

REVIEW

C₄ photosynthesis and water stress

Oula Ghannoum*

Centre for Plant and Food Science, University of Western Sydney, Locked Bag 1797, South Penrith DC, NSW 1797, Australia

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- **Background** In contrast to C₃ photosynthesis, the response of C₄ photosynthesis to water stress has been less well studied in spite of the significant contribution of C₄ plants to the global carbon budget and food security. The key feature of C₄ photosynthesis is the operation of a CO₂-concentrating mechanism in the leaves, which serves to saturate photosynthesis and suppress photorespiration in normal air. This article reviews the current state of understanding about the response of C₄ photosynthesis to water stress, including the interaction with elevated CO₂ concentration. Major gaps in our knowledge in this area are identified and further required research is suggested.
- **Scope** Evidence indicates that C₄ photosynthesis is highly sensitive to water stress. With declining leaf water status, CO₂ assimilation rate and stomatal conductance decrease rapidly and photosynthesis goes through three successive phases. The initial, mainly stomatal phase, may or may not be detected as a decline in assimilation rates depending on environmental conditions. This is because the CO₂-concentrating mechanism is capable of saturating C₄ photosynthesis under relatively low intercellular CO₂ concentrations. In addition, photorespired CO₂ is likely to be refixed before escaping the bundle sheath. This is followed by a mixed stomatal and non-stomatal phase and, finally, a mainly non-stomatal phase. The main non-stomatal factors include reduced activity of photosynthetic enzymes; inhibition of nitrate assimilation, induction of early senescence, and changes to the leaf anatomy and ultrastructure. Results from the literature about CO₂ enrichment indicate that when C₄ plants experience drought in their natural environment, elevated CO₂ concentration alleviates the effect of water stress on plant productivity indirectly via improved soil moisture and plant water status as a result of decreased stomatal conductance and reduced leaf transpiration.
- **Conclusions** It is suggested that there is a limited capacity for photorespiration or the Mehler reaction to act as significant alternative electron sinks under water stress in C₄ photosynthesis. This may explain why C₄ photosynthesis is equally or even more sensitive to water stress than its C₃ counterpart in spite of the greater capacity and water use efficiency of the C₄ photosynthetic pathway.

Key words: C₃ and C₄ photosynthesis, stomatal and non-stomatal limitation, high CO₂, water stress.

INTRODUCTION

Water stress is one of the most limiting environmental factors to plant productivity worldwide, and can be caused by both soil and atmospheric water deficits. The response of C₃ photosynthesis to water stress has been well studied and reviewed, as indicated by the large number of research (e.g. Sharkey and Seemann, 1988; Ortiz-López *et al.*, 1991; Cornic *et al.*, 1992; Tezara *et al.*, 1999; Cornic and Fresneau, 2002) and review (e.g. Cornic, 2000; Lawlor, 1995, 2002; Lawlor and Cornic, 2002; Flexas *et al.*, 2004) articles published on this topic. In general, C₃ photosynthesis is negatively affected by water stress measured as changes in leaf water potential (Ψ_{leaf}) or relative water content (RWC). In the early phase of water stress, when leaf RWC is still greater than 70 %, the decline in CO₂ assimilation rates (*A*) is largely the result of reduced intercellular CO₂ concentration (*C_i*) due to decreased stomatal conductance (*g*). Under these conditions, maximal photosynthetic capacity and quantum yield remain unaffected when measured under saturating irradiance and carbon dioxide concentration ([CO₂]). In addition, photosynthetic inhibition usually recovers relatively quickly when plants are re-hydrated.

If water stress persists and leaf RWC falls below 70 %, the loss of photosynthetic activity becomes increasingly less responsive to high [CO₂] and *A* fails to recover to pre-stress values following the removal of water stress. The exact mechanisms underlying this non-stomatal phase, also termed metabolic inhibition, are diverse and less well understood (for more details and reviews on this topic, see Cornic, 2000; Lawlor and Cornic, 2002; Lawlor, 2002; Flexas *et al.*, 2004; and references therein).

In contrast, the response of C₄ photosynthesis to water stress has been less well studied. This is in spite of the fact that C₄ plants make a significant contribution to the global carbon budget, and C₄ crops, such as maize and sorghum, are pivotal to current and future global food security (Lloyd and Farquhar, 1994; Ehleringer *et al.*, 1997; Brown, 1999; Pingali, 2001). Moreover, C₄ plants predominate in hot, arid regions which are prone to frequent drought. This fact is likely to be exacerbated by global climate change in three main ways: (1) global warming and changes in precipitation patterns are likely to expose many ecosystems, including C₄-dominated ones, to increasing soil and atmospheric water stresses (IPCC, 2007); (2) the impact of rising atmospheric [CO₂] on the productivity of C₄ plants is greatly influenced

* E-mail o.ghannoum@uws.edu.au

by soil water availability (Ghannoum *et al.*, 2000, 2006); and (3) global warming may lead to an increase in the proportion of land area covered by C_4 plants, especially in grasslands and rangelands (Archer, 1993; Henderson *et al.*, 1994; Crimp *et al.*, 2002). Therefore, it is important to understand how water stress influences the primary processes of CO_2 fixation in C_4 plants. In this article, I review the evidence related to the response of C_4 photosynthesis to water stress and attempt to summarize the current state of understanding in this area, including the interaction of elevated $[CO_2]$ with the effects of water stress on C_4 photosynthesis.

SIGNIFICANCE AND DISTRIBUTION OF THE C_4 PHOTOSYNTHETIC PATHWAY

Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), the primary CO_2 -fixing enzyme in plants, has poor kinetic properties. Compared with other enzymes, Rubisco is a slow catalyst with a low affinity for its substrate CO_2 . Most importantly, Rubisco has a low ability to discriminate between molecular CO_2 and O_2 (Jordan and Ogren, 1981; Andrews and Lorimer, 1987). The latter feature is particularly problematic because O_2 is the by-product of the light reactions of photosynthesis (Edwards and Walker, 1983) and is in high concentration in the atmosphere. By reacting RuBP with O_2 , Rubisco fixes less CO_2 and initiates a series of reactions, photorespiration, which culminates in the release of CO_2 back to the atmosphere (Edwards and Walker, 1983). Under the current atmospheric $[CO_2]$ and a temperature of 25 °C, photorespiration runs at about 20–30 % of photosynthesis in C_3 leaves (Sage, 2001, 2004). With increasing temperature, photorespiration increases faster than photosynthesis (Jordan and Ogren, 1984; Sage and Kubien, 2007). The C_4 photosynthetic pathway has evolved as an adaptation to high photorespiratory pressures resulting from various combinations of stresses which include low atmospheric $[CO_2]$, high temperature, aridity and/or salinity (Ehleringer *et al.*, 1991, 1997; Sage, 2001, 2004; Tipple and Pagani, 2007). According to carbon isotope discrimination records, plant fossils and molecular taxonomy studies, it is likely that C_4 plants formed a minor component of the world's flora for a long time before the recent expansion of C_4 grasslands some 5–10 million years ago (Cerling, 1999; Kellogg, 1999; Sage, 2004). It is estimated that the C_4 photosynthetic pathway has evolved independently some 45 times in three monocot and 16 dicot lineages (Kellogg, 1999; Sage *et al.*, 1999; Sage, 2004).

Although C_4 plants represent a mere 4 % of the world's flora, they contribute about 20 % of global primary productivity, mainly because of the high productivity of C_4 grasslands (Lloyd and Farquhar, 1994; Ehleringer *et al.*, 1997). The C_4 photosynthetic pathway is strongly represented in the grass (Poaceae) family, comprising about 50 % of total grasses (Hattersley, 1992; Sage *et al.*, 1999). C_4 plants are grouped into three biochemical subtypes [NAD malic enzyme (NAD-ME), NADP malic enzyme (NADP-ME) and phosphoenolpyruvate carboxykinase (PCK)] following the major C_4 acid decarboxylation enzyme in the bundle sheath (Hatch, 1987; Hattersley, 1992). The major C_4 crops, such as maize, sugarcane and sorghum belong to the NADP-ME subtype. At the regional level, the geographic distribution of C_4

grasses is strongly influenced by rainfall level. With decreasing rainfall (from 900 mm to 50 mm per annum), the abundance of NAD-ME grasses increases while that of NADP-ME grasses decreases. The distribution of PCK grasses is weakly correlated with rainfall gradient (Ellis *et al.*, 1980; Hattersley, 1992; Taub, 2000). This distribution suggests that C_4 grasses with different biochemical subtypes may have different water use efficiency (WUE) or drought tolerance. The first attribute has been validated with NAD-ME grasses having a greater whole-plant WUE under water stress than their NADP-ME counterparts (Ghannoum *et al.*, 2002). However, there is no evidence suggesting that the three C_4 biochemical pathways have different sensitivities to water stress. Hence, in the context of the current review, it is possible to discuss the effects of water stress on C_4 photosynthetic metabolism in general.

THE CO_2 -CONCENTRATING MECHANISM IN C_4 LEAVES

The key feature of C_4 photosynthesis is the operation of a CO_2 -concentrating mechanism in the leaves of C_4 plants, which consists of a series of biochemical and structural modifications around the ancestral C_3 photosynthetic pathway (Hatch, 1987). Although there are many ways, biochemically and anatomically, of achieving C_4 photosynthesis, the most common C_4 syndrome in higher plants involves the operation of two photosynthetic cycles (C_3 and C_4) across two photosynthetic cell types (mesophyll and bundle sheath), which are arranged in concentric layers around the vascular bundle (Fig. 1; Hatch, 1987). The first steps of C_4 photosynthesis occur in the mesophyll and involve the hydration of CO_2 into bicarbonate, which reacts with phosphoenolpyruvate (PEP) with the aid of PEP carboxylase (PEPC) to produce oxaloacetate, a C_4 acid, hence the terms C_4 cycle and C_4 photosynthesis. Oxaloacetate is converted into other C_4 acids (malate, aspartate or alanine) which diffuse into the bundle sheath cells where they are decarboxylated, releasing CO_2 for fixation by Rubisco and the rest of the C_3 cycle. The C_3

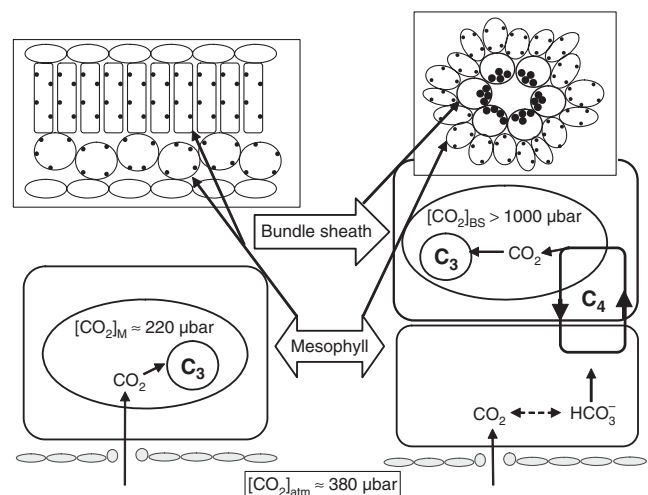


FIG. 1. A simplified, schematic representation of C_3 (left) and C_4 (right) photosynthesis.

product of the decarboxylation reaction returns to the mesophyll, completing the C₃ cycle (Fig. 1). The C₄ cycle acts like a CO₂-concentrating mechanism for two main reasons: (1) PEPC is faster than Rubisco and insensitive to O₂; and (2) the bundle sheath cell wall presents a significant gaseous diffusion barrier (Hatch, 1987; Brown and Byrd, 1993). Consequently, the high [CO₂] generated by the C₄ CO₂-concentrating mechanism in the bundle sheath leads to the suppression of apparent photorespiration in air as well as the saturation of C₄ photosynthesis at a lower ambient [CO₂] than for C₃ plants (Fig. 2). In addition, photorespired CO₂ is released within the bundle sheath, and either is refixed or contributes to increasing bundle sheath [CO₂] ([CO₂]_{BS}), which in turn, leads to reducing photorespiration. High [CO₂]_{BS} gives rise to the characteristic A/C_i curve of C₄ leaves. Relative to C₃ photosynthesis, the C₄ A/C_i curve is characterized by abrupt saturation at a relatively low C_i (Fig. 2). This constitutes the basis of a number of advantages conferred by the C₄, relative to C₃, photosynthetic pathway, chief of which is higher WUE (Osmond *et al.*, 1982; Long, 1999).

C₄ PHOTOSYNTHESIS AND WATER STRESS

C₃ and C₄ photosynthesis share most of the fundamental photosynthetic processes such as the C₃ cycle, light harvesting complexes and electron transport components. Hence, the two photosynthetic pathways may be expected to show, by and large, similar responses to water availability. Nevertheless, significant differences exist between the two photosynthetic types, which could make their response to water stress differ at a number of levels. A cursory examination of the literature reveals that the observed responses of C₄ photosynthesis to water stress are as diverse as those reported for C₃ photosynthesis. Some studies concluded that inhibition of C₄ photosynthesis under water stress is mainly due to stomatal closure, while others concluded that non-stomatal factors play a

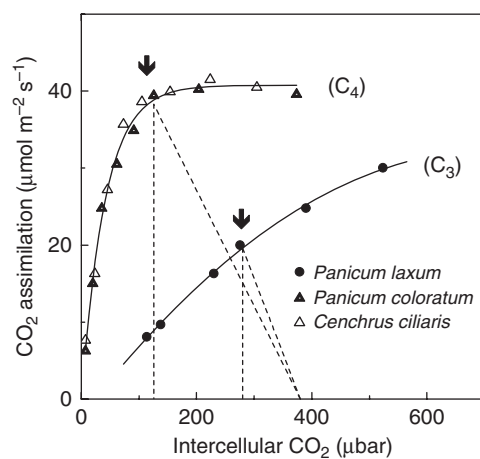


FIG. 2. The response of CO₂ assimilation rates (*A*) to intercellular CO₂ concentration (*C_i*) in one C₃ (*Panicum laxum*) and two C₄ (*Cenchrus ciliaris* and *Panicum coloratum*) grasses. Gas exchange measurements were made at 30 °C and 1200 μmol quanta m⁻² s⁻¹. The dotted lines represent the slope of stomatal conductance, $g = A/(C_a - C_i)$, where C_a and C_i are the ambient and intercellular [CO₂], respectively. The arrows indicate *A* at the operational *C_i* (i.e. *C_i* at normal air [CO₂]; O. Ghannoum, unpubl. res.).

major role (e.g. Lawlor and Fock, 1978; Becker and Fock, 1986; Loreto *et al.*, 1995; Lal and Edwards, 1996; Saccardy *et al.*, 1996; Maroco *et al.*, 2000; Ghannoum *et al.*, 2003; Marques da Silva and Arrabaça, 2004a; Ripley *et al.*, 2007; Carmo-Silva *et al.*, 2008). These studies used different C₄ species subjected to different severities and methods of inducing water stress (e.g. withholding watering, using an osmotic agent or drying of detached leaves) and made photosynthetic measurements using different techniques (e.g. various gas exchange instruments or O₂ electrodes) and under different conditions of light intensity and leaf temperature. Consequently, the different responses could be attributed to any combination of these factors. Therefore, there is a need to dissect the available evidence in order to draw a more comprehensive picture of the mechanisms underlying the response of C₄ photosynthesis to water stress. These mechanisms are summarized in Fig. 3 and, as commonly argued in the literature, are divided into stomatal and non-stomatal factors. The stomatal factors refer to the downstream effects of CO₂ limitation on photosynthetic activity. The non-stomatal factors encompass everything else, including the direct effects of reduced leaf and cellular water status on the activity of enzymes involved in the CO₂ fixation and electron transport reactions, induction of early senescence, and changes to leaf anatomy and ultrastructure (Fig. 3).

THE ROLE OF STOMATAL FACTORS IN THE INHIBITION OF C₄ PHOTOSYNTHESIS UNDER WATER STRESS

Similarly to what has been reported in C₃ plants, stomatal conductance of C₄ plants decreases with declining leaf water status, and this invariably coincides with reduced photosynthetic rates (e.g. Kalapos *et al.*, 1996; Maroco *et al.*, 2000; Ghannoum *et al.*, 2003; Carmo-Silva *et al.*, 2008). The concomitant decline of *A* and *g*, particularly under mild water stress (i.e. for leaf RWC >70 %), has been interpreted in a causal way in C₃ and C₄ plants alike, based on four main lines of

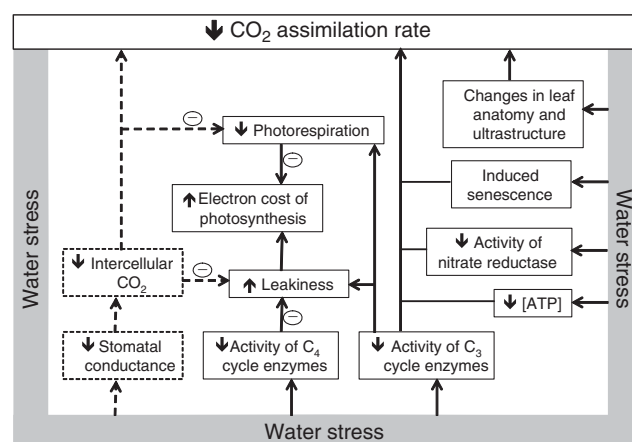


FIG. 3. Summary of the main effects of water stress on the photosynthetic parameters of C₄ leaves. Stomatal and non-stomatal factors are indicated by dashed and continuous lines, respectively. The (-) sign indicates an effect in the opposite direction. The term leakiness (Φ) is defined as the fraction of CO₂ fixed by PEPC which leaks out of the bundle sheath.

evidence: (1) reduced C_i , (2) recovery of A at high $[\text{CO}_2]$, (3) occurrence of photorespiration, and (4) recovery of A following re-hydration.

Intercellular CO_2 of C_4 plants subjected to water stress

Decreased C_i due to reduced stomatal conductance has been taken as a proof of CO_2 limitation for C_4 photosynthesis. The operation of a CO_2 -concentrating mechanism during C_4 photosynthesis introduces additional layers of complexity to this otherwise straightforward argument. A closer look at the literature shows that C_i decreases only during the early phases of water stress as has been reported for maize (Becker and Fock, 1986; Lal and Edwards, 1996; Leakey *et al.*, 2004), sorghum (Williams *et al.*, 2001), sugarcane (Du *et al.*, 1996), amaranthus (Lal and Edwards, 1996) and a non-crop C_4 grass species (Marques da Silva and Arrabaça, 2004a). During the later stages of drought, it is often observed that C_i increases while A continues its decline (e.g. Becker and Fock, 1986; Du *et al.*, 1996; Kalapos *et al.*, 1996; Lal and Edwards, 1996). In contrast, some studies using various C_4 plants reported no change in C_i under water stress (e.g. Saliendra *et al.*, 1996; Ripley *et al.*, 2007) or for most of the water stress period, with C_i increasing under severe stress at the end of the drying cycle (Kalapos *et al.*, 1996; Lal and Edwards, 1996).

By raising $[\text{CO}_2]$ at the sites of Rubisco, the C_4 CO_2 -concentrating mechanism serves to CO_2 -saturate A and virtually suppress photorespiration in normal air (Hatch, 1987). This is illustrated in Fig. 4 which uses the C_4 model of von Caemmerer (2000) to simulate the response of some key photosynthetic parameters to C_i in a mature C_4 leaf measured under optimal light and temperature. The shaded area highlights the range of C_i measured in well-watered and moderately water-stressed leaves (Fig. 4). The modelling predicts little change in A with C_i declining down to 50 μbar (Fig. 4A). Thus, based on our theoretical understanding, the CO_2 -concentrating mechanism endows C_4 photosynthesis with a significant buffering capacity against short-term fluctuations in C_i down to a certain concentration, such as those usually observed in mildly water-stressed C_4 leaves. This is supported by the results of Lal and Edwards (1996) who found that the initial decline in C_i , up to 50% of control values, had no effect on A during the early phases of water stress in both maize and amaranthus. Hence, it may be concluded that during the early stages of water stress, stomatal closure may not always reduce C_i enough to cause a detectable decline in A . In addition to inter-species variations amongst C_4 plants, whether or not a decline in C_i will elicit a reduction in A depends largely on growth and measuring conditions that influence the position of the operational C_i (i.e. C_i at normal air $[\text{CO}_2]$; Fig. 2). For example, conditions of high irradiance and nutrition tend to shift the operational C_i down to the CO_2 -responsive part of the A/C_i curve (Ghannoum *et al.*, 1997; Ghannoum and Conroy, 1998). In contrast, low irradiance tends to shift C_i to the flat part of the A/C_i curve, which necessitates a large decline in C_i before A is affected (e.g. Lal and Edwards, 1996). The interaction between environmental conditions (such as irradiance, nutrition, temperature) and the response of C_4 photosynthesis to water stress has not yet received its due attention.

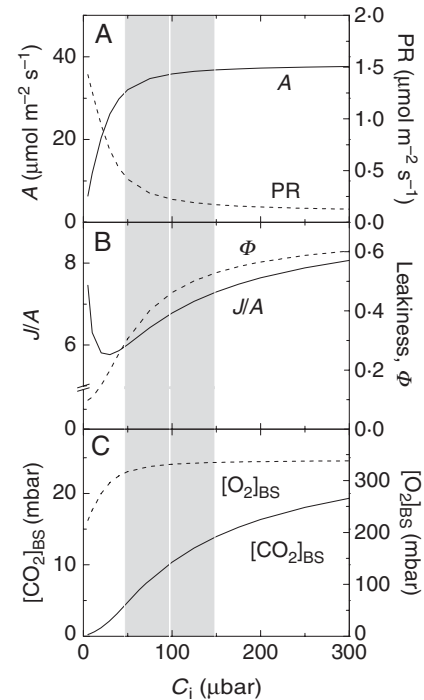


FIG. 4. Modelling the response of C_4 photosynthesis to intercellular $[\text{CO}_2]$, C_i , using the C_4 photosynthesis model developed by von Caemmerer (2000). (A) CO_2 assimilation rates (A , continuous line) and photorespiration (PR, dotted line); (B) the ratio of the rates of electron transport to CO_2 assimilation (J/A , continuous line) and leakiness (Φ , dotted line); and (C) bundle sheath $[\text{CO}_2]$ ($[\text{CO}_2]_{\text{BS}}$, continuous line) and $[\text{O}_2]$ ($[\text{O}_2]_{\text{BS}}$, dotted line). The modelling simulates a mature C_4 leaf with maximal PEPC and Rubisco activities of 120 and 40 $\mu\text{mol m}^{-2} \text{s}^{-1}$, respectively; a bundle sheath conductance to CO_2 per leaf area of 3 $\text{mmol m}^{-2} \text{s}^{-1}$. Other parameters are similar to those described in table 2 of von Caemmerer and Furbank (1999). The shaded area represents the likely range of C_i experienced by well-watered and mildly water-stressed C_4 leaves.

Recovery of photosynthetic rates by high $[\text{CO}_2]$ in C_4 plants subjected to water stress

If C_4 photosynthesis is limited by CO_2 supply due to stomatal closure under water stress, then increasing $[\text{CO}_2]$ should restore A either fully or partially to pre-stress values. Surprisingly, very few studies (apart from the literature about CO_2 enrichment, which is discussed in a later section) have attempted to specifically measure A under physiologically high $[\text{CO}_2]$. In a study where four C_4 grass species were exposed to a drying cycle, increased $[\text{CO}_2]$ up to 2500 ppm had no effect on A at any stage of the drying cycle in any of the four species (Fig. 5). Similar results were reported with three C_4 grasses exposed to severe water stress and measured at a $[\text{CO}_2]$ of 1000 ppm (Carmo-Silva *et al.*, 2008). When amaranthus was grown and measured at four $[\text{CO}_2]$ (18, 27, 35 and 70 Pa), elevated $[\text{CO}_2]$ alleviated slightly the negative impact of drought on A through the indirect effects of high $[\text{CO}_2]$ on Ψ_{leaf} , and only under mild but not severe water stress (Ward *et al.*, 1999). Rather than adjusting ambient $[\text{CO}_2]$, Du *et al.* (1996) maintained C_i at control values. Their results showed that raising C_i in water-stressed sugarcane leaves enhanced A fully to control values for $\Psi_{\text{leaf}} > -0.4$ MPa, and partially for Ψ_{leaf}

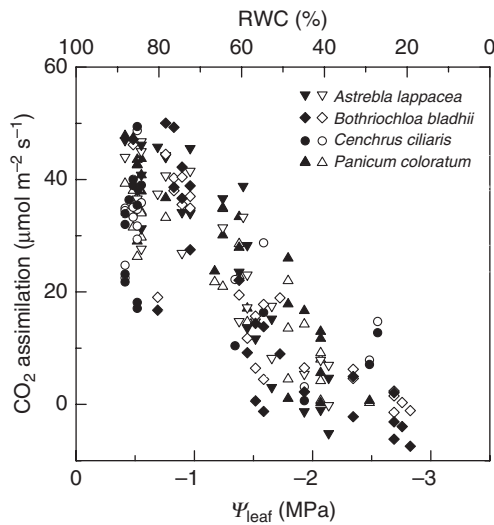


FIG. 5. CO_2 assimilation rates as a function of leaf relative water content (RWC) and water potential (Ψ_{leaf}) in four C_4 grasses growing in a drying soil: *Astrebla lappacea*, *Bothriochloa bladhii*, *Cenchrus ciliaris* and *Panicum coloratum*. Measurements were made at 28°C , $1000 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$, and ambient $[\text{CO}_2]$ of either 350 (open symbols) or $2500 \mu\text{bar}$ (closed symbols). Adapted from Ghannoum *et al.* (2003).

between -0.4 and -0.85 MPa. For $\Psi_{\text{leaf}} < -0.85$ MPa, C_i manipulations had no influence on A .

It has been argued that a mere doubling or tripling of ambient $[\text{CO}_2]$ is not enough to overcome the stomatal limitation caused by water stress, and that very high $[\text{CO}_2]$ ($>1\%$) is needed in order to force CO_2 to diffuse across the whole leaf surface and not just the near closed stomata. Super-saturating $[\text{CO}_2]$ may also be needed to overcome potential increases in mesophyll conductance in response to water stress (Cornic, 2000). For these reasons, some researchers used O_2 electrodes to measure CO_2 -dependent rates of O_2 evolution under super-saturating $[\text{CO}_2]$. In some studies, the use of these high $[\text{CO}_2]$ overcame part of the inhibitory effects of water stress on O_2 evolution rates (Saccardy *et al.*, 1996; Marques da Silva and Arrabaça, 2004a), but not in others (Ghannoum *et al.*, 2003). Super-saturating $[\text{CO}_2]$ was reported to restore O_2 evolution rates to control values in only one instance, using slowly dehydrated maize leaves (Saccardy *et al.*, 1996). A firmer conclusion regarding this line of evidence awaits further studies using more diverse C_4 species under well-defined conditions.

Photorespiration in C_4 plants subjected to water stress

In C_3 plants, low C_i causes a decrease in A and an increase in the rate of photorespiration due to a decreased $[\text{CO}_2] : [\text{O}_2]$ ratio at the sites of Rubisco. Increased photorespiration (e.g. due to reduced g under drought) causes an increase in the electron cost of CO_2 fixation (J/A , the ratio of electron transport to CO_2 assimilation rates), and indicates that A is CO_2 -limited. In C_4 plants, the relationship between C_i , photorespiration and J/A is more complex (Fig. 4A and B). Photorespiration in C_4 leaves remains very low under a range of environmental and genetic conditions, and runs at about $3.5\text{--}6\%$ of A (Lacuesta *et al.*, 1997; Carmo-Silva *et al.*, 2008). On the one hand, photorespiration may increase – from a very low base – with decreasing C_i

without any measurable impact on A (Fig. 4A). This is because photorespired CO_2 is most likely refixed within the bundle sheath before escaping to the atmosphere. The modelling results are supported by work on the oxygen sensitivity of C_4 photosynthesis. In an early study using maize subjected to osmotic stress, Lawlor and Fock (1978) found that A changed little in response to increasing $[\text{O}_2]$ from 1.5% to 21% . The decline of A with Ψ_{leaf} was almost indistinguishable between 1.5% and 21% $[\text{O}_2]$ (Lawlor and Fock, 1978). In a recent study using three C_4 grasses subjected to mild and severe water stress, Carmo-Silva *et al.* (2008) observed no changes in A with increasing $[\text{O}_2]$ above an optimum of 10% , and estimated photorespiration rates were small under all water stress conditions (Carmo-Silva *et al.*, 2008). On the other hand, if water stress were to reduce Rubisco activity independently of C_i , then both the carboxylation and oxygenation reactions of Rubisco would decrease in equal proportions. Accordingly, photorespiration is predicted to decrease rather increase under water stress (Fig. 3). This is in line with findings by Carmo-Silva *et al.* (2008). They found that photorespiration increased slightly between well-watered and moderate water-stress conditions, then decreased under severe water stress in two C_4 grasses (Carmo-Silva *et al.*, 2008). Consequently, while a small CO_2 -limitation may occur in the early phases of water stress, severe water stress tends to inhibit both photosynthesis and photorespiration in C_4 plants (Fig. 3).

In contrast to the aforementioned works, Lal and Edwards (1996) reported increased J/A in maize and amaranthus exposed to water stress, and concluded that A was CO_2 -limited in these two C_4 species under water stress (Lal and Edwards, 1996). In this study, the ambient $[\text{CO}_2]$ used for the low $[\text{CO}_2]$ comparison (fig. 4 in Lal and Edwards, 1996) was much lower than the C_i observed in the water-stressed leaves (fig. 1 in Lal and Edwards, 1996). Hence, the two situations, water stress and low C_i , were not comparable in their study. In the modelling example presented in Fig. 4, J/A showed a biphasic response to C_i (Fig. 4B). Below a C_i of $\sim 20 \mu\text{bar}$, J/A increases with decreasing C_i due to increasing photorespiration (Fig. 4A and B). This is comparable to the low $[\text{CO}_2]$ and moderate water stress treatments in Lal and Edwards (1996) and Carmo-Silva *et al.* (2008), respectively. Above a C_i of $\sim 20 \mu\text{bar}$, J/A increases with increasing C_i , which may be due to increased leakiness (Φ , the fraction of CO_2 fixed by PEPC which leaks out of the bundle sheath). It should be noted that, although leakiness is predicted to increase with C_i (Fig. 4B), this was not confirmed experimentally (Henderson *et al.*, 1992). Nevertheless, there is some evidence in the literature suggesting that leakiness increases under water stress (Bowman *et al.*, 1989; Saliendra *et al.*, 1996; Williams *et al.*, 2001). Increased J/A as a result of increased leakiness could explain the water stress results of Lal and Edwards (1996). Conclusive testing of this proposition requires the use of sophisticated techniques such as on-line measurement of carbon and oxygen isotopes discrimination by mass spectrometry or tube diode laser.

Recovery of photosynthetic rates following re-hydration of C_4 plants subjected to water stress

In addition to the aforementioned arguments, there remains one related to the recovery of A following re-hydration. Some

studies reported that when plants which have been deprived of water for 3–10 d were re-hydrated, photosynthetic rates, measured in normal air, returned to near control values (i.e. well-watered plants) relatively quickly (Lal and Edwards, 1996; Saccardy *et al.*, 1996; Foyer *et al.*, 1998). This has been interpreted as proof that the photosynthetic capacity remains intact under water stress. However, most of these studies measured the recovery of photosynthetic rates using the C₄ crop maize, which has been exposed to relatively mild stress such as withholding watering for several days (Lal and Edwards, 1996; Saccardy *et al.*, 1996; Foyer *et al.*, 1998). In a study using sorghum, recovery of A was only partial in response to re-hydration (Loreto *et al.*, 1995). Hence, it is important to undertake these measurements using C₄ species other than maize, exposed to different degrees of water stress. In these future studies, it is also important to distinguish whether the recovery of A occurs at the level of the same stressed leaf or the plant.

CONTRIBUTION OF NON-STOMATAL FACTORS TO THE INHIBITION OF C₄ PHOTOSYNTHESIS UNDER WATER STRESS

As for stomatal factors, arguments related to non-stomatal inhibition of A are very similar to those advanced for C₃ photosynthesis subjected to water stress (Lawlor, 2002). They include reduced activity of photosynthetic enzymes, decreased ATP concentration, inhibition of nitrate assimilation, induction of early senescence, and changes to the leaf anatomy and ultrastructure amongst others (Fig. 3 and Table 1). These metabolic factors have been reviewed recently by Lawlor (2002), Flexas and Medrano (2002) and Flexas *et al.* (2004). Therefore, in this review, my discussion is limited to evidence from the C₄ literature for the operation of such factors under water stress. In particular, I focus on the main point of difference with C₃ photosynthesis, which is the differential impact of water stress on the activity of C₃ and C₄ cycle enzymes.

Impact of water stress on the activity of C₃ and C₄ cycle enzymes

A number of studies have reported significant changes in the activity of photosynthetic enzymes in C₄ plants subjected to water stress (Table 1). For Rubisco, most studies reported decreased activity under water stress, while a couple of studies found no change (Table 1). In contrast, the response of the key C₄ cycle enzymes appears to be less consistent, with some studies reporting a decrease in activity, while others report no change or even increased activity under water stress (Table 1). This makes it difficult to draw firm conclusions about the role of these enzymes in water stress-induced photosynthetic inhibition in C₄ plants. This is further complicated by the fact that the literature offers only patchy data on a limited number of C₄ species. Nevertheless, a number of observations can be made regarding this aspect. In particular, there seems to be a more consistent inhibition of the activity of C₃ (e.g. Rubisco) than C₄ (e.g. PEPC) cycle enzymes in response to water stress (Table 1). In other words, the available, albeit limited, data suggest that water stress may lead to a decrease in the activity ratio of C₃/C₄ cycle enzymes in C₄ plants. This argument is supported by studies which reported increased leakiness in water-stressed C₄ plants (Bowman *et al.*, 1989; Saliendra *et al.*, 1996). Increased

TABLE 1. A literature survey of the effects of water stress on the activity of selected enzymes in a number of C₄ species

Species	Assay basis	Stress indicator	Effect						Reference
			Rubisco	PEPC	NADP-ME	PPDK	NR		
<i>Zea mays</i>	Leaf area	$\Psi_{\text{leaf}} = -1.17$ MPa	↓ by 20 %	↓ by 30 %	↓ by 33 %				Becker and Fock (1986)
	Chlorophyll	$\Psi_{\text{leaf}} = -1.2$ MPa		↑ slightly				↓ by 98 %	Foyer <i>et al.</i> (1998)
	Leaf area	RWC = 50 %	↓ by 75 %					↓ by 90 %	Lal and Edwards (1996)
<i>Saccharum</i> sp.	Leaf area	RWC = 50 %	↓ by 50 %	↔	↔				Saccardy <i>et al.</i> (1996)
	Leaf area	$\Psi_{\text{leaf}} = -1.61$ MPa	↓ by 50 %	↓ by 55 %	↓ by 73 %		↓ by 89 %		Du <i>et al.</i> (1996)
	Dry weight	$\Psi_{\text{leaf}} = -1.36$ MPa	↔	↑ slightly					Saliendra <i>et al.</i> (1996)
<i>Amaranthus cruentus</i>	Leaf area	RWC = 50 %	↓ by 75 %						Lal and Edwards (1996)
	Total protein	RWC = 40 %	↓ by 30 %						Marques da Silva and Arrabaca (2004a)
<i>Paspalum dilatatum</i>	Dry weight	RWC = 50 %	↔	↔					Carmo-Silva <i>et al.</i> (2007)
		RWC = 80 %	↓ by 18 %	↔					
		RWC = 80 %	↓ by 18 %	↓ by 50 %					
<i>Zoysia japonica</i>									

↑, increase; ↓, decrease; ↔, no significant change.

leakiness may be caused by a number of factors, one of which is reduced activity of C₃, relative to C₄, cycle enzymes (von Caemmerer and Furbank, 1999). In particular, if the carboxylation activity decreases more than the decarboxylation activity, CO₂ consumption will fall in the bundle sheath, leading to an increase in [CO₂]_{BS}. A greater [CO₂]_{BS} leads to a greater [CO₂] gradient across the bundle sheath cell walls, and hence a greater leakage of CO₂. In the study by Saliendra *et al.* (1996), increased leakiness was related to a decrease in Rubisco/PEPC activity ratio as a result of no change in Rubisco activity and a slight increase in PEPC activity. Bowman *et al.* (1989) concluded that a decrease in the C₃/C₄ activity ratio was the likely factor behind increased leakiness based on two main reasons. First, the changes in leakiness in response to water stress underwent diurnal fluctuations. This indicated that increased leakiness was caused by biochemical rather than anatomical factors (e.g. changes in the properties of bundle sheath cell wall and membranes). Secondly, there was a linear relationship between changes in leakiness and photosynthetic inhibition in response to water stress. This indicates that activities of C₃ cycle enzymes are more sensitive to water stress, assuming that this cycle is limiting C₄ photosynthesis (Bowman *et al.*, 1989). The differential response of C₃ and C₄ cycle enzymes to water stress and their eventual impacts on leakiness in C₄ plants is an important aspect which awaits further work.

Other non-stomatal factors

For C₄ plants, there is good evidence indicating that nitrate assimilation and nitrate uptake are strongly reduced under water stress (Table 1; Becker and Fock, 1986; Foyer *et al.*, 1998). This may explain the reported decreases in chlorophyll and protein content in a number of C₄ species subjected to water stress (Du *et al.*, 1996; Foyer *et al.*, 1998; Marques da Silva and Arrabaça, 2004b; Carmo-Silva *et al.*, 2007). The decrease in chlorophyll and protein contents under water stress may also be due to generalized protein degradation as a result of induced senescence as suggested by increased contents of amino acids (Becker and Fock, 1986). The induction of senescence under water stress – its timing and the factors which trigger it – is poorly understood.

Using light microscopy, Lal and Edwards (1996) observed ultra-structural distortions (e.g. changes in chloroplast position, distortion of intercellular spaces) in leaves of C₄ species under water stress. Such changes may have significant impacts on CO₂ diffusion inside the leaf as well as light penetration (Flexas *et al.*, 2004). However, due to the lack of data, it is not possible to make much of this aspect at this stage. It is hoped that with the proliferation of more sophisticated microscopic and imaging techniques, especially those which allow the observation of live tissue, there will be more studies published on the effects of water stress on the anatomy of C₃ and C₄ leaves alike.

THE ROLE OF ALTERNATIVE ELECTRON SINKS IN C₄ PHOTOSYNTHESIS EXPOSED TO WATER STRESS

Photorespiration results in the release of CO₂ and NH₃ into the atmosphere, and the consumption of ATP and other

reducing equivalents. Consequently, photorespiration may act as an alternative electron sink in C₃ plants exposed to water stress. By doing so, photorespiration can reduce the over-reduction of the photosynthetic electron transport chain (Osmond and Grace, 1995), and allow photosynthesis to recover more quickly after the removal of water stress. For C₃ plants, there is some evidence showing that photorespiratory electron transport increases under mild to moderate water stress, thus maintaining electron flow (e.g. Cornic and Fresneau, 2002; Haupt-Herting and Fock, 2002). Such data is lacking for C₄ plants. However, and as discussed earlier, photorespiration remains very low in C₄ plants under a wide range of physiological conditions. Therefore, unlike the case for C₃ photosynthesis, the scope for photorespiration acting as a protective electron sink is minimal during C₄ photosynthesis exposed to water stress.

Another photosynthetic, alternative electron sink is the Mehler reaction, which involves the direct reduction of molecular O₂ to superoxide radicals at photosystem I. Most studies involving C₃ plants exposed to moderate water stress indicate that the contribution of the Mehler reaction to total photosynthetic electron flow decreases or remains unchanged (Cornic and Fresneau, 2002; Haupt-Herting and Fock, 2002). For example, in tomato, the percentage of photosynthetic electrons dissipated by the Mehler reaction decreased from 13 % to 6 % in control and water-stressed leaves, respectively, while the contribution of photorespiration increased from 23 % to 40 % under water stress (Haupt-Herting and Fock, 2002). Direct measurements of O₂ exchange in leaves of well-watered C₄ grasses showed that O₂ uptake in the light depends on [CO₂] and light intensity (Siebke *et al.*, 2003). It was estimated that O₂ uptake associated with the Mehler reaction represents about 18 % of total light-dependent O₂ uptake in C₄ leaves (Siebke *et al.*, 2003). This is slightly greater than the rate of Mehler reaction measured in control C₃ leaves, indicating that the Mehler reaction has a slightly greater capacity in C₄ than C₃ leaves. However, it is likely that the Mehler reaction is insensitive – or even slightly suppressed – by water stress in C₄ as in C₃ leaves. Although there are no comprehensive measurements of O₂ exchange in C₄ plants exposed to water stress, a couple of indirect lines of evidence support this conclusion. First, in a study where maize was exposed to mild drought, it was observed that the activities of ascorbate peroxidase and glutathione reductase – enzymes involved in hydrogen peroxide detoxification – were unaffected by drought (Brown *et al.*, 1995). Secondly, in a review, Badger *et al.* (2000) argued that in higher plants excess light dissipation occurs mainly via non-radiative energy dissipation. Excess electron dissipation by Mehler O₂ uptake is significant mainly in photosynthetic organisms lacking well-developed non-photochemical quenching mechanisms, such as cyanobacteria (Badger *et al.*, 2000). The limited capacity of the Mehler reaction to act as a significant electron sink has been demonstrated by a study using tobacco with a genetically altered amount of Rubisco. Reducing the capacity for both photosynthesis and photorespiration in the transgenic, relative to the wild-type, plants did not lead to enhanced electron transport to free O₂ (Ruuska *et al.*, 2000). Consequently, the limited capacity for the Mehler reaction or photorespiration to act as significant alternative electron sinks may account for the

strong correlation between CO₂ assimilation and electron transport rates observed in C₄ leaves under a wide range of environmental conditions (e.g. Oberhuber and Edwards, 1993).

INTERACTIVE EFFECTS OF ELEVATED [CO₂] AND WATER STRESS ON C₄ PHOTOSYNTHESIS

Due to unprecedented rates of fossil fuel burning and deforestation since the start of the industrial revolution, atmospheric [CO₂] has been rising rapidly (IPCC, 2007). Understanding the effects of rising [CO₂] on C₄ plants is crucial given their significant contribution to the global carbon budget and food security. C₄ plants were not expected to respond to high [CO₂] because C₄ photosynthesis is mostly CO₂-saturated under current atmospheric [CO₂] due to the operation of the CO₂-concentrating mechanism. However, as more research was done on this topic, it became increasingly evident that C₄ plants can accumulate more biomass at elevated [CO₂], particularly if exposed to some form of water stress during growth. These findings were made consistently in controlled-environment and field studies alike (e.g. Samarakoon and Gifford, 1996; Seneweera *et al.*, 1998, 2001; Wand *et al.*, 1999; Wall *et al.*, 2001; LeCain *et al.*, 2003; Leakey *et al.*, 2004, 2006). Ghannoum *et al.* (2000, 2006) reviewed the mechanism underlying the response of C₄ plants to high [CO₂]. They concluded that elevated [CO₂] enhances biomass production in C₄ plants predominantly via the indirect effects on stomatal conductance. By reducing leaf and hence canopy transpiration, high [CO₂] leads to soil water conservation (Samarakoon and Gifford, 1996; Seneweera *et al.*, 1998, 2001; Wall *et al.*, 2001; LeCain *et al.*, 2003; Leakey *et al.*, 2006). In general, evidence from the literature about CO₂ enrichment argues against a substantial role for stomatal limitation in the observed decline of C₄ photosynthesis under water stress. In particular, high [CO₂] does not directly alleviate the adverse effects of water stress on C₄ photosynthesis (Ghannoum *et al.*, 2003). The latter conclusion was supported by results from free air [CO₂] enrichment (FACE) studies with the C₄ crops sorghum and maize (Wall *et al.*, 2001; Leakey *et al.*, 2004, 2006) and open-top chamber experiments with the C₄ grass *Bouteloua gracilis* (LeCain *et al.*, 2003). These experiments tested the interaction between elevated [CO₂] and water stress on C₄ photosynthesis in the field and, in the case of FACE, under natural growing conditions. A key advantage of these studies is that plants experience water stress at rates and severities normally experienced by field-grown plants, thus avoiding the need to get into discussions of whether drought occurred in a 'realistic' fashion in pots (or detached leaves). The other main advantage is that field studies allow for the impacts of soil feedbacks to be assessed. It is worth noting that some pot studies have attempted to measure changes in soil moisture with C₄ plants, and reported similar results (e.g. Samarakoon and Gifford, 1996; Seneweera *et al.*, 1998, 2001).

The FACE study undertaken in the North American Corn Belt with maize is particularly illuminating for the following main reasons: plants were grown in the field under rain-fed conditions; plants experienced both wet and dry seasons; and comprehensive diurnal and seasonal gas exchange and fluorescence analyses were carried out under growth conditions

(Leakey *et al.*, 2004, 2006). When the crop experienced a wet year due to above-average rainfall, A and all other measured photosynthetic parameters were not stimulated by high [CO₂] at any stage of the day or season (Leakey *et al.*, 2004). The failure of high [CO₂] to affect A during the course of the day is particularly interesting because it reveals that the diurnal drifts in A – particularly those brought about by fluctuations in leaf-to-air vapour pressure deficit – in this C₄ crop are not primarily stomatal in nature. Importantly, during the dry year, A was stimulated by elevated [CO₂] only intermittently during the course of the season. This stimulation was associated with improved soil water content as a result of the consistent reductions in g at high [CO₂] in maize (Leakey *et al.*, 2004). In an open top chamber study using a C₄ grass, Wall *et al.* (2001) reported a similar pattern of responses. Consequently, these studies indicate that when C₄ plants experience drought in their natural environment, elevated [CO₂] alleviates the effect of water stress almost entirely via the indirect effect of reduced stomatal conductance and subsequent improved soil moisture.

CONCLUSIONS

It is well-established that the physiological advantages, conferred by the higher photosynthetic efficiency of C₄, relative to C₃, photosynthesis under high light and temperature, are crucial for the ecological dominance of C₄ plants in open, hot and arid environments (Osmond *et al.*, 1982; Long, 1999). In particular, the presence of a CO₂-concentrating mechanism in C₄ leaves endows them with higher WUE than their C₃ counterparts when compared under standard conditions (Osmond *et al.*, 1982; Long, 1999). However, it remains questionable whether the higher WUE of C₄, compared with C₃, plants leads to greater tolerance to water stress. In this review, I argue that, although the C₄ CO₂-concentrating mechanism offers C₄ photosynthesis a greater buffering capacity against CO₂ shortages brought about by partial stomatal closure under water stress, the biochemistry of C₄ photosynthesis is as – or even more – sensitive than that of C₃ photosynthesis. The reasons are not clear. However, a greater sensitivity of the C₃, relative to the C₄, cycle emerges as a probable site of metabolic limitation under water stress.

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