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Utilization of O_2 in the metabolic optimization of C_4 photosynthesis

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ABSTRACT

The combined effects of O_2 on net rates of photosynthesis, photosystem II activity, steady-state pool size of key metabolites of photosynthetic metabolism in the C_4 pathway, C_3 pathway and C_2 photorespiratory cycle and on growth were evaluated in the C_4 species *Amaranthus edulis* and the C_3 species *Flaveria pringlei*. Increasing O_2 reduced net CO_2 assimilation in *F. pringlei* due to an increased flux of C through the photorespiratory pathway. However, in *A. edulis* increasing O_2 up to 5–10% stimulated photosynthesis. Analysis of the pool size of key metabolites in *A. edulis* suggests that while there is some O_2 dependent photorespiration, O_2 is required for maximizing C_4 cycle activity to concentrate CO_2 in bundle sheath cells. Therefore, the response of net photosynthesis to O_2 in C_4 plants may result from the balance of these two opposing effects. Under 21 versus 5% O_2, growth of *A. edulis* was stimulated about 30% whereas that of *F. pringlei* was inhibited about 40%.

Key-words: *Amaranthus edulis*; *Flaveria pringlei*; climate change; C_4 photosynthesis; oxygen requirement.

Abbreviations: *A*, net CO_2 assimilation rate; Ala, alanine; Asp, aspartate; Gly, glycine; Mal, malate; MPR, Mehler peroxidase reaction; NAD-ME and NADP-ME, NAD- and NADP-malic enzyme; PEP, phosphoenolpyruvate; PGA, 3-phosphoglycerate; PPDK, pyruvate Pi dikinase; PSII, photosystem II; Q_A, quinone A; Ru5P, ribulose 5-phosphate; RuBP, ribulose-1,5-bisphosphate; Rubisco, ribulose 1,5-bisphosphate carboxylase/oxygenase; Ser, serine; ϕ_{PSII}, quantum yield of PSII reaction centres.

INTRODUCTION

During photosynthesis, CO_2 fixation via ribulose-1,5-bisphosphate (RuBP) carboxylase yields two phosphoglycerate (PGA) molecules. Of the PGA formed, one-sixth are then converted primarily to carbohydrates while the other five-sixths are used to regenerate RuBP, the precursor of the C_3 cycle. However, the oxygenase reaction of RuBP carboxylase/oxygenase (Rubisco) with O_2 as substrate, which is competitive with CO_2, yields one PGA and one phosphoglycolate. As a result of the oxygenase reaction, plants evolved a metabolic process – photorespiration – to recover part of the carbon, in which phosphoglycolate is metabolized via the glycolate pathway to glycerate, which can then re-enter the C_3 cycle (Keys 1986; Andrews & Lorimer 1987; Leegood *et al*. 1995). Despite some evidence that photorespiration may play a protective role when excess energy is present (Andrews & Lorimer 1987; Kozaki & Takeba 1996), it is mainly a wasteful process since for every O_2 reacting with RuBP the equivalent of 2 NADPH are utilized (to re-assimilate the PGA and ammonia formed), the same as when one CO_2 reacts with RuBP. Furthermore, one-quarter of the carbon metabolized through the glycolate pathway is lost as CO_2 to the atmosphere (Keys 1986; Andrews & Lorimer 1987; Leegood *et al*. 1995). Indeed, in plants possessing the C_3 type of photosynthesis, reducing the O_2 levels from 21 to 2% or increasing CO_2 to 0·1% can increase the net CO_2 assimilation by up to 50% (Gerbaud & Andre 1987; Tolbert 1994) because of reduced oxygenase activity and photorespiration.

In C_4 plants, CO_2 is first hydrated to HCO_3^- in the cytoplasm of mesophyll cells and then fixed into C_4 acids via carboxylation of the C_3 acid phospho*enol*pyruvate (PEP). The C_4 acids, malate (Mal) and aspartate (Asp), are transported through the plasmodesmata to an adjacent bundle sheath cell where they are metabolized, releasing CO_2 and pyruvate (Pyr) in the malic enzyme-type species. The CO_2 released in this step is subsequently fixed by Rubisco in the conventional C_3 cycle, whereas Pyr or alanine (Ala) returns to mesophyll chloroplasts and is converted to PEP, reinitiating the C_4 cycle (Edwards & Walker 1983; Hatch 1987). Because of this additional metabolic pathway, C_4 plants are able to concentrate CO_2 in the Rubisco-containing bundle sheath cells (Edwards & Walker 1983) at levels up to three to 20 times higher than atmospheric CO_2 (Jenkins, Furbank & Hatch 1989; Dai, Ku & Edwards 1993; He & Edwards 1996; von Caemmerer & Furbank 1999). Thus, C_4 plants have been able to take advantage of the C_4 mechanism, especially in high light, high temperature and arid conditions (Ehleringer *et al*. 1991; Collatz, Berry & Clark 1998). However, the concentrating mechanism requires the generation of two ATP for conversion of Pyr to PEP with each turn of the C_4 cycle.

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Because of their CO_2 concentrating mechanism, C_4 plants greatly reduce the oxygenase reaction of Rubisco (Edwards & Walker 1983; Hatch 1987; Ogren 1994). In addition, since net CO_2 assimilation rates of C_4 plants are similar at O_2 concentrations of 2 and 20% (Dai *et al*. 1993; Ogren 1994), C_4 plants were thought to be O_2 insensitive (Edwards & Walker 1983; Cerling, Wang & Quade 1993; Dai *et al*. 1993). However, recent studies on single leaves demonstrated that C_4 photosynthesis in all three subtypes of C_4 plants has an optimal O_2 concentration of 5 to 10% (Maroco, Ku & Edwards 1997). At suboptimal O_2 concentrations, C_4 photosynthesis is limited by over-reduction of quinone A (Q_A) and a decrease in the efficiency of Photosystem II (PSII) (Maroco *et al*. 1997). Further studies on mutants of *A. edulis* lacking the C_4 cycle versus wild-type plants have shown that the requirement for O_2 is associated with function of the C_4 cycle (Maroco *et al*. 1998a).

In this study, analyses were made of the effects of O_2 on CO_2 fixation, the reductive state of the acceptor side and quantum yield of PSII (ϕ_{PSII}), photosynthetic metabolite pools, and plant growth in a C_3 versus a C_4 dicot. The results suggest O_2 benefits C_4 photosynthesis by increasing the activity of the C_4 cycle and the CO_2 concentrating mechanism, which limits photorespiration.

MATERIALS AND METHODS

Gas-exchange and fluorescence

Fully expanded leaves from 40 to 60-day-old plants of the C_3 species *Flaveria pringlei* Gandoger and the NAD-malic enzyme (NAD-ME) C_4 species *Amaranthus edulis* Speg., grown in a greenhouse with 25/35 °C night/day temperatures, were used for simultaneous measurements of chlorophyll *a* fluorescence (to measure Φ_{PSII} and the reduction state of Q_A) and gas exchange, as described elsewhere (Maroco *et al*. 1997; Maroco *et al*. 1998a). Measurements were made at a leaf temperature of $30 \pm 0{\cdot}2$ °C, PPFD of $1000 \pm 50\ \mu mol\ m^{-2}\ s^{-1}$ and a leaf to air vapour pressure difference of $19{\cdot}1 \pm 0{\cdot}1$ Pa kPa^{-1}.

For growth analysis, four plants per treatment were grown for 35 d in plexiglass boxes with supplemented irradiance from sodium vapour lamps (2000 $\mu mol\ m^{-2}\ s^{-1}$), day/night temperatures of 35/25 °C, 85% relative humidity and 350 $\mu L\ L^{-1}$ CO_2 and at 5 versus 21% O_2 during the daytime. Roots were kept outside the controlled atmosphere box to avoid the effects of low O_2 over root respiration and function (see Quebedeaux & Hardy 1973). Gas concentrations were obtained by mixing O_2 with N_2 and CO_2. Plants were separated into roots, stems and leaves and their biomass determined after drying in an oven at 50 °C for 62 h.

Metabolite extraction

Ten cm^2 leaf discs were cut from leaves undergoing steady-state photosynthesis at 0·035% CO_2 and different O_2 concentrations, and immediately frozen using a pressure-driven freeze-clamp apparatus with copper rods which had been cooled by immersion in liquid N_2. Half of each leaf disc was used to determine the size of the PEP, Pyr, RuBP and ribulose 5-phosphate (Ru5P) pools, and the other half was used for glycine (Gly), Ala, Asp, and serine (Ser) determination. Metabolites were extracted by grinding frozen leaf discs in liquid N_2 to a fine powder and resuspending the powder in cold (4 °C) 5% (v/v) perchloric acid (0·1 g leaf/1 mL). After centrifugation at 12000 *g* and 4 °C for 10 min, the pellet was washed and recentrifuged, and the supernatant fractions combined and neutralized to pH 7–8 with 5 M K_2CO_3. The neutralized extract was centrifuged again at 12000 *g* for 5 min at 4 °C and the remaining pigments in the supernatant were removed with activated charcoal (0·1 g mL^{-1}). The charcoal was removed by centrifugation at 2000 *g* for 2 min at 4 °C and the supernatant stored at –80 °C until assay.

Metabolite assays

Pyr and PEP

The pool sizes of Pyr and PEP were determined spectrophotometrically by following the decrease in absorbance at 340 nm using coupling enzymes. The assay mixture (final volume of 1 mL) contained 300 μL of the metabolite extract, 0·1 M Tris-HCl (pH 7·4), 7·5 mM $MgSO_4$/KCl and 0·16 mM NADH. For determining Pyr, the reaction was initiated by addition of 6 U of lactate dehydrogenase (Sigma, St. Louis, MO, USA) and the decrease in absorbance due to the reduction of Pyr to lactate was measured. Then, the PEP pool was determined in the same assay by adding ADP (0·24 mM) and 0·4 U of Pyr kinase (Holldorf 1984).

RuBP and Ru5P

The pool sizes of RuBP and Ru5P were determined radiometrically by following the incorporation of $^{14}HCO_3^-$ by Rubisco into acid-stable products. The assay mixture (final volume of 0·18 mL) contained 120 mM Tris-HCl (pH 8·0), 15 mM $MgCl_2$, 15 mM DTT and 12 mM $NaH^{14}CO_3$ (26·8 nCi μmol^{-1}). After a 2 min incubation at 30 °C, the reaction was initiated by adding purified spinach Rubisco (final concentration 2 U mL^{-1}). After incubation for 2·5 h at 30 °C, the reaction was stopped with addition of 0·25 mL of 20% (v/v) tricarboxylic acid. The samples were left 10 min at room temperature and then flushed with air for 10 min. Ten ml of scintillation liquid (Bio-Safe II, Research Products International Co., Mount Prospect, IL, USA) was added to the samples and the specific activity counted in a Beckman LS 700 Liquid scintillation counter (Beckman Instruments Co., Irvine, CA, USA). The RuBP pool size was calculated after correction for background counts and counting efficiency. The Ru5P pool was measured in parallel with the RuBP determination by adding 2·8 mM ATP and 2 U mL^{-1} of phosphoribulokinase (Sigma) to a duplicate assay mixture. The Ru5P pool size was calculated by subtracting the total count of this assay from that of the RuBP assay after correction for background and counting efficiency.

Asp, Ala, Gly and Ser

The amino acid analysis was performed by the Washington State University Bioanalytical Laboratory. Total soluble amino acids were extracted in cold (4 °C) 80% ethanol. After drying in a vacuum drier the residue was redissolved in high-performance liquid chromatography (HPLC) amino acid buffer and Asp, Ala, Ser and Gly were determined by HPLC using a Beckman 6300 automatic amino acid analyzer (Beckman Instruments Co.). The pool sizes of these four amino acids were then quantified against known amino acid standards.

Statistical analysis

Biochemical data presented are the means of two independent assays of three leaves each. Growth data are presented as the means of four plants per treatment and species. Statistical analysis was done by a GLM ANOVA with $\alpha = 0{\cdot}05$. Fisher LSD's with $\alpha = 0{\cdot}05$ are shown as error bars in the figures.

RESULTS AND DISCUSSION

Effects of O_2 on rates of CO_2 fixation and PSII efficiency

In the C_3 plant *Flaveria pringlei*, decreasing the O_2 concentration from 20 to approximately 2% increased net photosynthesis by 50% (Fig. 1) ($P = 0{\cdot}02$), whereas, at extremely low O_2 levels (0·3%), photosynthesis declined. Inhibition of photosynthesis by anaerobic conditions or very low levels of O_2 in C_3 plants has been reported in several C_3 species (Ziem-Hank & Heber 1980; Dietz, Schreiber & Heber 1985) with evidence that very low O_2 may limit PSII activity and mitochondrial respiration (Dietz *et al.* 1985) and starch synthesis Sharkey & Vassey 1989).

In the C_4 plant *A. edulis*, maximum rates of photosynthesis were obtained at 5 to 10% O_2 whereas at lower O_2 concentrations, net photosynthesis decreased (Fig. 1a) ($P = 0{\cdot}06$). The decline in photosynthesis at low O_2 is associated with decreased efficiency of PSII reaction centres and with over-reduction of Q_A (Fig. 1b) ($P < 0{\cdot}050$). The higher O_2 requirement for C_4 photosynthesis results from the activity of the C_4 cycle, since C_4 mutant plants with low C_4 cycle activity show an O_2 response similar to that for C_3 photosynthesis (Maroco *et al.* 1998a). Limited function of the C_4 cycle under low O_2 could account for the decrease in photochemistry and PSII activity and increase in non-photochemical dissipation of energy.

Maintenance of high rates of photosynthesis under atmospheric levels of O_2 (21%) is the result of the CO_2 concentrating mechanism in C_4 plants which greatly reduces photorespiration. Under the current atmospheric conditions, photorespiration in C_4 plants is estimated to be 3 to 7% of net photosynthesis (de Veau & Burris 1989; Dai *et al.* 1993). Our results are in accordance with these estimates (Fig. 1a).

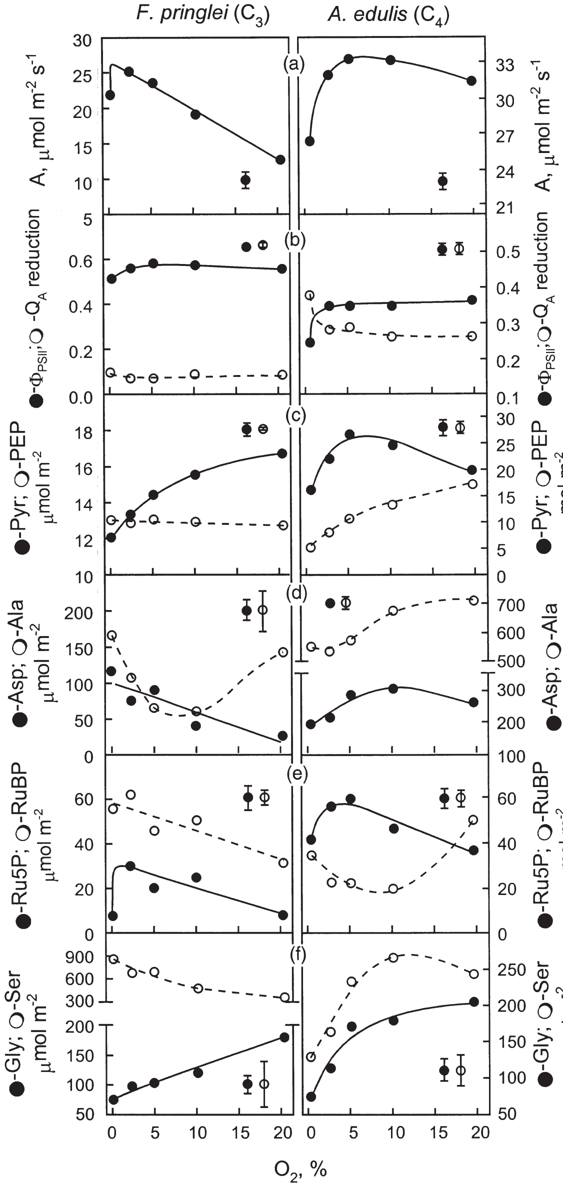

Figure 1. Net CO_2 assimilation (a), quantum yield of PSII (Φ_{PSII}) and reduction state of Q_A (b) and the pool sizes of Pyr and PEP (c), Asp and Ala (d), Ru5P and RuBP (e) and Gly and Ser (f) in *F. pringlei* (C_3) and *A. edulis* (C_4) as a function of O_2 concentration. Data points are the averages of two independent assays of three leaves each. Error bars are the Fisher LSD's with $\alpha = 0{\cdot}05$.

Effects of O_2 on pool sizes of photosynthetic metabolites

C_4 cycle metabolites

The effects of O_2 on changes in pool sizes of key photosynthetic metabolites in the C_4 *A. edulis* versus C_3 *F. pringlei* were investigated. While O_2 dependent changes in metabolite pools will not give quantitative information on flux through a pathway, it can give some qualitative information on pathway function. Very different patterns were observed in the pool sizes of several photosynthetic metabolites in the C_4 versus the C_3 species in response to changes in the level of O_2. In the C_4 *A. edulis,* increasing O_2 from 0 to 20% led to a 3·5-fold increase in PEP ($P = 0{\cdot}04$), the initial substrate for CO_2 fixation in the C_4 cycle (Fig. 1b). The size of the Pyr pool, the precursor for forming PEP, increased as O_2 increased from 0 to 5%, but then decreased at O_2 concentrations above 5% (Fig. 1c). Similarly, the Asp and Ala pools increased with increasing O_2 up to 10%, and then levelled off at higher O_2 concentrations (Fig. 1d). In NAD-ME type C_4 plants such as *A. edulis*, Asp is the primary C_4 acid formed in mesophyll cells, and Asp and Ala are the primary metabolites shuttled between the two photosynthetic cell types (Edwards & Walker 1983). Leegood & von Caemmerer (1988) showed that increasing rates of photosynthesis with increasing ambient levels of CO_2 in *A. edulis* was accompanied by a rise in the C_4 cycle metabolites PEP, Asp and Ala. Thus, the increase in the PEP and Asp pools with increasing O_2 suggests O_2 accelerates the C_4 cycle and increases the level of CO_2 pumped into the bundle sheath cells. This could explain the previously observed stimulatory effect of O_2 on C_4 photosynthesis (Glacoleva & Zalensky 1978; Dai *et al.* 1993; Maroco *et al.* 1997; Maroco *et al.* 1998a). In contrast to results obtained with *A. edulis*, in the C_3 *F. pringlei*, the pool size of PEP was independent of O_2 ($P = 0{\cdot}14$) although there was some increase in the Pyr pool with increasing O_2 concentrations ($P = 0{\cdot}02$). In leaves of C_3 plants, PEP is synthesized via glycolysis rather than by the ATP-dependent PPDK (pyruvate Pi dikinase), and it is not involved in primary carbon fixation. An increasing Pyr pool might reflect flow of some carbon from photorespiration through the glycolate pathway into Pyr (via hydroxypyruvate or glycerate) (Fig. 1c). Under low O_2 near anaerobic conditions Pyr formation by glycolysis may also be restricted. The linear decrease in the Asp pool with increasing O_2 (Fig. 1d) could occur by increased utilization of Asp as an amino donor in glycolate pathway metabolism.

Further evidence to support the hypothesis that O_2 enhances C_4 cycle activity in *A. edulis* is provided by plotting the effect of O_2 on the ratio of PEP/Pyr (Fig. 2). In *A. edulis* there is a significant linear increase in the PEP/Pyr ratio with increasing O_2 up to 21% ($P < 0{\cdot}05$), whereas in *F. pringlei* the ratio slowly decreased with increasing O_2, but was not statistically significant ($P > 0{\cdot}10$). The increasing PEP : Pyr ratio in response to increasing O_2 (Fig. 2) further suggests O_2 causes an increased efficiency of the C_4 cycle in concentrating CO_2 in the bundle sheath cells. As more PEP is regenerated from Pyr, more atmospheric CO_2 can be fixed and pumped into the bundle sheath cells. The results specifically suggest a limitation on PPDK activity under low O_2. In this respect, it is of interest that O_2 can stimulate the rate of conversion of pyruvate to PEP via PPDK (Huber & Edwards 1975; Furbank, Badger & Osmond 1983) and induce pseudocyclic electron flow via the Mehler peroxidase reaction (MPR) (Ivanov & Edwards 1997). This suggests O_2 may increase the PEP/Pyr ratio *in vivo* via generating ATP for PPDK in the mesophyll chloroplasts through the MPR. There is also evidence that the state of activation of PPDK falls under very low O_2 (less than 2%) which could also limit the function of the cycle. The activation state of PPDK in maize (C_4) leaves is inhibited by 60 to 70% under anaerobic conditions, compared to the activation state at 2 versus 21% O_2 (Nakamoto & Edwards 1983). Either means of restricting the C_4 pathway under low O_2, i.e. by limited ATP or decreased activation of PPDK, would limit the delivery of CO_2 to the C_3 cycle, and the associated utilization of reductive power for carbon assimilation. This could explain the observed decrease in PSII activity and increased non-photochemical dissipation of energy under low O_2.

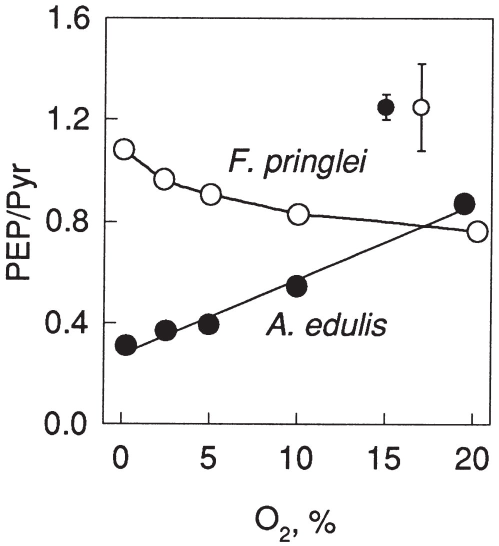

Figure 2. The ratios of PEP to Pyr as a function of O_2 concentration in the C_3 *F. pringlei* and in the C_4 *A. edulis*. Data points are the averages of two independent assays of three leaves each. Error bars are the Fisher LSD's with $\alpha = 0{\cdot}05$.

Amaranth is an NAD-ME type species, and there is evidence that the MPR is active *in vivo* and in other species of this subgroup based on O_2 isotope exchange analysis (Furbank & Badger 1982) and O_2 dependent increase in PSII activity under high CO_2 (Laisk & Edwards 1998; Maroco *et al.* 1998b). However, results of these studies also indicate that the MPR is not sufficient to provide all the additional ATP needed to support the C_4 cycle and that cyclic photophosphorylation must contribute. In this respect, it is of interest that O_2 is also required for the optimum function of cyclic photophosphorylation around Photosystem I as O_2 is required to prevent over-reduction of electron carriers on the acceptor side of PSII, which can inhibit cyclic electron flow (Ziem-Hank & Heber 1980; Dietz *et al.* 1985). In NAD-ME type species such as *A. edulis* the only energy requirement for conversion of Ala and CO_2 to Asp in mesophyll cells is 2 ATP through the combined activities of PPDK and adenylate kinase. This results in a high demand for ATP which can be met through the MPR and cyclic photophosphorylation.

C_3 cycle metabolites: Ru5P, RuBP

In *A. edulis*, the RuBP pool decreased as O_2 increased from 0 to 5–10% (Fig. 1e), where the rate of photosynthesis reached a maximum. This is consistent with an O_2-dependent increase in C_4 cycle activity and in the CO_2 level in bundle sheath cells. In support of this interpretation, the RuBP pool is known to decrease as the rate of photosynthesis in *A. edulis* and *Zea mays* increases with increasing atmospheric levels of CO_2 (Leegood & von Caemmerer 1988, 1989). The basis for the increase in RuBP pool at 20% O_2 in *A. edulis,* which occurs as the rate of photosynthesis slightly decreases, is uncertain (Fig. 1e). It could be explained if there were some restriction of metabolism through the glycolate pathway with increasing O_2 which resulted in feedback inhibition of catalysis by Rubisco.

The increase in the RuBP and Ru5P pools under decreasing O_2 in the C_3 plant *F. pringlei* is consistent with other evidence on the O_2 effects on C_3 photosynthesis. A similar effect on the RuBP pool was reported with decreasing O_2 from 21 to 2% in the C_3 species *Raphanus sativus* (von Caemmerer & Edmondson 1986), *Arabidopsis thaliana* (Chastain & Ogren 1985), *Spinacea oleracea* (Chastain & Ogren 1985) and *Phaseolus vulgaris* (Badger, Sharkey & von Caemmerer 1984). An increase in the RuBP pool under low O_2 may be a general phenomenon associated with a reduction in photorespiration, but in some cases it may be related to feedback inhibition of photosynthesis (Sun, Edwards & Okita 1999).

Photorespiratory metabolites: Gly and Ser

In the C_4 *A. edulis*, increasing O_2 caused an increase in the Gly and Ser pool which reached a maximum at about 5% O_2, consistent with the occurrence of some photorespiration (Fig. 1f). Relevant to this, in a study with maize (C_4) leaf discs when O_2 was increased from 1 to 50% there was an increase in the Gly pool (Marek & Stewart 1983); and, in *Flaveria bidentis* (C_4) decreasing CO_2 caused a rise in Gly (Leegood & von Caemmerer 1994), which was taken as evidence for the occurrence of photorespiration in C_4 plants. Direct evidence for photorespiration in maize was obtained using $^{18}O_2$ where the rate of synthesis of glycolate and Gly increased as O_2 was increased from 2, to 21, to 40%; as expected, rates of synthesis of these metabolites were much lower in maize than in the C_3 species wheat (de Veau & Burris 1989). With increasing O_2, photorespiration in C_4 plants may be limited in part by an O_2-dependent enhancement of the C_4 cycle and increased CO_2 levels in the bundle sheath cells, limiting RuBP oxygenase activity.

In *F. pringlei*, there was a linear increase in the Gly pool with increasing O_2 up to 21%. This corresponds to a linear decrease in rates of CO_2 fixation and in the RuBP pool in *F. pringlei* with increasing levels of O_2 above 2% (Figs 1a & e), which is consistent with reduction of photosynthesis due to increased photorespiration. *Flaveria pringlei* has a high pool of Ser at low O_2, where photorespiration is expected to be low. In wheat (C_3) the rate of synthesis of Ser, measured by incorporation of $^{18}O_2$, is five-fold higher at 21% O_2 compared with 2% O_2 as expected for O_2-dependent synthesis of Ser by photosynthesis; yet the size of the Ser pool was similar at the two O_2 levels (de Veau & Burris 1989). Thus, changes in the Gly pool may be a better indicator of the occurrence of photorespiration than changes in the Ser pool.

In summary, these results from analysis of metabolite pools suggest that while some photorespiration occurs in *A. edulis* in response to increasing O_2, O_2 is also utilized for maximizing the C_4 cycle activity. The net CO_2 assimilation response of *A. edulis* with respect to varying O_2 may result from the balance of these two opposing effects of O_2. This is illustrated in Fig. 3, where O_2 is proposed to increase the production of ATP in mesophyll chloroplasts of NAD-ME type species and to increase the conversion of Pyr to PEP.

Among the three C_4 subgroups, including C_4 monocots and dicots, there is a similar requirement for O_2 (5–10%) to obtain maximum rates of photosynthesis, and at lower O_2 levels there is a decrease in PSII yield and over-reduction of Q_A (Maroco *et al.* 1997; Fig. 1). A common feature may be O_2 dependence for C_4 cycle function in relation to PPDK (common to all subgroups) and an O_2 requirement in relation to cyclic/pseudocyclic photophosphorylation, which is considered necessary to meet the extra demands for ATP in C_4 plants (Edwards & Walker 1983; Hatch 1987; Furbank, Jenkins & Hatch 1990). However, there are obvious differences in the way the additional demands for ATP are met in the C_4 subtypes. For example, in NADP-ME type species, there is a relatively high demand for ATP in bundle sheath chloroplasts which is provided by cyclic photophosphorylation. In this case, the C_4 cycle primarily shuttles malate to bundle sheath cells and Pyr to mesophyll cells. Malate decarboxylation via NADP-ME donates both CO_2 and reductive power to bundle sheath chloroplasts. The primary photochemical function of bundle sheath chloroplasts is generation of ATP by cyclic photosphosphorylation for CO_2 fixation in the C_3 pathway. Further studies on the effect of O_2 on photochemistry and metabolite pools (both *in vivo* and with isolated cells) will be required to elucidate the mechanism of

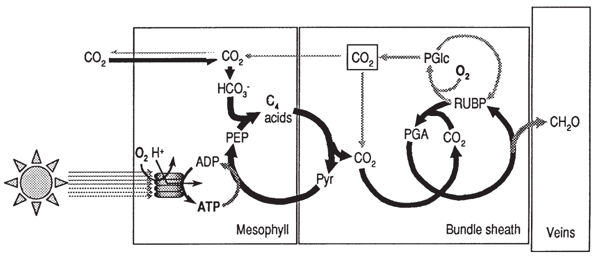

Figure 3. Schematic integration of the O_2 effects on C_4 photosynthesis. NAD-ME type C_4 plants like *A. edulis* are proposed to make use of O_2 to effectively supply the extra ATP required by the C_4 cycle in mesophyll chloroplasts to concentrate CO_2 in the bundle sheath cells and to reduce the reaction of O_2 via RuBP oxygenase.

O_2 enhancement of C_4 photosynthesis in different C_4 species.

Effect of 5 versus 21% O_2 on biomass

The effects of growth under 5 versus 21% O_2 on accumulated biomass of *F. pringlei versus A. edulis* were evaluated (Fig. 4). In *F. pringlei*, the biomass of the low-O_2 grown plants was 40% higher compared to that of the high-O_2 grown plants ($P < 0\cdot01$), which can be interpreted as low O_2 enhancement of growth in this C_3 species by restriction of photorespiration. In contrast, an opposite response was observed in *A. edulis*. Growth under 21% O_2 was enhanced by about 30% compared with that at 5% O_2 ($P = 0\cdot01$). In a study with another Amaranth species, *A. paniculatus*, growth under 21% O_2 was stimulated up to 75% compared with that under 4% O_2 (Knacker & Schaub 1982); although, the C_4 species *Panicum miliaceum* (another NAD-ME type species) had similar vegetative growth under 10, 21 and 40% O_2 (Quebedeaux & Chollet 1977). In *Sorghum bicolor* (NADP-ME type) there was no apparent effect of O_2 on vegetative growth over a range of 5 to 21%, although there was considerable variation between individual treatments (Quebedeaux & Hardy 1973). Enhancement of growth, or lack of inhibition of growth, of C_4 plants by O_2 levels above 5 or 10% may, in part, be the result of O_2 having a positive effect on the C_4 cycle.

The negative effect of O_2 on photosynthesis and growth in the C_3 *F. pringlei* is in contrast to the positive effect of O_2 on photosynthesis and growth in *A. edulis*. However, in *A. edulis* the maximum rates of photosynthesis were obtained at 5 to 10% O_2, whereas growth under 21% O_2 was higher than under 5% O_2. The measurements on photosynthesis were made on individual mature leaves over the course of a few hours, whereas the long-term exposure to different O_2 levels during growth included whole plants and leaves at different stages of development. Thus, the optimum level of O_2 for enhancement of photosynthesis during growth of whole plants of *A. edulis* may be different than that under short-term experiments with individual leaves. Alternatively, there may be some other positive effects of O_2 on growth which are not linked to photosynthesis, as previously reported for reproductive growth (Quebedeaux & Hardy 1973). Even the O_2-dependent rise in PEP might have consequences other than in the C_4 cycle. PEP is also a substrate for the shikimic acid pathway, leading to the formation of secondary and defence compounds that may render these plants more resistant to environmental stresses and thus improve growth (Weaver & Hermman 1997).

In conclusion, in this study we show that while increasing O_2 results in some photorespiration in the C_4 species *A. edulis*, it is also required for maximizing the function of its C_4 cycle. C_4 plants evolved in response to O_2-dependent losses through photorespiration, which was a selection pressure (a function of the concentration of CO_2 and O_2 in the atmosphere, the temperature-dependent CO_2/O_2 solubility ratio and Rubisco kinetic properties). The positive effect of O_2, which is suggested to improve C_4 cycle function, may contribute to the ability of C_4 plants to grow without O_2 inhibition, or even with O_2 enhancement, under current, ambient levels of O_2.

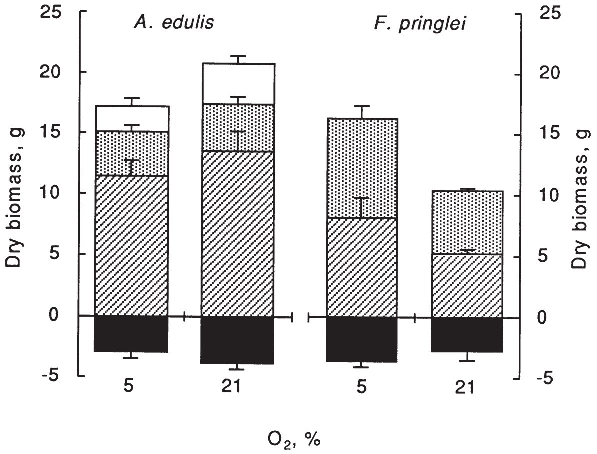

Figure 4. Accumulated dry biomass in *A. edulis* (C_4) and *F. pringlei* (C_3) grown at 5 and 21% O_2. ■, roots; ▨, stems; ⊡, leaves; □, inflorescences. Error bars are the SE of the means.

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