b*).

Drought had an effect on the CO_2 response curves of all of the species as characterized by more shallow initial slopes (lower carboxylation efficiencies) and lower saturated values in the progressive drought curve as compared to the well-watered curve (Figure 3.3 *a-f*). Carboxylation efficiency decreased by 61.7% from the control values in the C_4 type as compared to a 43.5% decrease in the C_3 type. The saturated values of the C_4 type decreased by 53.6% during the progressive drought as compared to the control, whereas the C_3 type only had a 35.8% reduction in value.

The average photosynthetic rates of all the species declined with each drought treatment (Figure 3.3 *a-f*). The photosynthetic rates during the progressive drought decreased by 50.7% relative to the control rates in the C_3 type and were about 53.1% lower in the C_4 type. During the severe drought treatment, photosynthetic rates decreased by 88.4% and 92.0% in the C_3 and C_4 types respectively.

Stomatal and metabolic limitations

The C_3 type had increased stomatal limitation during the progressive drought relative to the well-watered treatment, whereas the C_4 type maintained similar responses during both treatments (significant type and treatment interaction Table 3.2 and Figure 3.4 *left*). The C_3 type had significantly higher stomatal limitation than the C_4 type in the well-watered and progressive drought treatments (Figure 3.4 *left*). This limitation accounted for a 15.8% decrease in photosynthetic rate in the C_3 type under well-watered conditions as compared to only 9.2% in the C_4 type. The C_3 type had three times greater stomatal limitation during the progressive drought treatment as compared to the C_4 type, however the two types were similarly inhibited during the severe drought treatment.

The C_4 species showed similar responses to the well-watered and progressive drought treatments, but during the severe drought, stomatal limitation increased significantly (Figure 3.4 *right*). This is in contrast to the response of the C_3 species where stomatal limitation progressively increased as drought intensified (significant species and treatment interaction Table 3.2 and Figure 3.4 *right*).

Relative stomatal limitation depicts the effect of stomata on photosynthesis during either the progressive or severe drought treatments relative to the well-watered treatment (Figure 3.5). For the C_3 type, relative stomatal limitation remained the same during the progressive and severe drought treatments. However, it increased in the C_4 type during the severe drought in relation to the progressive drought (significant type and treatment interaction Table 3.2). The stomata were responsible for lowering photosynthetic rates by 24.4% from well-watered values during the progressive drought treatment in the C_3 type and accounted for only 6.3% decrease in photosynthesis in the C_4 type (Figure 3.5 *a*). During severe water stress, the photosynthetic rate of the C_4 type was similarly inhibited by the diffusion of CO_2 into the intercellular airspaces as the C_3 type.

For most of the species, relative stomatal limitation increased as drought progressed, except for *A. semialata* and *P. ecklonii* whose values decreased in the severe drought treatment relative to the progressive drought. *T. leucothrix* maintained similar values in

both drought treatments (significant species and treatment interaction Table 3.2 and Figure 3.5 *b*).

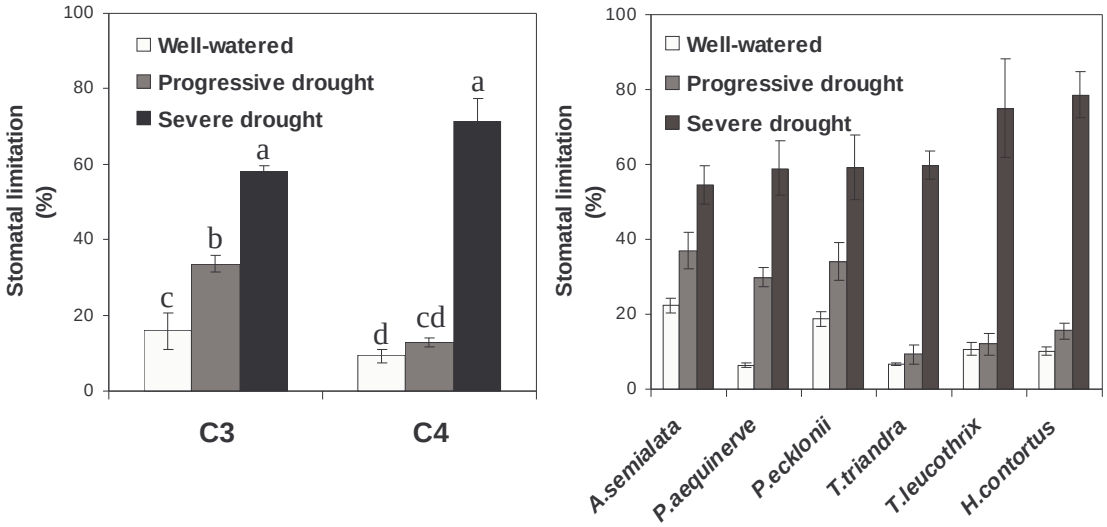

Figure 3.4: The stomatal limitation (L_s) to photosynthesis of well-watered, progressively drought stressed and severely drought stressed leaves of pot-cultivated Panicoid grasses, using data from the CO_2 response curves. Data are grouped by individual species (*right*) and according to photosynthetic type (*left*) (refer to Figure 3.2 for equations). (n = 6 for the well-watered treatment, n = 4-5 for the progressive drought and n = 4-5 for the severe drought, except for *T. triandra* which had n = 3. Vertical bars represent standard errors. Different letters indicate significant differences between means on the different days at $P < 0.05$ (Tukey HSD test).

The reduction in photosynthesis observed in the drought stressed leaves of the C_4 type can be attributed to metabolic factors rather than CO_2 diffusion limitation. Metabolic limitation was responsible for a 48.0% decrease in photosynthesis in the C_4 type as opposed to only a 26.3% decrease observed in the C_3 type during the progressive drought relative to the well-watered treatment (Figure 3.5 *c*). The severe drought treatment had a huge metabolic effect on photosynthesis in all of the species (n.s. species and treatment interaction Table 3.2 and Figure 3.5 *d*). Metabolic limitations accounted for the over 70% decrease in photosynthesis of both types.

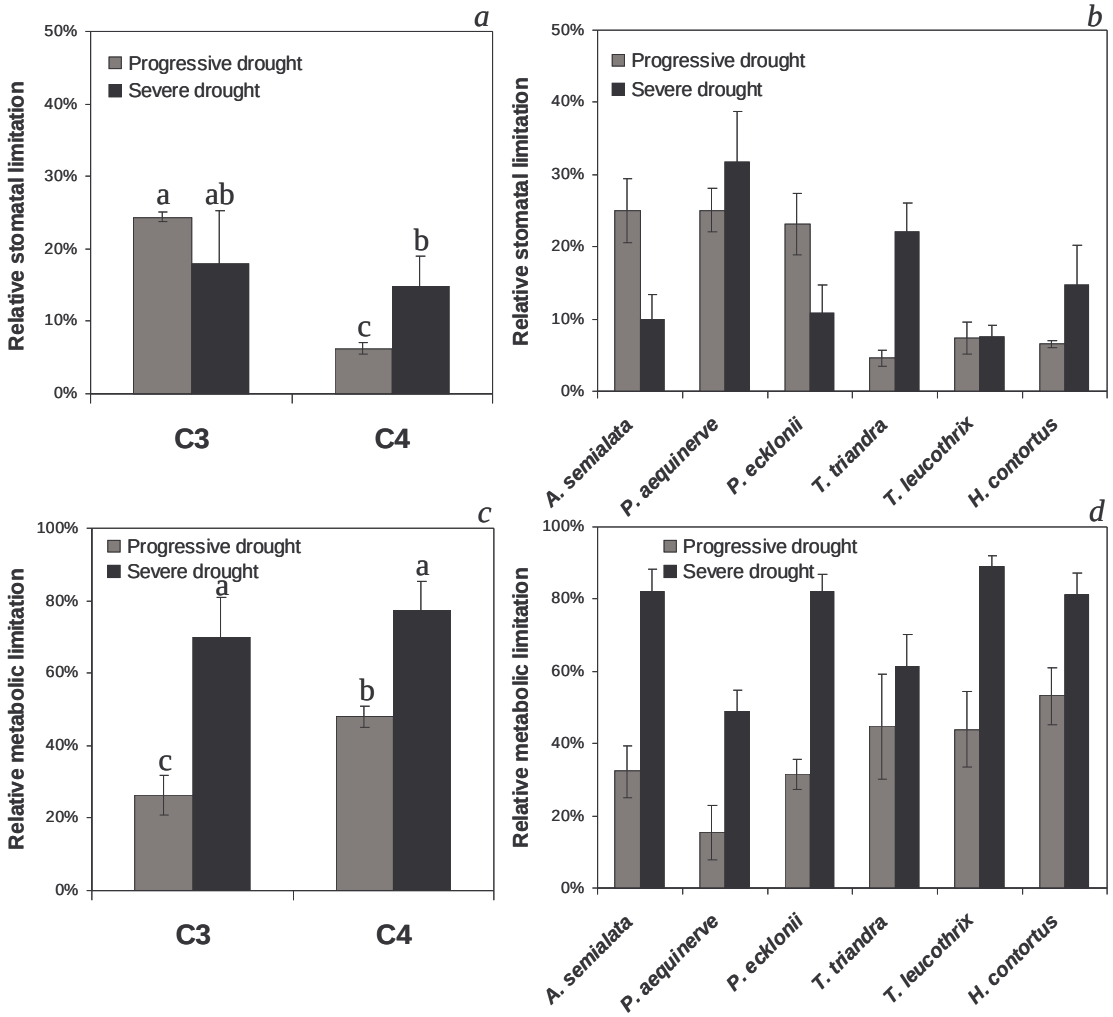

Figure 3.5: The relative stomatal (R_{sl}) and metabolic (R_{ml}) limitations of photosynthesis for pot-cultivated Panicoid grasses subjected to progressive and severe drought treatments as determined from CO_2 response curve data. Values are means of 4 –5 replicates, except for *T. triandra*, which had three replicates for the severe drought. Vertical bars represent standard errors. Different letters indicate significant differences between means on the different days at $P < 0.05$ (Tukey HSD test).

Table 3.2: Summary of statistical significance of photosynthetic type, species (represented by species nested in type) and two drought treatments: progressive and severe on stomatal limitation, relative stomatal limitation and relative metabolic limitation of pot-cultivated Panicoid grasses. n.s., not significant; P>0.05; *, P<0.05; **, P<0.01; ***, P<0.001.

	Type	Species (type)	Treatment	Type x treatment	Species (type) x treatment
Stomatal limitation	*** $F_{1,68}$= 36	*** $F_{4.68}$ = 6.1	*** $F_{2,68}$ = 200	*** $F_{2,68}$ = 23	** $F_{4,68}$ = 3.5
Relative stomatal limitation	*** $F_{1,38}$ = 7.0	**n.s.** $F_{4,\ 38}$ = 0.82	**n.s.** $F_{1,38}$ = 0.13	*** $F_{1,38}$ = 6.4	* $F_{4,38}$ = 0.88
Relative metabolic limitation	** $F_{1,38}$ = 1.2	** $F_{4,38}$ = 4.8	*** $F_{1,38}$ = 73	**n.s.** $F_{1,38}$ = 2.8	**n.s.** $F_{4,38}$ = 1.2

Photosynthetic rates decreased steadily in all species during drought, except for *H. contortus*, which did not lose much photosynthetic capacity during the first 20 days of drought (Figure 3.6 *a*). By day 48, all of the species were showing physical signs of severe water stress: wilting, curled or folded leaves and leaf mortality. All of the species lost considerable photosynthetic capacity at this time (Figure 3.6 *b*).

Plants assigned to the drought treatment were watered on 1st June 2007 to the soil water contents of the control pots. Gas exchange measurements were used to monitor the photosynthetic recovery of these plants. *A. semialata* recovered the quickest (only three days after watering). The C_3 species recovered full photosynthetic capacity sooner than most of the C_4 species (Figure 3.6 *b*). *H. contortus* recovered twenty days after watering, while *T. triandra* and *T. leucothrix* did not recover until day 75, which was three and half weeks after watering. The recovery pots of *A. semialata* and *P.aequinerve* had higher photosynthetic rates than their control pots at the end of the experiment.

Photosynthetic recovery

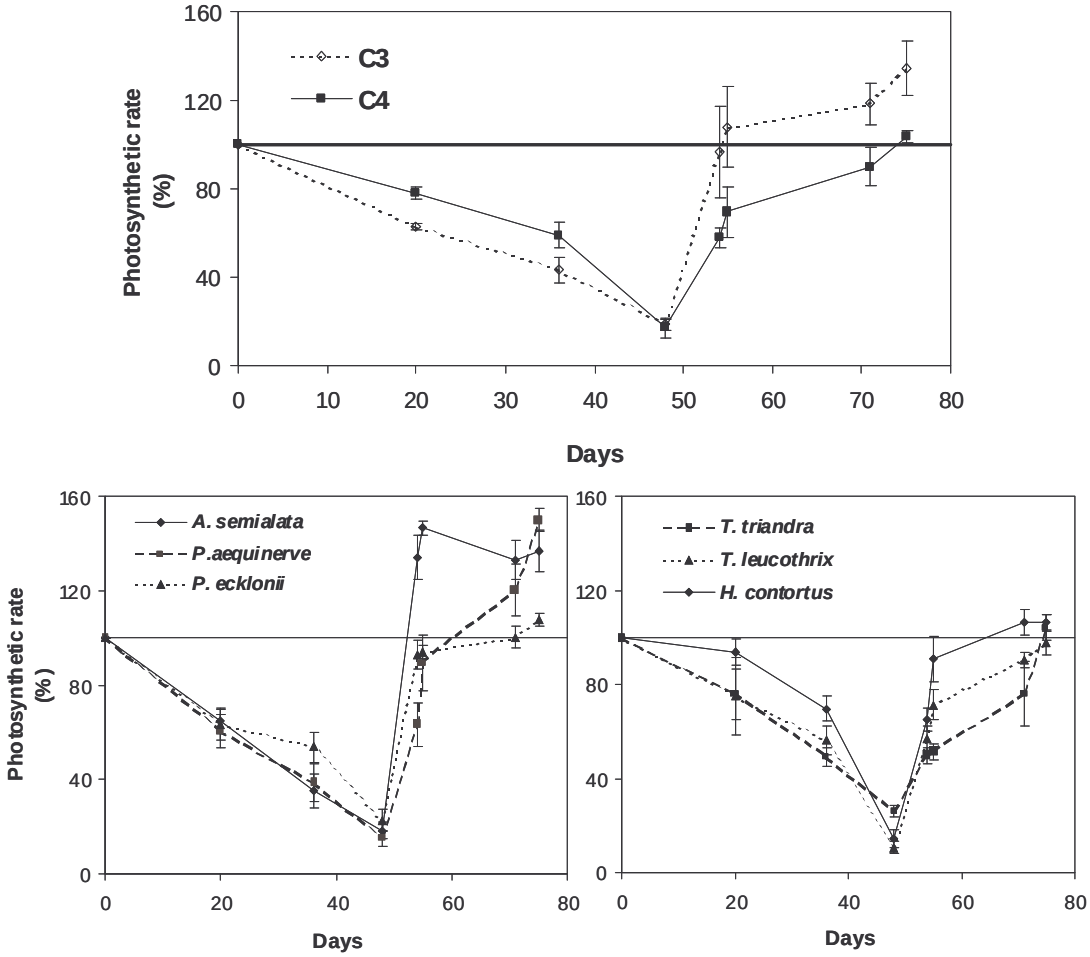

Figure 3.6: The average photosynthetic rates of plants assigned to the drought treatment as a percentage of the control pot averages for the individual Panicoid species and then grouped according to photosynthetic type from gas exchange measurements made during a dry down / watering experiment. Gas exchange measurements were made on control and drought plants on the same days. Vertical bars represent standard errors.

Refer to Chapter 6 and the sections titled “Limitations to photosynthesis” and “Photosynthetic recovery” on page (**85**) for the discussion of this chapter’s results.

Chapter 4: Whole plant water use efficiency

Introduction

The gas exchange measurements of Chapter 2 showed that the C_4 plants had higher instantaneous water use efficiencies (WUE_{leaf}) than the C_3 plants under natural and simulated well-watered conditions, but this advantage was lost during the drought imposed in the pot experiment. The loss of WUE_{leaf} advantage of the C_4 type corresponded to a greater decrease in photosynthesis rather than transpiration or stomatal conductance. One of the aims of this chapter was to determine whether whole plant water use efficiency (WUE_{plant}) would exhibit similar trends to those observed in WUE_{leaf}. Some researchers have demonstrated that WUE_{plant} does not correlate with WUE_{leaf} due to the difference in time scale of the two processes, the additional energy expenditure of respiration during long term growth and the partitioning of photoassimilates into non-photosynthetic tissues (Maroco *et al.* 2000 and Ghannoum *et al.* 2001). This topic is pursued along with the following questions: 1) Does the C_4 type have a greater WUE_{plant} than the C_3 type under well-watered conditions? 2) Is this hypothesized advantage of the C_4 type a result of increased productivity, low water usage or both? 3) Is WUE_{plant} of the C_4 type susceptible to drought as was observed in WUE_{leaf}? 4) Is growth or water usage affected most by drought?

Methods and Materials

Measuring whole plant water use and growth

Whole plant water use efficiency (WUE_{plant}), whole plant relative leaf expansion (RGR_{area}) and whole plant water loss per leaf area (E_{plant}) were monitored during three watering treatments of differing duration. See Figure 2.1 for the SWC of the pots during the treatments. The well-watered treatment lasted for one week; a slowly induced moderate drought was imposed over three weeks and when prolonged for a further week was considered the severe drought treatment. In addition to the treated pots, a set of equivalent well-watered pots (control) was monitored throughout the experiment. RGR_{area} was calculated from the weekly measurements of leaf area made during the three treatment periods. Leaf area was tracked by measuring the lengths of ten randomly selected leaves and counting the total number of leaves per plant. The correlation between leaf length and leaf area was determined from a preliminary study that was conducted on each species. Small, medium and large plants of each species were collected from the Faraway farm field site and brought back to the lab. The lengths and areas of 10-15 leaves of each plant were measured. Leaf areas were analyzed using the computer program WinDIAS (Delta-T Devices, Cambridge, U.K.). The average leaf area for each pot-cultivated plant was calculated by taking the average length of ten leaves per plant and applying it to the linear correlation equation between leaf length and leaf area. Whole plant leaf area was calculated by multiplying average leaf area by the total number of leaves per plant. RGR_{area} was calculated according to Ghannoum *et al*. 2001:
$(\ln A_2 - \ln A_1) / (T_2 - T_1)$, where A represents whole plant leaf area at two points in time (T_1 and T_2).

On 10th April and 26th of June, specific leaf areas (*SLA*) were determined on thirty-five leaves of each species, five leaves per plant per treatment (*SLA* = leaf area / dry weight). Leaf areas were measured using the computer program WinDIAS (Delta-T Devices, Cambridge, U.K.) and the leaves were dried in a 60º C oven for two days before weighing. *SLA* was used to calculate plant dry leaf mass production during the three watering treatments using the following equation:

Leaf dry mass = 1 / *SLA* * plant leaf area

For each treatment, WUE_{plant} was calculated as grams of leaf dry mass accumulated / kg of total water transpired by each plant. Evaporation from the soil was accounted for by subtracting the average weight of the water lost from four pots that did not contain plants from the calculated water loss value for each of the pots. (Please refer to Chapter 2 'Materials and Methods' on page 34 for explanation of the pot experimental set-up). E_{plant} was calculated for each treatment as the average amount of water transpired per day per plant divided by the average calculated plant leaf area.

Data analysis

The effects of species, type and treatment on E_{plant} were tested using the statistical design described in Chapter 2 (page 35). The treatments referred to in this experiment were well-watered, moderate drought and severe drought. Levene's test was used to determine homogeneity of variance between groups. Transformations of the data were performed when needed. In the case of WUE_{plant} *and* RGR_{area}, between treatment effects consistently failed the homogeneity test and thus were dealt with in a different way.

The effects of the three treatments on WUE_{plant} *and* RGR_{area} were tested with a nested general linear model that had type and species nested within type as independent variables.

Results

Whole plant water use efficiency (WUE_{plant})

Under high soil moisture, the C_4 type had significantly higher WUE_{plant} than the C_3 type (Table 4.1 and Figure 4.1 *a*). This advantage continued through the three weeks of moderate drought (Figure 4.1 *b*), but was eventually lost under severe drought conditions (Figure 4.1 *c*). The severe drought induced negative WUE_{plant} values for both types due to huge reductions in leaf area production through leaf mortality. Species had significantly different WUE_{plant} during well-watered conditions due to the C_3 species, *A. semialata* having a similar value to the C_4 species (significant species effect Table 4.1 and Figure 4.1 *d*). Moderate drought caused *P. aequinerve* and *H. contortus* to have negative WUE_{plant} (Figure 4.1 *e*) while all of the species had negative values under severe water stress primarily due to leaf death (Figure 4.1 *f*).

Whole plant relative leaf expansion (RGR_{area})

The C_4 type had significantly higher RGR_{area} than the C_3 type under well-watered conditions and this was consistent for all species within a photosynthetic type (n.s. species effect) (Table 4.1 and Figure 4.2 *a, b*). The C_4 type also maintained higher RGR_{area} than the C_3 type under moderate drought conditions (Table 4.1 and Figure 4.2 *c*). *P. aequinerve* and *H. contortus* were the only species to have negative RGR_{area} during the moderate drought treatment. (Figure 4.2 *d*). Loss of whole plant leaf area in these species was a result of leaf senescence and leaf death. There were no significant differences between the types or species during the week of severe water stress (Table 4.1 and Figure 4.2 *e, f*). All of the species showed reductions in leaf size and numbers of leaves, which resulted in negative RGR_{area} values.

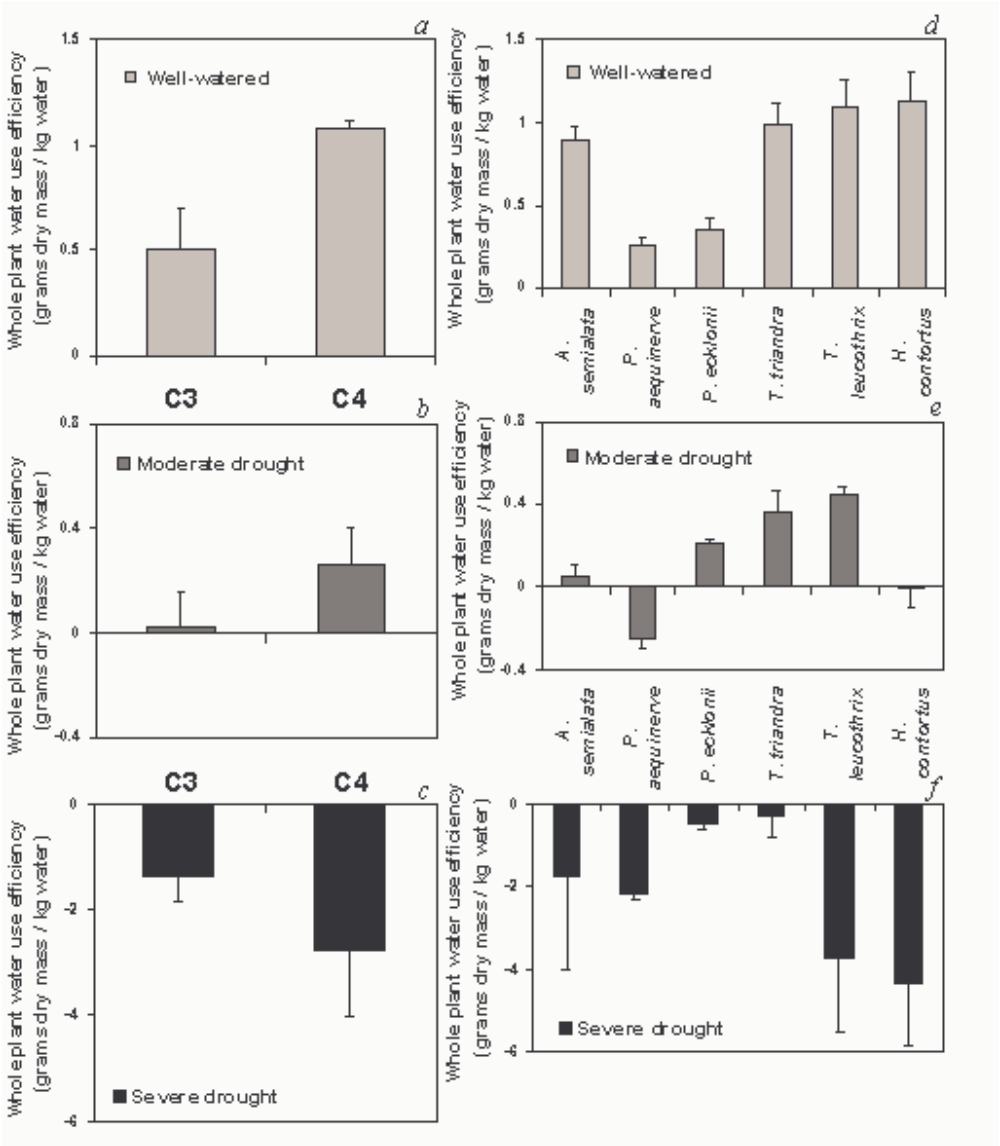

Figure 4.1: Whole plant water use efficiency during three drought treatments: one well-watered week, three weeks of moderate drought and one week of severe drought of pot-cultivated Panicoid grasses, grouped as individual species (*right*) and according to type (*left*). (n = 14 for the well-watered treatment, n = 5 – 7 for the moderate drought treatment and n = 3 – 4 for the severe drought treatment). Vertical bars represent one standard error.

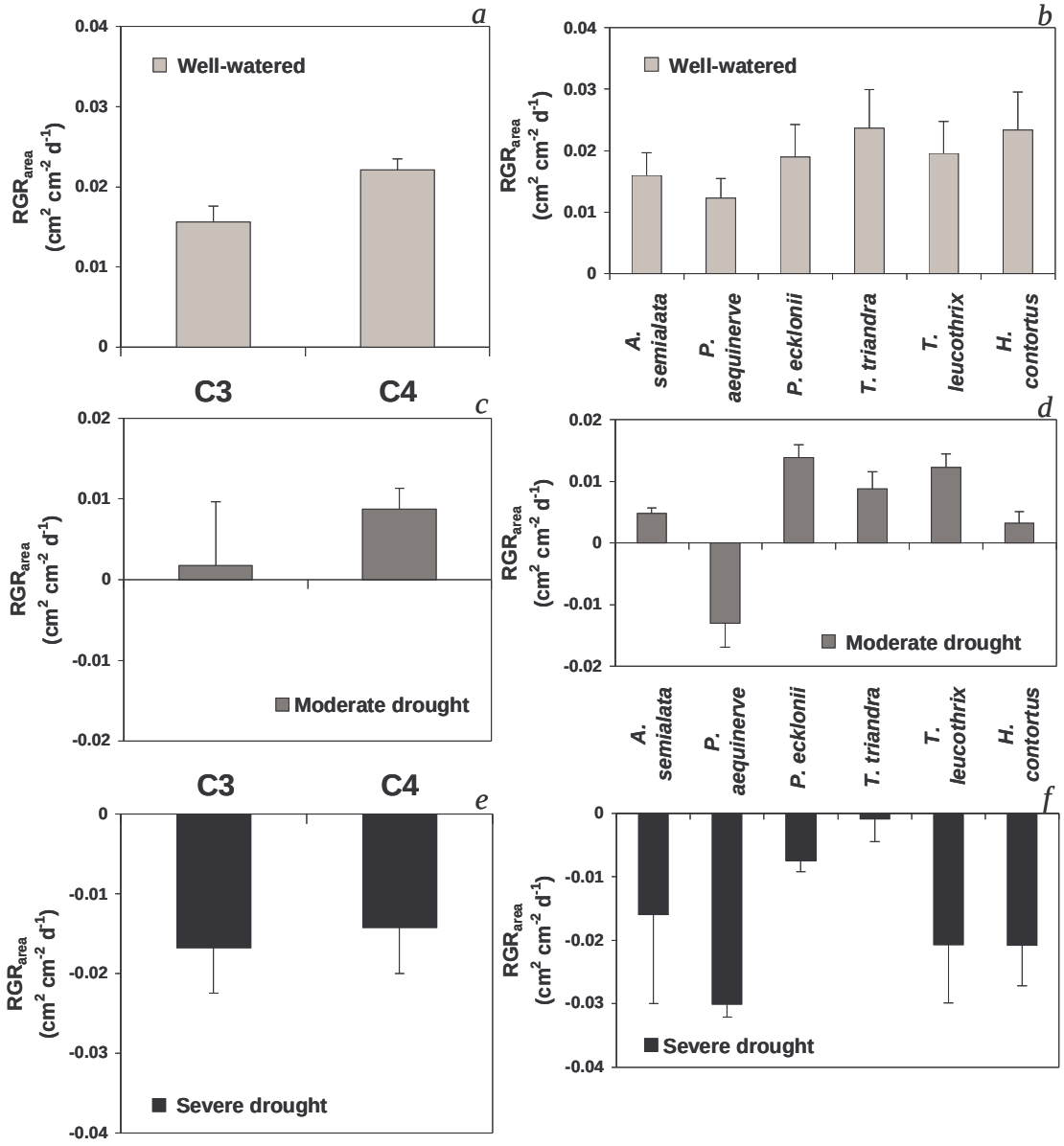

Figure 4.2: Whole plant relative leaf expansion during three drought treatments: one well-watered week, three weeks of moderate drought and one week of severe drought of pot-cultivated Panicoid grasses grouped as individual species (*right*) and according to photosynthetic type (*left*). (n = 14 for the well-watered treatment, n = 5 – 7 for the moderate drought treatment and n = 3 – 4 for the severe drought treatment). Vertical bars represent one standard error.

Table 4.1: Summary of statistical significance of photosynthetic type and species (represented by species nested in type) on whole plant water use efficiency and whole plant relative leaf expansion during three watering treatments imposed on pot-cultivated Panicoid grasses. n.s., not significant; P>0.05; *, P<0.05; **, P<0.01; ***, P<0.001.

	Type	Species (type)
WUE_{plant} Well-watered	*** $F_{1,77} = 43$	*** $F_{4,77} = 9.2$
WUE_{plant} Moderate drought	*** $F_{1,27} = 23$	*** $F_{4,27} = 14$
WUE_{plant} Severe drought	**n.s.** $F_{1,13} = 0.90$	**n.s.** $F_{4,13} = 1.1$
RGR_{area} Well-watered	** $F_{1,81} = 8.6$	**n.s.** $F_{4,81} = 0.68$
RGR_{area} Moderate drought	* $F_{1,27} = 5.2$	*** $F_{4,27} = 21$
RGR_{area} Severe drought	**n.s.** $F_{1,14} = 1.8$	**n.s.** $F_{4,14} = 0.29$

Table 4.2: Summary of statistical significance of photosynthetic type, species (represented by species nested in type) and three imposed watering treatments (well-watered, moderate drought and severe drought) on whole plant water loss per leaf area for pot-cultivated Panicoid grasses. n.s., not significant; P>0.05; *, P<0.05; **, P<0.01; ***, P<0.001.

	Type	Species (type)	Treatment	Type x treatment	Species (type) x treatment
E_{plant}	*** $F_{1,142} = 38$	*** $F_{4,142} = 45$	*** $F_{2,142} = 540$	**n.s.** $F_{2,142} = 0.10$	*** $F_{8,142} = 7.7$

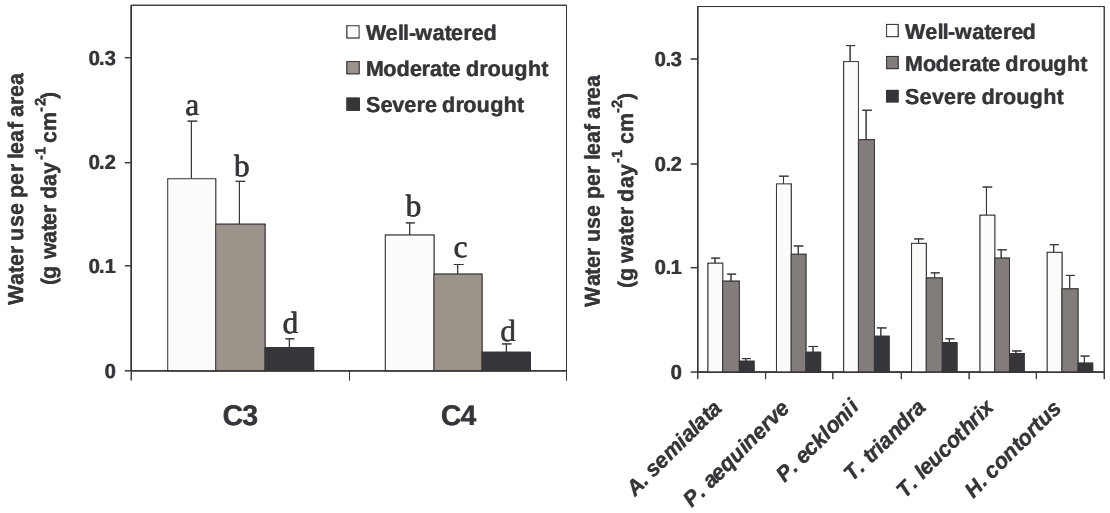

Figure 4.3: Whole plant water loss per leaf area during three drought treatments: one well-watered week, three weeks of moderate drought and one week of severe drought of pot-cultivated Panicoid grasses grouped as individual species (*right*) and according to photosynthetic type (*left*). Values for the well-watered and moderate drought graphs are means of 7-9 replicates and values for the severe drought are means of 5 replicates. The vertical bars represent standard errors. Different letters indicate significant differences between means during the different treatments at $P < 0.05$ (Tukey HSD test).

Whole plant water loss per leaf area (E_{plant})

Both photosynthetic types decreased E_{plant} during the moderate and severe droughts relative to the well-watered conditions (n.s. type and treatment interaction Table 4.2 and Figure 4.3). The C_4 type maintained significantly lower E_{plant} than the C_3 type under well-watered and moderate drought conditions; however both types had similar E_{plant} during the severe drought (significant type effect Table 4.2). E_{plant} of the individual species were only significantly differently to each other during the well-watered and moderate drought treatments (significant species and treatment interaction Table 4.2). *P. ecklonii* had the highest E_{plant} under well-watered and moderate drought conditions, but the severe drought caused all the species to have similar E_{plant}.

Refer to Chapter 6 and the section titled “Whole plant water use efficiency” on page (**89**) for the discussion of this chapter’s results.

Chapter 5: Whole plant hydraulic conductance, leaf water potential and anatomy

Introduction

Water stress makes it increasingly difficult for a plant to maintain the hydraulic continuum between the soil and leaf. This imposes a physical limitation on the rate at which water can be supplied to the leaves to sustain transpiration. Stomata respond to changes in leaf water potential caused by alteration in the liquid phase conductance from soil-to-leaf. (Sperry 2000). According to Sperry (2000), a controlled decline in hydraulic conductance may be advantageous for a plant during drought because it increases the sensitivity response of the stomata, thereby avoiding complete hydraulic failure.

Water flow in plants can be viewed as a catenary process, where each catena (chain) element is viewed as a hydraulic conductance across which water flows. The total conductance of a plant can be represented as the conductance of the roots, stems and leaves in series (van den Honert 1948). Water flow is driven by the water potential difference between the soil and the evaporating surfaces, created by the evaporation of water from the leaves (Tyree 1999). Whole plant hydraulic conductance can be calculated as: $K_{plant} = E / \Delta\Psi$, where E = transpiration rate and $\Delta\Psi$ = is the component of water potential driving the flow (the pressure differential between the soil and the sites of evaporation). Thus WUE_{leaf} is affected by water potential and plant hydraulic conductance through the above mechanisms.

Hydraulic conductance is dependent upon the size and shape of xylem vessels. Vessels that are long and wide can rapidly supply water to transpiring leaves because flow capacity increases with the fourth power of a vessel's radius. A second influence of vessel shape on K_{plant} is that longer vessels have fewer inter-vessel pit membranes which cause resistance to water flow. However, highly conductive xylem vessels are susceptible to cavitations that develop under high xylem tensions during drought. Shorter, narrower

vessels that are able to withstand high xylem tensions are less vulnerable in these environments. When water is limiting, hydraulic safety is selected for at the expense of reduced flow rate (Hacke and Sperry 2001).

Kocacinar and Sage (2003) suggested (for eudicots) that a secondary consequence of C_4 plants having a higher WUE_{leaf} advantage over C_3 plants is the ability to modify xylem structure and function to improve either hydraulic safety or to enhance photosynthetic potential depending on the growth environment. C_4 plants growing in an arid environment may be selected to have safer xylem at the cost of having lower water flow capacity, whereas a mesic C_4 plant will enhance photosynthetic potential by allowing a larger leaf area per unit of xylem than a C_3 plant.

Hydraulic differences between the photosynthetic types arise from the fact that C_4 plants are less sensitive to stomatal closure and require less water than C_3 plants for a given photosynthetic rate. This could potentially translate into the C_4 type having a lower plant hydraulic conductance than the C_3 type. This relaxation in hydraulic demand of the C_4 type may also be reflected in its anatomical characteristics. The C_4 type could potentially have smaller xylem or a lower number of vascular bundles than the C_3 type.

The questions posed for this chapter are: 1) Does the C_4 type have a lower plant hydraulic conductance than C_3 type because of its lower water requirement? 2) Does whole plant hydraulic conductance of the photosynthetic types decrease under drought? 3) Does whole plant hydraulic conductance correlate with anatomical characteristics? 4) Do drought treated grasses have safer xylem compared to the well-watered grasses? 5) Does the C_4 type have less vulnerable xylem than the C_3 type, characterized by shorter and narrower xylem vessels? 6) Are anatomical and hydraulic characteristics modified as a result of water stress?

Methods and Materials

Whole plant hydraulic conductance, leaf water potential and pre-dawn leaf water potential

Transpiration was measured using a Li-6400 photosynthesis system (Li-Cor Inc., Lincoln, NE, USA) on field-grown and pot-cultivated plants on the days specified in Chapter 2 'Materials and Methods'. The leaf used to measure transpiration was immediately excised and put into a Schölander model pressure chamber (Schölander *et al.* 1965) to determine midday leaf water potential (Ψ_{leaf}). For the field experiment, pre-dawn leaf Ψ_{leaf} were made on 5-7 leaves of different plants of each species on the mornings after midday gas exchange measurements giving the stressed leaves time to rehydrate. Pre-dawn Ψ_{leaf} was measured on the same plants as midday Ψ_{leaf} for the pot-cultivated plants. Leaves were excised from the plant and immediately placed into a Schölander model pressure chamber to determine if leaf water potentials recovered overnight. Measurements ceased at sunrise. Whole plant hydraulic conductance was calculated as E / (pre-dawn Ψ_{leaf} – midday Ψ_{leaf}).

Xylem vessel length

Leaves (fully expanded non-apical) of each species were collected in the field on the 5th, 7th and 12th September 2006. The leaves were immediately brought back to the lab. The leaves were pressurized under water and a series of 1 cm segments were cut starting from the apex. The first appearance of bubbles signified the length of the longest xylem vessel. 17-30 leaves of each species were measured. Average vessel length per leaf length was also calculated for each species.

Vascular bundle size class frequency, average maximum vessel diameter, total xylem lumen area and theoretical leaf hydraulic conductance

Four leaves (first fully-expanded non-apical) of each species were harvested from plants in the field on 5th September 2006. Leaf areas were measured with the computer program WinDIAS (Delta-T Devices, Cambridge, U.K.). On 15th May 2007 one leaf (first non-apical) was tagged on each of the pot-cultivated plants belonging to the drought treatment

and then harvested one week later. A one cm segment was cut from the middle of each leaf (field and pot-cultivated leaves). These segments were fixed in FAA for 24 hours. A series of 12 hour rinses were conducted in the following sequence: 50% ethanol, 70% ethanol, 35% n-butanol, 55% n-butanol, 75% n-butanol, 100% n-butanol (three times). Segments were put into vials containing 100% n-butanol and 10% paraplast embedding medium (Sigma-Aldrich Inc., St. Louis, MO, USA) and wax chips and placed into an oven (65ºC) for 12 hours. The vials were then put into the oven for 12 hours with paraplast and wax. This was repeated three times. The specimens were molded into blocks allowed to set, trimmed and cut into 15 µm sections with a microtome (Ernest Leitz Wetzlar GmbH, Germany). The sections were stained by the following protocol: xylol 5 minutes, xylol 5 minutes, xylol/absolute ethanol 3minutes, absolute ethanol 3 minutes, 95% ethanol 3 minutes, 70% ethanol 2 minutes, safranin for 4 hours, 70% ethanol 1 minute, 90% ethanol 30 seconds, 95% ethanol + picric acid 10 seconds, 95% ammoniacal alcohol 10 seconds, 100% ethanol 2 minutes, 100% ethanol 2 minutes, fast green 30 seconds, clove oil 30 seconds, clove oil: absolute ethanol: xylol (1:1:1) 10 seconds, xylol 1 minute, xylol 2 minutes, xylol 2 minutes and mounted on microscope slides with Canada balsam.

Sections were viewed under a light microscope and images were analyzed using WinDIAS (Delta-T Devices, Cambridge, U.K.). Vascular bundle size class frequency was determined by counting the total number of small, intermediate and large bundles in each cross section and dividing by the width of the leaf blade. The determination of vascular bundle size class is based on the descriptions of Ellis (1976). In this study, the terms large, intermediate and small correspond to Ellis (1976) terms first order, second order and third order, respectively. Three representative vascular bundles of each size class (large, intermediate and small) were chosen in each section. The lengths of the major and minor axes of all vessels in each representative bundle were measured. Each vessel was assumed to be an ellipse. Each vessel's diameter was calculated using the formula: $\sqrt{(a^2)} + (b^2) / 2$, where a and b are the short and long axes respectively. The three largest vessel diameters for each leaf per treatment were recorded and averaged. The lumen area of each vessel in each representative bundle for the well-watered samples was

calculated using the formula for the area of an ellipse: (a * b* π). Total lumen area was calculated as the sum of all the vessel lumen areas in each bundle size class multiplied by the frequency of the bundle class size. Theoretical leaf hydraulic conductance was calculated for the well-watered samples as:

$$K_t = \frac{\pi * a^3 * b^3}{64 * \mu * (a^2 + b^2)}$$ (Lewis and Boose 1995)

where K_t is the volume flow rate; μ is the viscosity of water; a and b are the short and long axes, respectively. Both total xylem lumen area K_t were normalized by leaf area.

Data analysis

The statistical design used in Chapter 2 was applied to the data for K_{plant}, Ψ_{leaf} and pre-dawn Ψ_{leaf} (see page 35 for the specifics).

A general linear model with a nested design was used on the data for average length of longest xylem vessel, vessel length / leaf length, total xylem lumen area and theoretical leaf hydraulic conductance to determine differences between photosynthetic type and species when type was taken into account. Levene's test was used to determine homogeneity of variance. Raw data was log transformed. Statistical differences between means were determined by Tukey HSD post-hoc tests if the general linear model effect was significant.

A nested general linear model was used to analyze the data for vascular bundle size class frequency and average maximum vessel diameter to determine the differences between the species (when accounting for type), treatment and their interaction. The two treatments relate to leaves grown naturally under well-watered conditions and leaves that were severely drought stressed during the pot experiment. A one-way ANOVA was used to analyze treatment effects on the number of intermediate bundles in the leaves of *Tristachya leucothrix*. Raw data were log transformed. Levene's test was used to determine homogeneity of variance. Statistical differences between means were determined by Tukey HSD post-hoc tests if the general linear model effect was significant.

Results

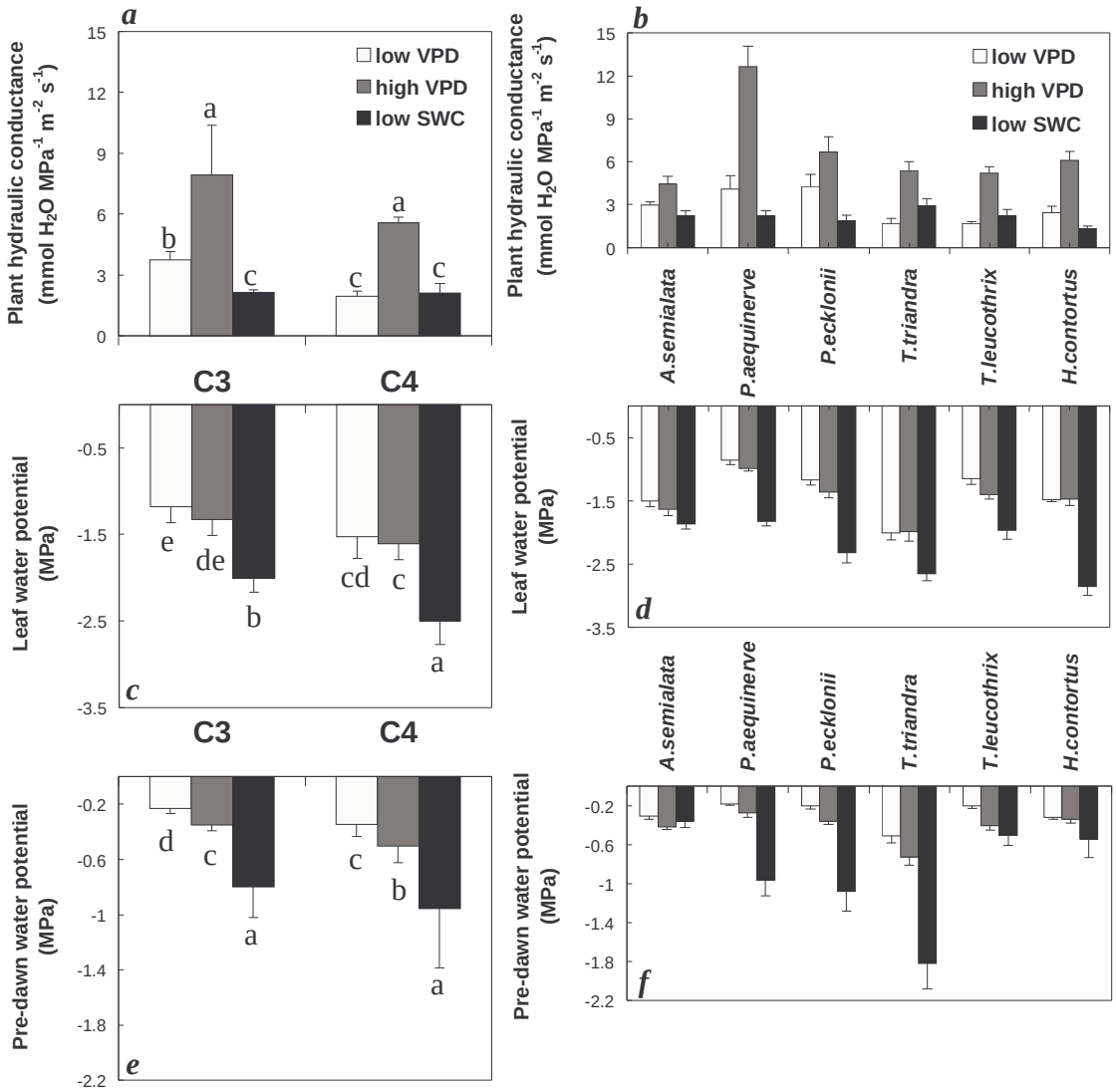

Figure 5.1: Midday leaf water potential, pre-dawn leaf water potential and whole plant hydraulic conductance, calculated as E / (pre-dawn Ψ_{leaf} – midday Ψ_{leaf}) for field-grown Panicoid grasses during three naturally occurring treatments in the field. Data are grouped according to type (*left*) and individual species (*right*). Midday leaf water potential and whole plant hydraulic conductance: n = 9-11 for all three treatments. Pre-dawn water potential: n = 5 for low VPD day, n = 7-9 for high VPD day and n = 8 for the low SWC day. Vertical bars represent standard errors. Different letters indicate significant differences between means on the different days at $P < 0.05$ (Tukey HSD test).

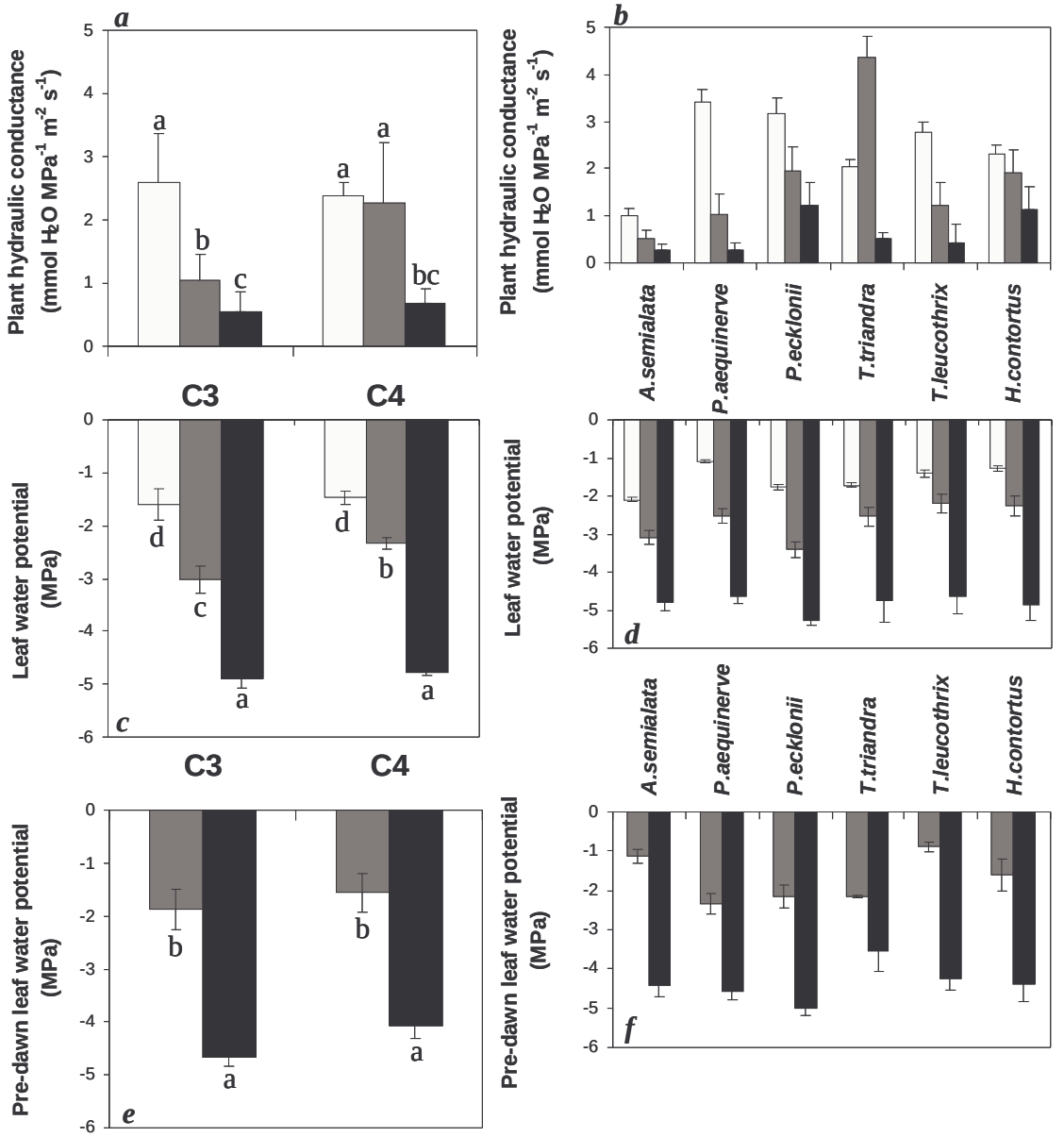

Figure 5.2: Midday leaf water potential, pre-dawn leaf water potential and whole plant hydraulic conductance for pot-cultivated Panicoid grasses during three watering treatments imposed by the pot experiment. Data are grouped as individual species (*right*) and according to type (*left*). White bars represent well-watered conditions of day 0, gray bars represent progressive drought of day 36 and black bars represent severe drought of day 48 ($n \geq 14$ for day 0 and $n = 7$ for day 36 and 48). Vertical bars represent standard errors. Different letters indicate significant differences between means on the different days at $P < 0.05$ (Tukey HSD test).

Table 5.1: Summary of statistical significance of photosynthetic type, species (represented as species nested in type) and treatment as specified for the field data as: low VPD, high VPD or low SWC and as three watering treatments during the pot experiment: well-watered, progressive drought and severe drought for the pot data, on whole plant hydraulic conductance, midday leaf water potential and pre-dawn leaf water potential of the Panicoid grasses. n.s., not significant; P > 0.05; *, P< 0.05; **, P < 0.01; ***, P < 0.001.

	Type	Species (type)	Treatment	Type x treatment	Species (type) x treatment
K_{plant} field	*** $F_{1,163}$= 17	* $F_{4,163}$= 3.2	*** $F_{2,163}$= 98	*** $F_{2,163}$= 7.2	*** $F_{8,163}$= 4.1
K_{plant} pot	* $F_{1,162}$= 6.2	*** $F_{4,162}$= 11	*** $F_{2,162}$= 83	** $F_{2,162}$= 5.3	*** $F_{8,162}$= 4.7
Ψ_{leaf} field	*** $F_{1,164}$= 65	*** $F_{4,164}$= 33	*** $F_{2,164}$= 130	**n.s** $F_{2,164}$= 0.63	*** $F_{8,164}$= 5.8
Ψ_{leaf} pot	*** $F_{1,386}$= 36	*** $F_{4,386}$= 19	*** $F_{2,386}$= 1100	*** $F_{2,386}$= 7.9	*** $F_{8,386}$= 5.6
Predawn Ψ_{leaf} field	*** $F_{1,232}$= 16	*** $F_{4,232}$= 31	*** $F_{2,232}$= 85	* $F_{2,232}$= 3.3	*** $F_{8,232}$= 12
Predawn Ψ_{leaf} pot	** $F_{1,72}$= 7.1	* $F_{4,72}$= 2.6	*** $F_{1,72}$= 240	**n.s** $F_{1,72}$= 0.63	** $F_{4,72}$= 3.6

Whole plant hydraulic conductance for field-grown grasses

The two photosynthetic types responded differentially to the three environmental treatments (significant type and treatment interaction Table 5.1). Both types increased K_{plant} during the high VPD treatment relative to the low VPD treatment (control). However the C_4 type had similar K_{plant} during both the low SWC and control treatments, while the C_3 type had significantly lower K_{plant} during the low SWC treatment as compared to the control. The C_4 type had a lower K_{plant} than the C_3 type during the low VPD treatment, but both types had similar values during the high VPD and low SWC treatments (Figure 5.1 *a*).

The species also responded differentially to the treatments (significant species and treatment interaction Table 5.1). *A. semialata* and *P. ecklonii* were the only species to not adjust K_{plant} between the control and high VPD treatments. *P. ecklonii* significantly decreased K_{plant} during the low SWC treatment relative to the control treatment, while the other species did not (Figure 5.1 *b*).

Whole plant hydraulic conductance for pot-grown grasses

The three watering treatments induced differential effects on the two types (significant type and treatment interaction Table 5.1). At the start of the greenhouse experiment, both types had similar $K_{plant,}$ but K_{plant} significantly decreased in the C_3 type as drought progressed, whereas K_{plant} of the C_4 type remained unaltered (Figure 5.2 *a*). Both photosynthetic types had similar K_{plant} during the severe drought, which were significantly lower than each type's initial values.

The significant species and treatment interaction is a result of *P. aequinerve* having significantly decreased K_{plant} during the progressive drought relative to the control. *T. triandra* increased K_{plant} during this period and the remaining species maintained similar K_{plant} during these treatments (Table 5.1 and Figure 5.2 *b*).

Midday leaf water potential for field-grown grasses

The photosynthetic types responded similarly to the three treatments (n.s. type and treatment interaction Table 5.1). Both types maintained similar Ψ_{leaf} during high and low VPD, however each type had significantly lower Ψ_{leaf} during the low SWC treatment relative to the control (Figure 5.1 *c*). The C_4 type maintained significantly more negative Ψ_{leaf} than the C_3 type during all three treatments (significant type effect Table 5.1).

The treatments induced differential effects on the species (significant species and treatment interaction Table 5.1). *A. semialata* and *T. triandra* had similar Ψ_{leaf} during the low SWC and control treatments, while the other species had significantly lower Ψ_{leaf} during the low SWC treatment relative to the control (Figure 5.1 *d*).

Midday leaf water potential for pot-grown grasses

The two types had similar Ψ_{leaf} at the start of the experiment (Figure 5.2 *c*). Ψ_{leaf} of the C_4 type decreased more gradually than the C_3 type during the progressive drought period (up to day 36), but by day 48 both types had similar Ψ_{leaf} that were significantly lower than the other days (significant type effect Table 5.1).

Initially *P. aequinerve* had the most favorable water status, but as the drought intensified, this species reached values similar to the other species (Figure 5.2 *d*). The severe drought induced very negative Ψ_{leaf} in all of the species.

Pre-dawn leaf water potential for field-grown grasses

The C_4 type had significantly lower pre-dawn Ψ_{leaf} than the C_3 subtype during the low and high VPD treatments, but both types had similar values during the low SWC treatment (Figure 5.1 *e*).

The species responded differently to the treatments (significant species and treatment interaction Table 5.1). *T. leucothrix* had a significantly lower pre-dawn Ψ_{leaf} during the high VPD treatment as compared to the control (Figure 5.1 *f*). The other species did not adjust their pre-dawn Ψ_{leaf} between these two treatments. *A. semialata* and *H. contortus* maintained very similar pre-dawn Ψ_{leaf} across all treatments, while the other species had significantly lower pre-dawn Ψ_{leaf} during the low SWC treatment.

Pre-dawn leaf water potential for pot-grown grasses

Both of the photosynthetic types significantly decreased pre-dawn Ψ_{leaf} during the severe drought treatment relative to the progressive drought (Figure 5.2 *e*). The C_4 type had consistently higher pre-dawn Ψ_{leaf} than the C_3 type in both drought treatments.

A. semialata and *T. leucothrix* maintained a more favorable water status than the other species during the progressive drought; however the severe drought had the greatest effect on the Ψ_{leaf} of *A. semialata* (Figure 5.2 *f*).

Anatomical measurements

Table 5.2: Average length of longest xylem vessel and vessel length per leaf length for six species of Panicoid grasses harvested from the field during well-watered conditions, grouped as individual species and according to photosynthetic type.

Species	Average length of longest xylem vessel (cm)	Vessel length / leaf length (%)
A. semialata	0.97 ± 0.05 *a*	5.58 ± 0.31 *c*
P. aequinerve	0.75 ± 0.06 *bc*	15.69 ± 1.2 *a*
P. ecklonii	0.85 ± 0.06 *ab*	9.03 ± 0.73 *b*
T. triandra	0.62 ± 0.03 *c*	5.70 ± 0.30 *c*
T. leucothrix	0.83 ± 0.05 *ab*	4.01 ± 0.28 *d*
H. contortus	0.83 ± 0.04 *ab*	5.24 ± 0.43 *cd*
C_3	**0.87 ± 0.06 *a***	**9.60 ± 2.99 *a***
C_4	**0.74 ± 0.07 *b***	**5.06 ± 0.50 *b***

All values are means ± s.e. Means within a column (for the individual species and photosynthetic types separately) that are not followed by the same letter are significantly different at P < 0.05 (Tukey HSD test). 20 - 30 leaves were used for each species.

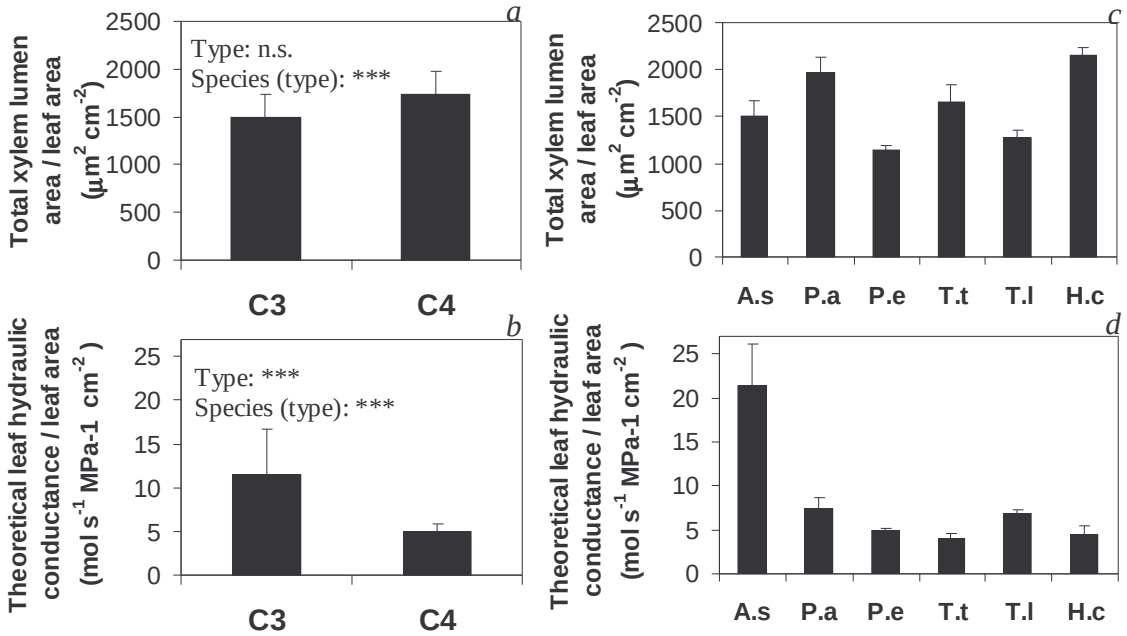

Figure 5.3: The total xylem lumen area (*a, c*) and theoretical leaf hydraulic conductance (*b, d*) of six species of Panicoid grasses grown naturally under well-watered conditions in the field. Data are grouped as individual species (*right*) and according to photosynthetic type (*left*) grown naturally under well-watered conditions in the field. Values are means (n = 4) and vertical bars represent standard errors. n.s., not significant; P > 0.05; *, P< 0.05; **, P < 0.01; ***, P < 0.001.

Table 5.3: Comparison of the vascular bundle size class frequency and the average maximum xylem vessel diameter for the six species of Panicoid grasses that were grown under well-watered conditions in the field (F) and under severe water stress (WS) imposed during the pot experiment**.**

Species	Bundle size class frequency (Number / cm blade width)			Average diameter of the three largest xylem vessels (μm)
	Large	Intermediate	Small	
A. semialata (F)	1.52 ± 0.08		5.10 ± 0.20	31.03 ± 1.23 *
A. semialata (WS)	1.67 ± 0.17		3.90 ± 0.22	22.53 ± 0.93
P. aequinerve (F)	1.92 ± 0.15		5.18 ± 0.65	16.59 ± 0.59
P. aequinerve (WS)	1.50 ± 0.17		4.40 ± 0.45	14.83 ±0.61
P. ecklonii (F)	2.20 ± 0.08 *		6.86 ± 0.11 *	18.27 ± 0.63
P. ecklonii (WS)	1.13 ± 0.05		3.71 ± 0.23	18.45 ± 1.18
T. triandra (F)	3.62 ± 0.21		16.13 ± 0.59	16.57 ± 0.33 *
T. triandra (WS)	3.28 ± 0.04		16.76 ± 1.00	11.95 ± 0.35
T. leucothrix (F)	2.22 ± 0.10	5.04 ± 0.49 *	8.60 ± 0.57	25.67 ± 0.92 *
T. leucothrix (WS)	2.12 ± 0.21	2.94 ± 0.37	6.63 ± 0.42	16.71 ± 0.73
H. contortus (F)	2.89 ± 0.07		14.87 ± 0.03	15.22 ± 0.75
H. contortus (WS)	2.73 ± 0.15		14.35 ± 0.41	16.11 ± 0.49
C_3 (F)	1.80 ± 0.20*b* *		5.45 ± 0.57*b* *	22.45 ± 4.56*a* *
C_3 (WS)	1.43 ± 0.16*c*		4.00 ± 0.20*c*	18.60 ± 2.22*b*
C_4 (F)	2.98 ± 0.41*a*		13.49 ± 2.33*a*	18.53 ± 3.25*b* *
C_4 (WS)	2.71 ± 0.33*a*		12.58 ± 3.00*a*	14.92 ±1.50*c*

Values are means of four replicates ± s.e. * indicates significant differences between the treatments. Different letters indicate significant differences between the types and treatment at $P < 0.05$ (Tukey HSD test).

Anatomical characteristics

Average length of longest xylem vessel and vessel length / leaf length

The C_3 type had significantly longer xylem vessels that also comprised a greater proportion of the total leaf length than the C_4 type (Table 5.2). *A. semialata* had the longest xylem vessels of all the study species, but this length was only significantly longer than the xylem lengths of *P. aequinerve* and *T. triandra*. The proportion of xylem length to leaf length was greatest in *P. aequinerve* and the smallest in *T. leucothrix*.

Total xylem lumen area and theoretical hydraulic conductance

The photosynthetic types had similar total xylem lumen areas when normalized for leaf area (Figure 5.3 *a*), however the C_3 type had significantly higher K_t per leaf area than the C_4 type (Figure 5.3 *b*) under well-watered field conditions. *A. semialata* had the greatest K_t of all of the species (Figure 5.3 *d*).

Vascular bundle size class frequencies

The C_4 type had significantly greater numbers of large and small vascular bundles per cm of leaf width than the C_3 type under well-watered field conditions (Table 5.3). Drought resulted in the C_3 type having significantly lower numbers of large and small vascular bundles relative to its well-watered values, while the C_4 type maintained the same number of vascular bundles in both treatments. *P. ecklonii* was the only species to significantly reduce the numbers of large and small vascular bundles during drought and *T. leucothrix* had a significantly lower number of intermediate bundles during the drought relative to well-watered treatment (Table 5.3).

Average maximum vessel diameters

The average maximum xylem vessels of the C_3 type are larger than the C_4 type under both treatments (Table 5.3). Both types decreased their maximum diameters under drought. *A. semialata* had the largest maximum vessels of all of the species when grown in a well-watered environment. *A. semialata*, *T. triandra* and *T. leucothrix* were the only species to significantly reduce the size of their maximum vessels under water stress

(Table 5.3). The vascular bundles of the C_3 type are larger and contain bigger vessels than the C_4 type.

Refer to Chapter 6 and the sections titled “Hydraulics” and “Kocacinar and Sage Hypothesis” on page (**94**) for the discussion of this chapter’s results.

Chapter 6: Discussion

The primary aim of this study was to determine the leaf level and whole plant level drought sensitivity of NADP-ME grasses relative to C_3 grasses by controlling for phylogeny. Physiological leaf-level responses of three C_4 (NADP-ME) and three C_3 Panicoid grasses were initially compared *in-situ* under natural drought conditions to investigate whether the NADP-ME species were able to maintain efficient water use through lower stomatal conductances while fixing CO_2 at rates equal to or greater than C_3 species. Drought conditions were replicated in a pot experiment to take a closer look at previously observed trends and to explain the susceptibility of C_4 (NADP-ME) photosynthesis and water use efficiency during water stress. Whole plant water use was also monitored to determine if the leaf level (WUE_{leaf}) advantage of the NADP-ME species during well-watered conditions and subsequent loss of advantage during severe water stress translated into similar whole plant (WUE_{plant}) trends. It was hypothesized that there would be a correlation between WUE_{leaf} and WUE_{plant} considering that both parameters are influenced by similar factors (Figure 6.1).

WUE_{leaf} is dependent on photosynthesis and leaf level transpiration (E_{leaf}) as indicated by the arrows in the figure below. E_{leaf} is governed by the resistance of the stomata and of the layer of unstirred air next to the leaf surface. The artificial flow rate induced by the gas analyzer cuvette greatly reduces leaf boundary layer resistance resulting in stomatal conductance (g_s) being the most important parameter in regulating water loss. Leaf boundary layer resistance becomes more important under drought conditions and when we scale up to the whole plant level. Leaf folding during drought will increase boundary layer resistance and thereby decrease E_{plant}. WUE_{plant} is directly affected by the changes associated with E_{plant}, but is also affected by changes in leaf biomass production, which is linked to photosynthetic rates. Photosynthetic rates are directly affected by g_s, which plays a dual role of regulating both CO_2 uptake and water loss (as mentioned previously). Changes in leaf water potential (Ψ_{leaf}) caused by alterations in plant hydraulic conductance (K_{plant}) induce a stomatal response that directly affects gas exchange.

Anatomical characteristics will influence K_{plant} and Ψ_{leaf}, which in turn will affect g_s. Thus it can be seen that the components of WUE_{leaf} and WUE_{plant} are closely linked.

The investigation of hydraulic parameters was included to determine their influences on gas exchange. g_s is usually positively correlated with hydraulic conductance of the soil-leaf continuum (Sperry 2000). Anatomical characteristics were measured to determine their influences on K_{plant} and Ψ_{leaf} and to test the Kocacinar and Sage (2003) hypothesis on monocots. The results of this present study are compared to various studies and are discussed here.

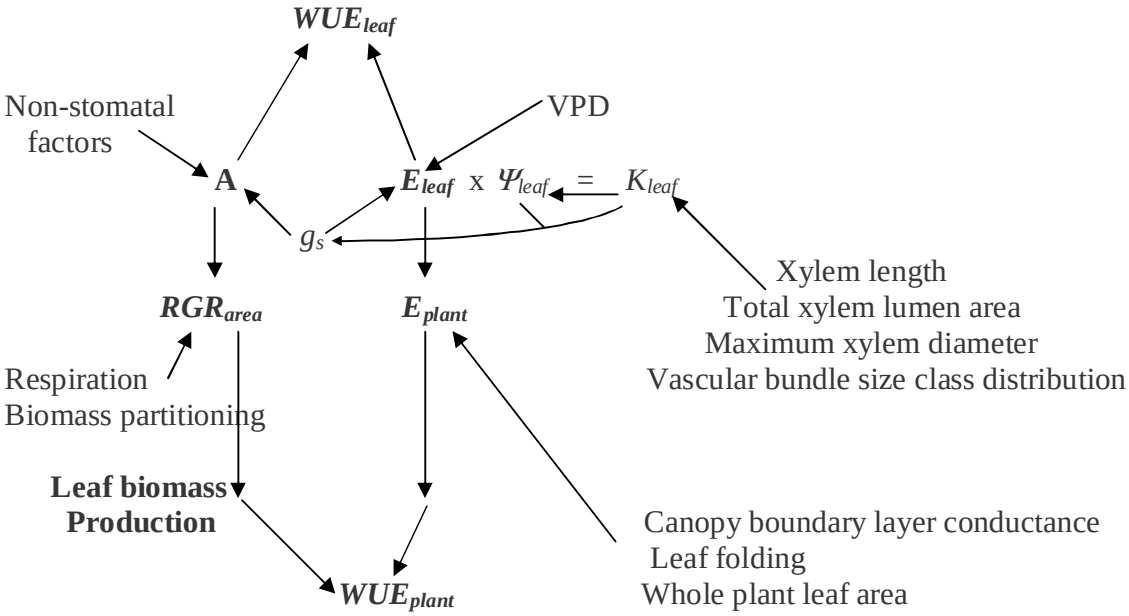

Figure 6.1: A schematic of the interactions between leaf and whole plant water use efficiency characteristics. Arrows indicate direct relationships between parameters. Adjustments to one component will influence and affect the other components**.** WUE_{leaf} is leaf water use efficiency, WUE_{plant} is whole plant water use efficiency, A is photosynthetic rate, E_{leaf} is leaf transpiration rate, Ψ_{leaf} is leaf water potential, g_s is stomatal conductance, K_{leaf} is leaf hydraulic conductance, RGR_{area} is whole plant relative leaf expansion, and E_{plant} is whole plant water loss.

Leaf level water use efficiency

The C_4 type had significantly higher instantaneous water use efficiency (WUE_{leaf}) than the C_3 type under well-watered conditions in both the field and the greenhouse experiments (Figure 2.3 *a* and Figure 2.4 *a*). The WUE_{leaf} advantage of the C_4 type was maintained in the field, but this advantage was lost during the pot experiment at an average SWC of 4.3% (day 36). Xu *et al.* (2006) also demonstrated how WUE_{leaf} decreases with decreasing SWC in the three C_4 grass species *Panicum virgatum, Setaria italica* and *Bothriochloa ischaemum*. In addition, Marques da Silva and Arrabaça (2004) showed how WUE_{leaf} of three C_4 grasses *Setaria sphacelata*, *Paspalum dilatatum* and *Zoysia japonica* were sensitive to decreasing relative water content (RWC). Alternatively, Maroco *et al*. (2000) showed that WUE_{leaf} of two Sahelian C_4 grasses was not changed by drought. Thus, the response of WUE_{leaf} to drought in C_4 grass species is highly variable.

The reduction in WUE_{leaf} of the C_4 type during the pot experiment corresponded to a greater decrease in photosynthesis rather than transpiration (Figures 2.4 *a, b, c*). The C_4 type had a similar transpiration rate and stomatal conductance to the C_3 type under well-watered conditions. The C_4 type was able to achieve a higher photosynthetic rate than C_3 type at this time because photorespiration is nearly suppressed in C_4 species. As drought progressed (days 20-48), the C_4 type did not decrease transpiration to save water as was observed in the C_3 type. Rather transpiration rates were maintained during the drier periods. However it was shown that the C_4 type lost photosynthetic advantage at a g_s of 0.1 mol H_2O m^{-2} s^{-1} during the pot experiment intimating that factors other than the limitation of CO_2 diffusion were at play in the reduction of photosynthesis (Figure 2.6 *right*). Ripley *et al.* (2007) demonstrated during a common garden experiment that drought caused the C_4 subspecies of *A. semialata* (NADP-ME) to lose photosynthetic advantage to the C_3 subspecies at a g_s of about 0.08 mol H_2O m^{-2} s^{-1}, implying a possible NADP-ME-specific photosynthetic vulnerability.

Carmo-Silva *et al.* (2007) showed similar photosynthetic sensitivities to drought in their comparison of three grass species belonging to the three biochemical types of C_4 photosynthesis. The photosynthetic rates of *Paspalum dilatatum* (NADP-ME) and *Zoysia japonica* (PCK) were the most sensitive to drought showing rapid decline with decreasing leaf RWC, while the photosynthetic rate of *Cynodon dactylon* (NAD-ME) was the least sensitive to water deficit and was sustained even below 40% RWC. However, the stomata of *P. dilatatum* were the most sensitive to water stress, whereas *C. dactylon* was able to sustain stomatal conductance at the lowest recorded RWC. The NADP-ME species of this study differed in that they maintained measurable stomatal conductances at very low SWC (Figure 2.5 *d*). This observation could mean that the stomata of the NADP-ME species are insensitive to plant water status or the technique used to measure transpiration was invalid (see later discussion).

Limitations to photosynthesis

The photosynthetic advantage of the C_4 type was maintained throughout most of the drought experiment, up until the average SWC was reduced to 3% (Figure 2.4 *b*). At this point, water stress was so severe that both types lost over 88% of their photosynthetic capacity. Decreased photosynthetic rates during moderate water stress can be attributed to stomatal closure (Jones 1973 and Downton *et al*. 1988), whereas metabolic (non-stomatal) factors become more important under severe drought conditions (Flexas *et al.* 2006a, Lawlor 2002, Ghannoum *et al.* 2003). Saccardy *et al*. (1996) showed that these effects were also dependent on whether the drought was rapidly or slowly induced. Photosynthesis of *Zea mays* was limited by non-stomatal factors during a rapid drought, but stomatal closure accounted for the decrease in photosynthesis during slow dehydration. Conversely, Marques da Silva and Arrabaça (2004) showed that photosynthesis of *Setaria sphaecelata* was limited by non-stomatal factors in slow stress experiments, but stomatal limitations were more relevant during rapidly imposed water stress. Since slow drought is more ecologically relevant (Table 2.2), non-stomatal factors may be more important than stomatal factors in wild plants growing naturally in the field.

Flexas *et al*. (2006a) characterized the phases of photosynthetic response to water stress by daily maximum stomatal conductances (g_s). Above g_s of 0.05-0.1 mol H_2O m^{-2} s^{-1}, photosynthesis is mostly limited by CO_2 diffusion as characterized by reduced stomatal and mesophyll conductances. Below this g_s threshold, general metabolic impairment occurs. The C_3 species studies here did not decrease g_s into the low end of the range (<0.05) until the late stages of drought (day 36, Figure 2.5 *d*). By day 48, the C_3 type was below the threshold specified by Flexas *et al*. (2006a), whereas g_s of the C_4 type was maintained at the high end of the range (Figure 2.4 *d*). According to the argument of Flexas *et al*. (2006a), the C_4 type should have less general overall impairment than the C_3 type at this stage of drought, but it was determined this was not the case. The two types were equally inhibited by metabolic limitations of photosynthesis (Figure 3.5 *c*). The more interesting result of this experiment was that the C_4 type showed greater metabolic rather than stomatal limitations of photosynthesis during the progressive drought treatment at a g_s of 0.16 mol H_2O m^{-2} $s^{-1.}$ This is well above the range where photosynthesis is thought to be limited primarily by CO_2 diffusional resistances, according to Flexas *et al.* (2006a).

CO_2 response curves were constructed to analyze the specific effects of drought stress on photosynthesis of the two types. The progressive drought treatment, characterized by an average SWC of 3.8%, caused reductions in the carboxylation efficiencies and the saturated photosynthesis values of both types (Figures 3.3 *a-f*). However, the C_4 type had a 61.7% decrease in carboxylation efficiency as compared to only a 43.5% decrease in the C_3 type. The C_4 type also had a greater decrease in the CO_2 saturated portion of the curve than the C_3 type. This amounted to a 53.6% decrease in C_4 type and a 35.8% decrease in the C_3 type. These results agree with Ripley *et al*. (2007), who found that the carboxylation efficiency of the C_4 subspecies of *Alloteropsis semialata* decreased by 76% during drought as compared to only a 39% decrease in the C_3 subspecies implying a greater vulnerability of the C_4 cycle to maintain maximum activity than the C_3 cycle during drought (von Caemmerer 2000).

The C_4 type was more susceptible to the progressive drought treatment showing metabolic effects, rather than stomatal effects on photosynthesis that reduced photosynthetic capacity more than was observed for the C_3 type (Figures 3.5 *a*, *c*). Metabolic factors reduced the photosynthetic rate of the C_4 type by almost half as compared to a reduction of only 26% in the C_3 type during the progressive drought treatment relative to the control. These results are in agreement with Ripley *et al*. (2007) who found that the C_4 subspecies of *A. semialata* had significantly higher relative metabolic limitation values than the C_3 subspecies, accounting for a 36%, as opposed to a 19% reduction in photosynthesis during drought. Alternatively, the stomata had four times greater effect on photosynthesis of the C_3 type in this study relative to the C_4 type (Figure 3.5 *a*). Ultimately the photosynthetic capacities of severely drought stressed plants were inhibited mostly by metabolic factors regardless of photosynthetic type (Figure 3.5 *d*).

The mechanisms as to why the C_4 type had a higher metabolic limitation to photosynthesis under progressive drought conditions relative to the C_3 type are unclear. At high CO_2 concentrations, PEP carboxylase activity is limited by PEP regeneration in C_4 plants. The activities of C_4 cycle enzymes or alternatively the capacity of the chloroplastic electron transport chains can limit PEP regeneration at high irradiance (von Caemmerer 2000). The initial slope of the CO_2 response curve for the C_4 type is affected by different maximal PEP carboxylase activities. Low PEP carboxylase activity causes a reduction in the saturated portion of the curve due to Rubisco not being completely saturated with CO_2 in the bundle sheath (von Caemmerer 2000). Curvature of the CO_2 response curve is affected by bundle sheath conductance (von Caemmerer 2000). Drought caused a decrease in the initial slopes and CO_2 saturated regions of the $A{:}c_i$ curves of the C_4 species indicating low PEP carboxylase activity and a decrease in the rate of PEP regeneration, respectively. The curvature of the graphs also decreased during drought indicating an increase in bundle-sheath conductance to CO_2.

The validity of $A{:}c_i$ curves in analyzing drought-related loss of biochemical photosynthetic capacity of C_3 species has been questioned by some researchers.

Heterogeneous stomatal closure and cuticular conductance are two factors that could invalidate measured c_i values (Lawlor 2002). Determining the carboxylation efficiency of C_3 species using A:c_i curve analyses is dependent upon the assumption that the CO_2 concentration of the internal airspaces (c_i) is equal to the CO_2 concentration of the chloroplast (c_c). Ethier and Livingston (2004) and Manter and Kerrigan (2004) have argued that if mesophyll conductance were low then c_c would be considerably lower than c_i, resulting in the underestimation of carboxylation efficiency. Flexas *et al*. (2002) confirmed this in their assessment of the photosynthetic capacity of severely water stressed grapevine. They demonstrated the carboxylation efficiency and CO_2 compensation point remained unchanged under severe drought when analyzed using c_c instead of c_i indicating that photosynthesis was limited by increased resistance of the mesophyll to CO_2 diffusion. However, Ripley *et al.* (2007) demonstrated that the decrease in carboxylation efficiency of the C_3 subspecies of *A. semialata* during drought was similar whether expressed on the basis of c_c or c_i. This further suggests that decreases in mesophyll conductance did not account for the drought limitation of photosynthesis in the C_3 subspecies. Since fluorescence measurements were not made during this experiment, we cannot estimate mesophyll conductance or c_c. It is unlikely that these issues will invalidate the results as changes in mesophyll conductance is still a non-stomatal effect and only the interpretation of what a non-stomatal response is will change.

Photosynthetic recovery

The C_3 type recovered full photosynthetic capacity faster than the C_4 type (Figure 3.6 *a*). In general, recovery of photosynthetic capacity after mild stress (g_s above 0.1 mol H_2O m^{-2} s^{-1}) is rapid, usually occurring the day of re-watering, and complete (Flexas *et al.* 2004). Alternatively, the recovery of photosynthesis after severe water stress takes several days and pre-drought photosynthetic rates are not always attained (Souza *et al*. 2004, Flexas *et al.* 2004, Miyashita *et al.* 2005). Rapid recovery of photosynthesis in drought-stressed plants upon re-watering indicates that the decrease in net CO_2 uptake is a result of stomatal closure (Cornic 2000). However, if photosynthesis does not recovery immediately than non-stomatal factors may be limiting the process. Current knowledge

about the physiological limitations of photosynthetic recovery after drought is sparse. Lal and Edwards (1996) demonstrated that *Amaranthus cruentus* and *Zea mays* made a full photosynthetic recovery within 2 - 4 days of re-watering after photosynthesis had dropped to 5 - 10% of the original rate. Miyashita *et al.* (2005) showed that kidney bean reached the photosynthetic levels of the control pots when re-watered after 2 –3 days without watering. However, when re-watering started on day 7 of drought, photosynthesis only recovered to half of the control pots values. The photosynthetic rates of the current study species declined to 11- 26% of the control pot rates after 48 days of a slowly induced drought, and it took 3 - 15 days for these rates to recover upon re-watering (Figure 3.6 *b*). There were differential recovery rates between the photosynthetic types as the C_3 species recovered more quickly than the C_4 species.

Whole plant water use efficiency

One of the aims of these experiments was to determine if the WUE_{leaf} advantage of the NADP-ME species during naturally and simulated well-watered conditions and their subsequent loss of this advantage during severe water stress translated into similar WUE_{plant} trends. Long (1999) acknowledged that WUE_{leaf} values will only translate into equivalent whole plant water use efficiency (WUE_{plant}) values if the plants have the same degree of coupling with the environment. Canopy structure, stature, leaf orientation, leaf curling and folding affect this coupling. WUE_{plant} is also dependent on the physical environment including: air temperature, humidity, irradiance and wind speed during the growth period. In addition, the conditions of the gas exchange cuvette used to determine WUE_{leaf} are such that these natural conditions are disturbed. Differences in biomass portioning to non-photosynthetic tissues and carbon loss from respiration also affect WUE_{plant} independently of WUE_{leaf} (Ghannoum *et al.* 2002). Thus the clear C_4 WUE_{leaf} advantage of field grown and pot-cultivated plants may not actually translate into a WUE_{plant} advantage.

Water stress caused WUE_{leaf} and WUE_{plant} to decrease in the two types relative to the control. The C_4 maintained higher WUE_{plant} than the C_3 type under well-watered and

moderate drought conditions, but this advantage was lost during the severe drought treatment (Figures 4.1 *a, b, c*). WUE_{leaf} was significantly higher in the C_4 type than the C_3 type during the low VPD treatment in the field and at the start of the pot experiment (Figure 2.3 *a* and Figure 2.4 *a*). The severe water stress induced by the pot experiment caused WUE_{leaf} to decline in both types, but it ultimately resulted in the C_4 type having a significantly lower WUE_{leaf} than the C_3 type (Figure 2.4 *a*). This is in contrast to the results of the field experiment, which showed that even though WUE_{leaf} decreased in the C_4 type under low SWC relative to the low VPD treatment, this value was still significantly higher than the value for the C_3 type (Figure 2.3 *a*). The soil water content during the low SWC treatment was 5% as compared to only 3% during the severe drought treatment of the pot experiment. This difference may explain why the C_4 type was able to maintain WUE_{leaf} advantage over the C_3 type in the field and not during the pot experiment. If pre-dawn Ψ is an estimate of soil Ψ, then the 5% SWC measured in the field corresponded to an average soil Ψ of –0.89 MPa, whereas the 3% SWC measured on day 48 of the pot experiment corresponded to an average soil Ψ of -4.4 MPa. Based on this relationship, the interval between 3-5% SWC marks the area where soil Ψ rapidly declines. So it makes sense that this small difference had a huge effect on photosynthesis and WUE_{leaf} of the plants. The SWC and soil water potential of the field site from late winter until mid-summer never reached the values simulated in the pot experiment (Figure 2.2). The potential for this type of intensive soil drying is possible in the field as Grahamstown is considered a semi-arid area; however, this study was conducted during an unusually long mesic period.

The same environmental conditions must exist in order to compare WUE_{plant} of different species. Comparing results of various studies is complicated by the fact that the growing conditions of each experiment are variable. In addition, some studies (Maroco *et al.* 2000, Ghannoum *et al.* 2002, Xu *et al.* 2006) consider carbon allocation to roots in their calculations of whole plant biomass when determining $WUE_{plant,}$ whereas this study monitored carbon allotted to photosynthetic leaf area.

For grasses grown in the same environment at 25°C, Downes (1969) determined the ratio of dry weight production per unit of water lost was almost twice as great in the C_4 species relative to the C_3 species. For the grasses of this study grown under similar well-watered conditions at an average temperature of 25°C, the WUE_{plant} of the C_4 type was also twice as high as the C_3 type in pot experiment (Figure 4.1 *a*). Drought caused WUE_{plant} to decrease in both photosynthetic types from well-watered values (Figures 4.1 *b, c*). However the species showed more variation in their responses to drought regardless of their photosynthetic type. *H. contortus* (C_4) and *P. aequinerve* (C_3) had negative WUE_{plant} under moderate drought conditions as a consequence of leaf senescence (Figure 4.1 *e*).

A range of results has been reported on how drought affects WUE_{plant} of various C_4 species. Xu *et al.* (2006) demonstrated that WUE_{plant} of *P. virgatum, S. italica* and *B. ischaemum* decreased with soil drying, but values were not significantly different to the well-watered values. Maroco *et al.* (2000) found mixed results in their comparison of two C_4 Sahelian grasses. WUE_{plant} of water stressed *Shoenefeldia gracilis* was significantly higher than the values for the well-watered plants, while *Dactyloctenium aegyptium* had only significant differences between treatments towards the end of the experiment. Ghannoum *et al.* (2002) showed a significant increase in WUE_{plant} in NADP-ME and NAD-ME grasses that were subjected to drought relative to the control plants. The severe drought induced by this current experiment resulted in the both photosynthetic types having negative WUE_{plant} due primarily to the reduction of leaf biomass production cause by leaf mortality (Figure 4.1 *c*).

In an attempt to compare actual values of WUE_{plant} with other studies, culms and below ground biomass were taken into account to determine the water use efficiency of the entire plant. At the end of the pot experiment, all plants were harvested and divided into live leaves, culms and roots. The percentage of live leaves to the sum of the other components was calculated and applied to the calculation of WUE_{plant} under well-watered conditions. The values for the C_3 and C_4 types were 2.5 ± 0.44 and 5.3 ± 0.62 g dry mass / kg water. The value for the C_4 type is similar to the values Xu *et al.* (2006) found for *B. ischaemum* and *P. virgatum* and (6.43 and 5.46 g dry mass / kg water, respectively), but

slightly lower than the average value for the NADP-ME subtype (7.3 g dry mass / kg water) determined by Ghannoum *et al*. (2002) and *D. aegyptium* (11 g dry mass / kg water) as determined by Maroco *et al.* (2000).

The components of WUE_{plant}: whole plant water loss per leaf area (E_{plant}) and leaf biomass production as represented by the relative leaf expansion (RGR_{area}) were investigated to determine whether growth or water usage had a greater effect on WUE_{plant} during drought. RGR_{area} decreased in all of grasses during drought due to a reduction in the size and number of leaves produced under drought and through leaf death (Figures 4.2 *c-f*). These effects were particularly extreme at the end of the dry down experiment resulting in negative RGR_{area} values for all of the species (Figure 4.2 *f*). Maroco *et al.* (2000) found that the RGR_{area} of two C_4 Sahelian grasses decreased with drought. *S. gracilis* had significantly lower values in the water stressed plants than in the well-watered plants, but there was no difference between treatments in *D. aegyptium*. E_{plant} of the two types also decreased steadily as drought progressed (Figure 4.3). In particular, the severe drought treatment caused huge reductions in E_{plant} of all of the species relative to the well-watered values. Lowering E_{plant} did not enhance the WUE_{plant} of the species during drought; rather reductions in leaf biomass production by means of leaf senescence were more influential on WUE_{plant}.

E_{plant} did not correlate with transpiration in the C_4 type for the pot experiment as was shown for the C_3 type. E_{plant} of the C_4 type decreased steadily with drought intensity while transpiration rates remained relatively unchanged during the more stressful periods (Figure 2.4 *c* and Figure 4.3). The differences between transpiration rate and E_{plant} trends may be explained by differences in the boundary layer conductance of a single leaf in a gas exchange cuvette and that of a whole plant. The conditions of the leaf cuvette are not representative of the actual growth environment. An artificial flow rate increases the boundary layer conductance of the individual leaf thereby increasing the rate of water vapor transfer above what may have been measured on a whole plant level. The resistance of the boundary layer in relation to other whole plant resistances to water vapor transfer, namely the stomata, is small. However, individual leaves may curl and fold further affecting boundary layer conductance (Redmann 1985). This parameter may

become more important than stomatal conductance if a complete seal is achieved of the rolled leaf edges, which may occur during severe water stress (Redmann 1985). Heckathorn and de Lucia (1991) found that leaf rolling reduced transpiration by 7-13% in water stressed plants of *Andropogon gerardii* and *Spartina pectinata* by lowering leaf temperatures and thus leaf to air vapor pressure deficit. Takahiro and Ryoichi (2000) studied the effects of leaf rolling on gas exchange in rice and determined that it inhibited transpiration only and that photosynthesis, stomatal conductance and leaf temperature were not affected. They concluded that leaf rolling improved the water use efficiency of a single leaf by decreasing transpiration through the reduction of boundary layer conductance.

The manual unfolding of leaves before taking gas exchange measurements may explain why E_{plant} did not correlate with transpiration in the pot experiment. This technique may have inflated the actual transpiration rates of the species by changing the leaf boundary layer resistance that developed as a result of folding. This was especially significant in *H. contortus* and *T. triandra*, which usually had tightly folded leaves even under moderate drought. When water stress was particularly extreme (day 48 of the pot experiment) most species had leaves that needed uncurling or unfolding before gas exchange measurements were taken. Still this fact does not explain why the leaves of the C_4 type commenced transpiration after being unfolded, but the leaves of the C_3 type did not.

O'Toole *et al.* (1979) showed how artificially curling the leaves of *Oryza sativa* reduced transpiration and that the degree of leaf rolling had a differential effect on transpiration. The more tightly folded, the less the leaf transpired. What is most interesting is that transpiration of a tightly folded leaf recovered quickly (a few minutes) to initial values upon unrolling. The stomata of the C_4 study species may have in fact been open under severe drought, but these species had created an "artificial" environment (i.e. leaf folding to increase the upper leaf surface area boundary layer resistance) that discouraged transpiration as a way to control plant water balance. Once this environment was disturbed, transpiration was able to proceed.

Hydraulics

There is often a strong correlation between g_s and the hydraulic conductance of the soil-plant-atmosphere continuum (Sperry *et al.* 2003). This correlation is partially due to stomata regulating plant water status through the adjustment of transpiration. The feedback response of stomatal closure ensures that stomata respond to the water status of cells within their immediate area (Buckley 2005). Changes in plant water status are prompted by changes in hydraulic conductance through cavitation and soil drying (Sperry *et al.* 2003). As was pointed out in the previous section, the potential insensitivity of the stomata of the C_4 species to drought may result from leaf folding or rolling occurring as an alternative mechanism to reduce transpiration. Investigating the relationship between g_s and K_{plant} confirmed that the stomata of the C_4 did not respond to changes in K_{plant} in either the field or pot experiments as suggested by Sperry *et al*. (2003). There was a slight increase in g_s of the C_4 type on day 36 as compared to day 20 of the pot experiment while K_{plant} was maintained (Figure 2.4 *d* and Figure 5.2 *a*). Similarly in the field, g_s decreased in the low SWC treatment compared to the low VPD treatment (control) while K_{plant} was maintained during both treatments (Figure 2.3 *g* and Figure 5.1 *a*). For the C_3 type, K_{plant} and g_s decreased steadily during the dry-down period of the pot experiment and also decreased during the low SWC treatment relative to the control indicating that the stomata of the C_3 type are sensitive to changes in K_{plant} (Figures 2.3 *g* 2.3 *d*, and Figures 5.1 *a* 5.2 *a*).

Hydraulic conductance is proportional to the number of xylem conduits in parallel and their diameters raised to the fourth power (Sperry *et al.* 2003). This is a rough estimation of water conducting capacity because this does not take into account the added resistance of water flow through the inter-vessel pit membranes of the xylem (Sperry *et al.* 2003). The C_3 type had lower hydraulic resistance than the C_4 type by having longer xylem vessels that comprised a greater percentage of its total leaf length. The lengths of the longest xylem for the C_3 and C_4 types were 8.7 and 7.4 mm respectively (Table 5.2). These values are similar to the mean xylem length (9 mm) in the leaf blade of *Festuca arundinacea* as reported by Martre and Durand (2001). However, these values are much

smaller than the ones reported for *Saccharum spp*, which ranged from 77 – 121 mm (Neufeld *et al.* 1992). The C_3 type also had greater capacity to water flow than the C_4 type by having average maximum xylem vessel diameters that were significantly larger than those of the C_4 type (Table 5.3). The range of maximum diameters described for *Saccharum spp* were 24.4 – 54.2 µm, whereas the values for the C_3 and C_4 species of this study were only 22.4 and 18.5 µm, respectively. The wide xylem vessels of the C_3 type resulted in this type having a higher theoretical leaf hydraulic conductance than the C_4 type (Figure 5.4 *b*). However, both photosynthetic types had similar total xylem lumen areas (Figure 5.4 *a*). This is a result of the C_4 type having greater numbers of smaller diameter vessels than the C_3 type. The total xylem lumen area of a few larger diameter vessels will equal the area of many smaller diameter vessels. However since K_t is a fourth power relationship as opposed to a second power relationship (transverse sectional area), the difference between a few larger vessels as compared to many smaller vessels is huge when accounting for water flow.

Long xylem vessels with large diameters are most beneficial under mesic conditions. The cost of having these particular xylem is a greater risk of hydraulic failure through cavitation caused by high xylem tensions during drought (Sperry 2000). Thus, shorter, narrower and mechanically stronger xylem are more beneficial in environments where water is limiting. The tradeoff of having these particular xylem is lower flow capacity. The C_3 type had xylem characteristics that enhanced water-conducting efficiency that resulted in this type having a higher K_{plant} than the C_4 type under well-watered field conditions (Figure 5.1 *a*). The more vulnerable xylem of the C_3 type caused K_{plant} to decrease during drought conditions in the field whereas the safer xylem of the C_4 type allowed it to maintain similar conductances on both days (Figure 5.1 *a*). These xylem also caused K_{plant} of the C_3 type to decrease more quickly during the dry-down pot experiment as compared to the C_4 type (Figure 5.2 *a*). This was result of the more rapid decline of Ψ_{leaf} in the C_3 type than the C_4 type (Figure 5.2 *c*). The safer xylem of the C_4 type permitted it to endure more negative Ψ_{leaf} than the C_3 type during the low SWC field treatment (Figure 5.1 *c*).

Drought caused both of the photosynthetic types to significantly reduce the size of their largest xylem vessels produced after the imposition of drought (Table 5.3). The strategy of the species to drought was not entirely dependent on photosynthetic type. *P. ecklonii* (C_3 species) was the only species to significantly decrease the numbers of large and small vascular bundles per cm of leaf width. In addition, *T. leucothrix* (C_4 species) decreased the number of its intermediate bundles. *A. semialata* (C_3 species), *T. triandra* (C_4 species) and *T. leucothrix* reduced the size of their largest vessels during drought. Thus, there was significant plasticity in the response of the species to drought.

Kocacinar and Sage hypothesis

Kocacinar and Sage (2003) suggested that the secondary consequence of the C_4 type having a higher WUE_{leaf} than the C_3 type is the ability to alter xylem structure and function to either improve hydraulic safety (decrease transpiration therefore less K_{plant} is needed) and / or enhance photosynthetic capacity with no extra loss of water, depending on the environment. In mesic environments, the C_4 type may increase photosynthetic potential by allowing an increase in leaf area per unit of xylem tissue relative to the C_3 type. In arid environments, the C_4 type may have safer xylem that has less flow capacity but is less vulnerable to cavitations. This hypothesis was tested on eudicot species of similar taxonomic and/or ecological distribution. The current study allowed a test of this hypothesis for wild grasses from a single site while controlling for phylogeny. Contrary to the mesic hypothesis, the leaf area to xylem area ratio was the same for the two types when grown under well-watered conditions in the field (the inverse of results of Figure 5.4 *a*). The strategies of the two photosynthetic types during drought were not always distinct; they were sometimes blurred by the strategies of the individual species. In general, it was shown that the C_4 had more smaller-sized xylem vessels than the C_3 type (Table 5.3). This redundancy in xylem increased hydraulic safety. Loss of function of a few small vessels does not affect overall water conduction as much as the loss of function of a few larger vessels. In addition, the average diameter of the three largest xylem vessels in the C_4 type was smaller than in the C_3 type (Table 5.3). However, this trend dissolves when you take the species into account. *A. semialata* had the largest average

xylem diameter, *T. triandra* had the smallest and the other species had similar diameters. Drought induced differential species responses. There was no clear C_3 / C_4 trend in the reduction of xylem vessels size during drought. *A. semialata*, *P. aequinerve*, *T. triandra* and *T. leucothrix* decreased the size of their largest vessels from well-watered sizes, thus enhancing xylem safety. *P. ecklonii* significantly decreased the total number of vascular bundles per centimeter of leaf width, but did not adjust the size of its largest vessels during drought, thus making this species more vulnerable to cavitations. *H. contortus* used the same strategy during both treatments. It slightly increased the size of its largest xylem vessel while maintaining a similar number of vascular bundles during drought compared to well-watered values. *T. leucothrix* significantly decreased the number of intermediate vascular bundles per cm of leaf width only while reducing the size of its largest vessels thereby increasing overall xylem safety. The C_4 species did not necessarily increase xylem safety during drought as was suggested by Kocacinar and Sage (2003) because they also had "safe" xylem under well-watered conditions relative to the C_3 species.

The extensive vascular system of the C_4 type may be an artifact of needing a shorter distance between the mesophyll and bundle sheath cells in order for metabolites to diffuse freely between the two cell types. Ueno *et al.* (2006) found that the C_4 grasses had shorter distances between the small longitudinal veins, long longitudinal veins and transverse veins than the C_3 grasses. Transverse cross-sections in this study revealed that the C_4 type had over 2.5 times the number of small vascular bundles and 1.5 times the number of large vascular bundles per centimeter of leaf width than the C_3 type under well-watered conditions. These numbers increased during drought. This denser hydraulic network of the C_4 type has been hypothesized as to why C_4 plants are able out-compete C_3 plants in hot environments (Sage 2004). Reducing interveinal distance reduces evaporative surface area relative to conduit size (Sage 2004). Having a greater number of small vascular bundles with smaller sized xylem vessels confers a higher degree of hydraulic safety in the C_4 type as compared to the C_3 type. The evolution of the C_4 pathway may have predisposed C_4 species to the secondary benefits of efficient water usage.

Species differences

There were many significant differences between the species for most parameters in this study. In comparison to the type differences, species effects accounted for more significant results in all of the general linear models analyzed. The differences between the six study species and even the differences between the three species within each type may explain how they are able to co-exist in the same grassland. Each species has a unique combination of strategies by which it is able carve out a specific niche in the field. Specific examples are mentioned below.

The large xylem vessels of *A. semialata* made this species vulnerable to drought and high evaporative demand. g_s decreased the most in this species during the high VPD field treatment, which caused a subsequent decrease in photosynthesis (Figures 2.3 *d*, *h*). The high VPD probably put too much hydraulic strain on its xylem even though the SWC was 25%. If the stomata had not closed, negative tensions could have developed in the xylem causing runaway cavitations. The sensitivity of the stomata allowed this species to maintain similar Ψ_{leaf} and pre-dawn Ψ_{leaf} during the three field treatments (Figures 5.1 *d, f*) and have similar WUE_{leaf} and WUE_{plant} to the C_4 species under well-watered conditions during the pot experiment (Figure 2.5 *a* and Figure 4.1 *d*).

The severe drought (day 48) took its toll on *A. semialata*. This species had one of the lowest g_s and photosynthetic rates on this day (Figure 2.5 *b, d*). Ψ_{leaf} reached a very low –4.8 MPa and K_{plant} and transpiration were barely measurable on this day (Figures 5.2 *b, d* and Figure 2.5 *c*). Despite of this, photosynthesis recovered the quickest in this species after re-watering (Figure 3.6 *b*).

P. aequinerve had the longest xylem relative to leaf length, resulting in fewer inter-vessel pit membranes by which water must flow through, thus decreasing hydraulic resistance (Table 5.2). This feature increased leaf hydraulic conductivity, but it also made this species vulnerable during drought. The ability to sustain such high K_{plant} during the high VPD treatment resulted in substantial increases in g_s and photosynthesis at this time

(Figures 2.3 *d, h* and Figure 5.1 *b*). Nevertheless K_{plant} decreased substantially during the severe drought of the pot experiment resulting in this species having the lowest g_s and photosynthetic rate of all the species (Figures 2.5 *b, d* and Figure 5.2 *b*). This reduction in photosynthesis led to a negative RGR_{area} during the moderate and severe drought treatments (Figures 4.2 *d, f*).

P. ecklonii had the third largest xylem vessels (Table 5.3). These vessels enhanced the water conducting efficiency of this species under well-watered conditions (in the field). They enabled high rates of transpiration during this period that resulted in a similar photosynthetic rate to the three C_4 species (Figures 2.5 *b, c*). Moreover, it permitted this species to attain the highest E_{plant} values of all the species during the well-watered and moderate drought treatments (Figure 4.3). *P. ecklonii* reduced the total number of vascular bundles per centimeter of leaf width but not the size of its largest vessels during drought (Table 5.3). This amplified this species vulnerability to water stress. The consequence of this strategy was that *P. ecklonii* decreased its photosynthetic rate more rapidly than the C_4 species as drought developed (Figure 2.5 *b*).

T. triandra had the greatest number of large and small vascular bundles per centimeter of leaf width and decreased the size of its largest vessels during drought (Table 5.3). This redundancy of narrow xylem provided a degree of hydraulic safety when water availability was low. *T. triandra* was able to endure the most negative midday Ψ_{leaf} during the low VPD and high VPD treatments as well as sustain the lowest pre-dawn Ψ_{leaf} on all three field days (Figures 5.1 *d, f*). This species was also able to increase K_{plant} during the progressive drought (day 36) while sustaining high g_s and transpiration (Figures 2.5 *c, d* and Figure 5.2 *b*). On the other hand, photosynthesis was more susceptible than hydraulics to drought. WUE_{leaf} decreased during the progressive drought (day 36) due to a greater decrease in photosynthetic rates rather than transpiration (Figures 2.5 *a, b, c*). In addition, the photosynthetic rate of this species was the slowest to recover after re-watering even though g_s recovered more quickly (Figure 3.6 *b*).

T. leucothrix is the only species that had intermediate vascular bundles (Table 5.3). These bundles gave this species more hydraulic flexibility. They contained larger xylem vessels than the small vascular bundles increasing hydraulic capacity when water was available. At the same time, these bundles increased the redundancy of xylem vessels and provided a degree of hydraulic safety when water was limiting. As a result, *T. leucothrix* was the only species to increase transpiration and sustain K_{plant} during the low SWC treatment as compared to the low VPD treatment (Figure 2.3 *f* and Figure 5.1 *b*). Having the second largest xylem vessels even under drought conferred a degree of vulnerability. This species had the lowest g_s and photosynthetic rate of the C_4 species on day 48 (Figures 2.5 *b, d*). However, the stomata of *T. leucothrix* seemed to be more responsive than the stomata of the other C_4 species as shown by its improvement in WUE_{leaf} on day 36 by decreasing transpiration more than the other C_4 species (Figures 2.5 *a, c*). This allowed *T. leucothrix* to maintain the most favorable water status of all the species on this day (Figure 5.2 *d*).

H. contortus had xylem vessels with the smallest diameters as well as the second highest number of large and small vascular bundles per centimeter of leaf width under well-watered conditions (Table 5.3). These particular xylem characteristics conferred a degree of hydraulic safety for this species. Photosynthesis and g_s were not as vulnerable to the high VPD treatment as was shown for *A. semialata* (Figures 2.3 *d, h*). However during drought, photosynthesis was more susceptible than hydraulics as was demonstrated for *T. triandra*. WUE_{leaf} decreased on day 36 due to a greater decrease in photosynthetic rates rather than transpiration (Figures 2.5 *a, b, c*). These results corresponded to what was happening on the whole plant level. *H. contortus* had a negative WUE_{plant} during the progressive drought treatment of the pot experiment because of high rates of leaf mortality reflected in its negative RGR_{area} (Figure 4.1 *e* and Figure 4.2 *d*).

Anatomical characteristics and species distribution

The anatomical and drought response characteristics of these study species may explain their distributions in Southern Africa. The C_3 type had xylem characteristics that

enhanced water-conducting efficiency. The cost of having efficient water conducting xylem is vulnerability to cavitations induced by very negative xylem pressures that develop under low soil moisture availability. It could be for this reason that the distributions of *A. semialata, P. aequinerve* and *P. ecklonii* are restricted to the more mesic environment of the east coast. The ability of *A. semialata* to extend northwards into Tanzania is achieved at high altitudes (Gibbs Russell *et al.* 1991). As a C_3 grass, this species is only more competitive than C_4 grasses at cooler temperatures. This is due to the fact that the additional investment of the C_4 cycle lowers the quantum yield of C_4 plants at low temperatures. Alternatively the safer xylem of *T. triandra* and *H. contortus* allow these species to extend their distribution into the drier western regions of South Africa and into the very low rainfall areas of Namibia (Gibbs Russell *et al.* 1991). At the same time, these grasses are abundant in the summer rainfall regions of South Africa where the higher temperatures make them more competitive than C_3 grasses in the low lying regions. The large diameter xylem vessels of *T. leucothrix* may restrict its distribution largely within the same range as the C_3 species. However, the hydraulic flexibility provided by its abundance of intermediate vascular bundles has allowed this species to extend into the fynbos biome of southwestern South Africa.

Key Points

Under well-watered conditions in the field and during the pot experiment, the C_4 type had significantly higher WUE_{leaf} than the C_3 type. This is in accordance to our initial hypothesis (Chapter 1). The WUE_{leaf} advantage of the C_4 type was due to having a higher photosynthetic rate than a C_3 type because both types had similar transpiration rates. Ultimately the severe drought treatment of the pot experiment caused the C_4 type to have a significantly lower WUE_{leaf} than the C_3 type (Chapter 2). This reduction in WUE_{leaf} of the C_4 type corresponded to a greater decrease in photosynthesis rather than transpiration. g_s of the C_4 type remained higher than the C_3 type during the latter stages of the dry-down period as photosynthesis continued to steadily decline until it reached a similar rate to the C_3 type on day 48. These results did not agree with the field data, which showed that the C_4 type maintained both photosynthetic and water use efficiency advantage over the C_3

type on the low SWC day. The 2% differential in SWC between the field and pot experiments occurred on the asymptotic section of the soil suction curve signifying a dramatic decrease in soil Ψ. This additional water stress resulted in the C_4 type losing photosynthetic advantage. CO_2 response curves showed that the susceptibility of C_4 photosynthesis to drought was a result of greater metabolic effects, rather than stomatal effects (Chapter 3). Metabolic factors reduced the photosynthetic rate of the C_4 type by almost 50% as compared to a reduction of only 26% in the C_3 type during the progressive drought treatment relative to well-watered conditions. However, the photosynthetic rates of both types were equally inhibited by metabolic factors during the severe drought treatment. This fact did not explain why the photosynthetic rate of the C_4 type took longer than the C_3 type to recover to control pot rates after re-watering. The carbon-concentrating mechanism of the C_4 type may have been dysfunctional and additional time was needed for repair. The fact that full photosynthetic capacity was eventually achieved in the C_4 species meant that there was a degree of resiliency in this mechanism.

WUE_{leaf} corresponded to trends in $WUE_{plant,}$ and photosynthesis corresponded to trends in RGR_{area} however transpiration did not correlate with E_{plant}. The C_4 lost its WUE_{plant} advantage over the C_3 type during severe drought due to decreases in leaf biomass production reflected in the negative values of RGR_{area} (Chapter 4). E_{plant} of the photosynthetic types decreased steadily as drought progressed particularly during the driest period. Reductions in E_{plant} during drought did not enhance WUE_{plant} rather loss of leaf biomass was more influential on WUE_{plant}.

The lack of correlation between transpiration and E_{plant} in the drought stressed leaves of the C_4 species may have been due to differences in boundary layer resistance of folded and unfolded leaves and whole plant canopies. The C_4 species folded their leaves during drought. This may have been a response to the potential insensitivity of their stomata. Leaf folding was a strategy used by these species to create an environment that reduced transpiration by increasing leaf surface boundary layer resistance, thereby bypassing the possible limitations of the stomata. The manual unfolding of these leaves and the artificial flow rate of the gas analyzer cuvette decreased this resistance and caused

transpiration rates to increase. Nevertheless, more work needs to be done on this. Transpiration rates of folded and unfolded drought stressed leaves need to be measured to determine if there are actual differences.

The ability of the C_4 species to transpire under severe water stress may have to do with the anatomy of their xylem (Chapter 5). The C_4 species had twice as many vascular bundles per centimeter of leaf width than the C_3 species, which increased xylem redundancy lessening the risk of complete hydraulic failure. The vasculature of *T. triandra* and *H. contortus* was comprised of five times as many small vascular bundles than large ones per centimeter of leaf width, while *T. leucothrix* had more hydraulic flexibility than the other species by having intermediate vascular bundles in addition to large and small ones. These combined qualities provided interesting trends in hydraulics between the photosynthetic types. Firstly, K_{plant} of the C_4 type decreased more slowly during the dry-down experiment than the C_3 type. The C_4 type was also able to maintain similar conductances during well-watered conditions and under low soil water content in the field, whereas K_{plant} of the C_3 type decreased under drought. Finally, the C_4 type was able to endure more negative Ψ_{leaf} than the C_3 type during the low SWC field treatment.

These anatomical characteristics may also explain the current distribution of the study species in Southern Africa. *T. triandra* and *H. contortus* seem to be hydraulically suited to withstand more negative xylem tensions and have greater resistance to cavitations than the C_3 species, thus making them the least vulnerable to drought conditions. These qualities may have allowed these species to extend their range from the more mesic east coast of Southern Africa, where the C_3 species of this study and *T. leucothrix* occur, into the more arid western regions of Namibia.

Unlike the C_3 species, the C_4 species investigated in this study (NADP-ME) are hydraulically tolerant of drought. However, photosynthesis of the C_4 species appears to be at risk to severe water stress. This finding corresponds to the inverse correlation between annual rainfall and the abundance of NADP-ME species around the world. There are some issues in using annual rainfall as the primary criterion for determining

species abundance especially for semi-arid environments. It does not take into account the frequency of rainfall events, the amount of water that falls during each event, the number of rainless days in between these events or the season of rainfall relative to growth. Precipitation of semi-arid environments is highly variable. These environments are usually characterized by many small rainfall events, but large and infrequent storms tend to bring most of the annual rain (Williams *et al.* 1998). In addition, it is not uncommon for semi-arid environments to experience frequent atmospheric drought (high VPD) even when soil water content is high (Maroco *et al.* 1997). Thus discerning the complexities of precipitation distribution is important in our understanding of the determinants of the productivity of grasslands and possibly the abundance of the C_4 subtypes around the world.

This experiment simulated a long, slow drought event that took 48 days for severe water deficits to develop. The C_3 species strategy was to avoid dehydration by stomatal closure, while the NADP-ME species seemed more able to tolerate the drought, as they were able to maintain higher rates of carbon fixation than the C_3 species for most of the dry-down period. Models of above-ground productivity and precipitation for three temperate southern African grasslands have shown that the interval between rainfall events was the most important variable at the wettest site, whereas the size of the rainfall event was most important at the driest site (Swemmer *et al*. 2007). If NADP-ME species were selected for / or competitive in habitats with small and frequent rainfall events then alternatives to stomatal closure (i.e. leaf folding) would be most beneficial during the short intermittent periods of water stress so that CO_2 uptake is not sacrificed while trying to reduce water loss. However, maximizing use of available soil moisture becomes hazardous if the next rainfall event happens later in the season. The cost of enduring such a severe drought for the NADP-ME species of this study was that full photosynthetic recovery was not achieved for over three weeks after re-watering.

In order to tease out the environmental variations affecting the distribution of the C_4 subtypes around the world, more questions need to be answered. 1) Do NADP-ME species respond differently to drought that happens quickly and / or for longer (than this

experiment)? 2) Do NAD-ME species have lower photosynthetic and stomatal sensitivities, sustain lower Ψ_{leaf} and have less vulnerable xylem characteristics than co-occurring or closely related to NADP-ME species? 3) What are the underlying mechanisms for the metabolic limitation of photosynthesis in NADP-ME species (need to investigate chlorophyll fluorescence, enzyme activities, on-line isotope analysis)?

In the end, it is the metabolic inhibition of photosynthesis during drought that makes these NADP-ME species susceptible to drought. Damage to the C_4 carbon-concentrating mechanism may have incurred as a result, which delayed the photosynthetic recovery of the NADP-ME species relative to the C_3 species. This susceptibility may be compounded by the fact that the NADP-ME species have less responsive stomata than the C_3 species (personal observation).

This study confirms the metabolic sensitivity in co-occurring NADP-ME Panicoid grasses and has demonstrated that photosynthesis is much more at risk than hydraulics to severe drought. These findings may explain why NADP-ME species abundance around the world decreases with decreasing rainfall.

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