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RESEARCH PAPER

Evidence for the presence of photorespiration in desiccation-sensitive leaves of the C₄ ‘resurrection’ plant *Sporobolus stapfianus* during dehydration stress

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Abstract

The possible role of photorespiration as a general stress protection mechanism, and in C₄ plant metabolism, is controversial. In particular, the potential involvement of photorespiration in the acquisition of desiccation tolerance in ‘resurrection’ plants is unknown. An investigation was carried out into whether photorespiration is present in leaves of the C₄ resurrection plant *Sporobolus stapfianus* Gandoger (Poaceae) and whether it functions as a mechanism of stress resistance in the desiccation-tolerant younger leaves (YL) of this plant. It is shown that the enzymes involved in the photorespiratory pathway maintain their activity until 88% relative water content (RWC) in both YL and desiccation-sensitive older leaves (OL). In subsequent stages of dehydration stress, the enzymatic activity declined similarly in both YL and OL. The content of the photorespiratory metabolite, serine, and ethanolamine, a direct product of serine decarboxylation, is higher in the early stages of dehydration (88% RWC) in OL, suggesting a transiently enhanced photorespiratory activity in these leaves. This was confirmed by simultaneous gas exchange and fluorescence measurements, showing suppression of the electron

transport rate in OL exposed to non-photorespiratory conditions (2% O₂) at 85% RWC. It is concluded that a higher photorespiratory electron transport occurs in desiccation-sensitive OL, and it is therefore proposed that the capacity to scavenge excess electrons through photorespiration does not contribute to protect leaves of the desiccation-tolerant YL of *S. stapfianus* during the stress.

Key words: Ethanolamine, glycine, photorespiratory enzymes, photosynthesis, poikilohydric plant, serine.

Introduction

The term photorespiration describes the light-dependent evolution of CO₂ due to the oxygenase activity of the enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco, EC 4.1.1.39). Photorespiration involves three different organelles: peroxisomes mitochondria, and chloroplasts (Fig. 1). The pathway is essential to avoid the rapid depletion of leaf carbohydrates since the first essential role of photorespiration in plant metabolism is the recovery of phosphoglycollate released by the Rubisco oxygenase reaction (Berry *et al.*, 1978).

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Abbreviations: Ci, intercellular CO₂ concentration; DR, dark respiration; GK, glycerate kinase; GOX, glycollate oxidase; GR-1, glyoxylate reductase; HPR-1, hydroxypyruvate reductase I; HPR-2, hydroxypyruvate reductase II; J_l, linear electron transport through PSII; J_o, alternative electron transport rate; LSU, large subunit; OL, older leaves; PCK, phosphoenolpyruvate carboxylase; PEPC, phosphoenolpyruvate carboxylase; Pn, net photosynthetic rate; Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase; RWC, relative water content; YL, younger leaves.

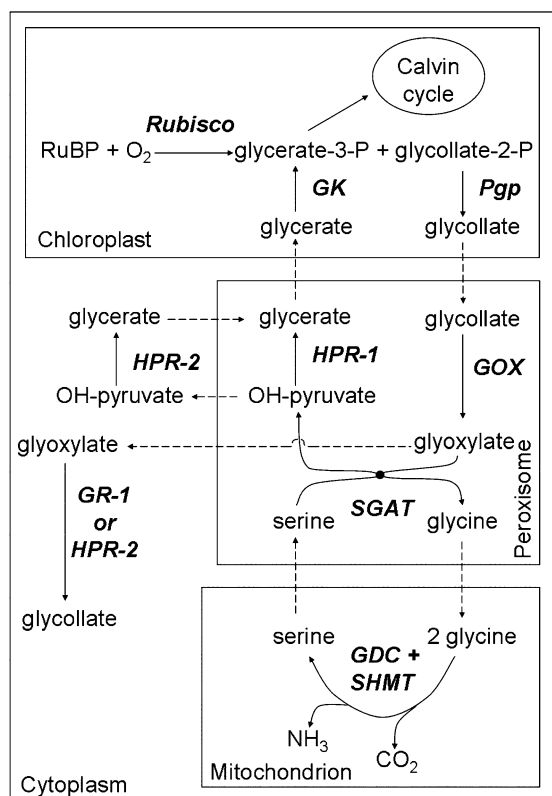


Fig. 1. Schematic diagram of the photorespiratory pathway. RuBP, ribulose-bisphosphate; GK, glycerate kinase (EC 2.7.1.31); Pgp, phosphoglycollate phosphatase (EC 3.1.3.18); HPR-1, hydroxypyruvate reductase I (EC 1.1.1.29); HPR-2, hydroxypyruvate reductase II (EC 1.1.1.81); GOX, glycollate oxidase (EC 1.1.3.15); SGAT, serine:glyoxylate aminotransferase (EC 2.6.1.45); GR-1, glyoxylate reductase I (EC 1.1.1.79); GDC, glycine decarboxylase (EC 1.4.4.2); SHMT, serine hydroxymethyltransferase (EC 2.1.2.1).

Despite the pivotal importance of phosphoglycollate ‘recycling’, photorespiration does not appear to be solely an energy-consuming process that plants use to cope with the oxygenase activity of Rubisco. Many authors have suggested different roles for the photorespiratory cycle other than carbon recovery. Tobacco plants grown for a long time in low O₂ to suppress photorespiration show decreased photosynthetic rates, reduced plant growth, and an alteration in chloroplast structure (Migge *et al.*, 1999). It has also been suggested that photorespiration is important for the biosynthesis of important metabolites such as serine and glycine (Madore and Grodzinski, 1984). Many studies have shown that photorespiration can be important for energy dissipation to prevent photo-inhibition (Osmond, 1981; Wu *et al.*, 1991; Osmond and Grace, 1995; Kozaki and Takeba, 1996; Osmond *et al.*, 1997; Igamberdiev *et al.*, 2001).

The oxygenase activity of Rubisco, and subsequently the magnitude of the photorespiratory flux, changes according to environmental conditions. The rate of photorespiration is dependent on the exposure of Rubisco to oxygen partial pressures. With increasing temperature,

both the specificity of Rubisco for CO₂ (Brooks and Farquhar, 1985) and the solubility of CO₂ decrease, resulting in higher O₂ partial pressures in the leaf and enhanced rates of photorespiration. Rising temperature and concurrent drought stress also induce stomatal closure, which may in turn result in a positive feedback on Rubisco oxygenase activity and photorespiration. First, stomatal closure reduces latent heat flux dissipation by leaves through transpiration, thereby indirectly leading to a further leaf temperature increase, and to a higher O₂/CO₂ ratio in the mesophyll. Secondly, stomatal closure reduces the intercellular CO₂ concentration (C_i). Despite the high Rubisco affinity for CO₂, a low C_i may dramatically limit carboxylation (Flexas *et al.*, 2004), in turn contributing to an increase in the oxygenase activity of Rubisco.

In drought-stressed leaves, the reduction of CO₂ assimilation driven by diffusive and metabolic constraints (Flexas *et al.*, 2004) reduces the electron consumption by photosynthesis. Photorespiration enhancement may, at least partially, use the excess of electrons generated by light capture through photochemical reactions. A direct involvement of the photorespiratory pathway, acting as an electron sink for the protection of the photosynthetic apparatus from electron induced photo-damage, has been shown in drought-stressed C₃ plants (Wingler *et al.*, 1999; Guan *et al.*, 2004).

Due to their ability to concentrate CO₂, plants with C₄ metabolism are assumed to have negligible photorespiratory activity (Edwards and Walker, 1983; Hatch, 1987; Dai *et al.*, 1993). However, C₄ plants possess a complete set of photorespiratory enzymes (Rehfeld *et al.*, 1970), and many studies have shown that the magnitude of photorespiration has been grossly underestimated in C₄ plants (Lawlor and Fock, 1978; Farineau *et al.*, 1984; De Veau *et al.*, 1989; Dai *et al.*, 1995; Lacuesta *et al.*, 1997; Kiirats *et al.*, 2002). In C₄ plants, photorespiration is also enhanced by both low C_i levels (Dai *et al.*, 1995) and drought stress (Lawlor and Fock, 1978).

Because work on photorespiration is often hampered by difficulties in quantifying the rate of photorespiration (Wingler *et al.*, 1999), the role of this pathway in the protection of C₃ and C₄ leaves against photo-damage is controversial (Brestic *et al.*, 1995; Loreto *et al.*, 1995; Laisk and Edwards, 1998; Wingler *et al.*, 1999). The present study investigates the role of photorespiration during dehydration stress in *Sporobolus stapfianus*, a ‘resurrection’ plant with C₄ phosphoenolpyruvate carboxykinase (PCK) anatomy (Dalla Vecchia *et al.*, 1998). Resurrection plants are known to possess vegetative tissues that can tolerate almost complete dehydration (Gaff, 1971). In angiosperms, desiccation tolerance is acquired during dehydration and is the result of morphological and physiological responses to dehydration stress (Oliver *et al.*, 1998; Farrant, 2000; Vicre *et al.*, 2004). The potential involvement of photorespiration as a protective

pathway, able to prevent photo-damage during dehydration, has never been investigated in resurrection plants. In *S. stapfianus* the majority of older leaves (OL) do not acquire desiccation tolerance during dehydration stress, whereas younger leaves (YL) are desiccation tolerant (Martinelli *et al.*, 2007). Therefore, using *S. stapfianus*, it is possible to compare the response to dehydration stress in genetically identical desiccation-sensitive and desiccation-tolerant leaf material. The comparison of photorespiratory activity between old, desiccation-sensitive, and young, desiccation-tolerant leaves provides a suitable means to investigate the role of photorespiration in the acquisition of desiccation tolerance.

To assess whether photorespiration is up-regulated as a protective mechanism during dehydration, the present investigation analysed in both YL and OL: (i) the *in vitro* enzymatic activity of key enzymes involved in the photorespiratory pathway; (ii) the content of amino acids known to be biological markers for enhanced photorespiratory flux (Foyer *et al.*, 2003); and (iii) the *in vivo* photorespiratory rate by simultaneous measurement of linear electron transport through PSII (J_p) and net photosynthetic rate (Pn) under different O₂ conditions (Di Marco *et al.*, 1994; Loreto *et al.*, 1995).

Materials and methods

Plant material and growing conditions

Plants of *S. stapfianus* were grown in pots and were maintained in a greenhouse according to Martinelli *et al.* (2007). Dehydration stress was imposed by withholding water for 12 d under the same conditions as described in Martinelli *et al.* (2007).

In *S. stapfianus*, each tiller is usually composed of four fully expanded leaves. Within each tiller, the innermost leaf is the youngest and the outermost is the oldest. In the applied growing conditions, the age difference between the outer and inner leaf is on average 4 months. The inner YL and the outer OL were used for the present experiments. Sampling was carried out following the procedure outlined by Martinelli *et al.* (2007). At each sampling point during dehydration stress, four leaf samples were utilized for the determination of leaf relative water content (RWC). The RWC was calculated according to the formula: $RWC = (\text{fresh weight} - \text{dry weight}) / (\text{full turgor weight} - \text{dry weight})$. Full turgor weight was determined following a 24 h incubation in the dark of the tissues in flasks containing distilled water. Dry weight was determined after oven drying at 80 °C for 2 d.

At the end of the dehydration stress, after 48 h rehydration, leaf viability was visually evaluated. Leaves were considered non-desiccation tolerant if they were not able to recover turgor and chlorophyll content. The sampling utilized for the following set of measurements was the same as utilized by Martinelli *et al.* (2007), i.e. YL 100% leaf viability; OL 35% viability.

Gas exchange and fluorescence measurements were performed on YL and OL during a separate dehydration experiment.

Gel electrophoresis and protein blot analysis

Proteins were extracted in a solution containing 25 mM phosphate buffer (pH 7.5), 14 mM β-mercaptoethanol, 1 mM dithiothreitol, and 5 mM EDTA. Proteins were separated using SDS-PAGE

(Laemmli, 1970). Equal amounts of protein (10 μg) were added to each lane. As a positive standard, 10 μg of protein from a leaf extract of *Arabidopsis thaliana* (L.) Heynh. was added to the last lane of each gel. The percentage of polyacrylamide in the running gel was 8%. Denatured proteins were stained with Coomassie blue. The analysis has been performed twice with similar results.

For phosphoenolpyruvate carboxylase (PEPC; EC 4.1.1.31) protein blots, proteins were extracted and separated as previously described for Rubisco. As a standard, 10 μg of protein from a mixture of different leaves (dry and fresh leaves) of *S. stapfianus* was added in the last lane of each gel. The standard was necessary to make possible the comparison between different gels. The percentage of polyacrylamide in the running gel was 8%. Denatured proteins were electrophoretically transferred to nitrocellulose membranes. Polypeptide detection was performed using a polyclonal antiserum raised in rabbit against a synthetic peptide encompassing the N-terminal domain of sorghum C₄ PEPC (Pacquit *et al.*, 1995). After incubation with the secondary antibody, conjugated with peroxidase, immunodetection was performed. Both proteins of the Coomassie blue gel and the PEPC protein blot were quantified using the ImageJ 1.34s application.

Enzyme assays

Enzymes were extracted from frozen leaf material stored at –80 °C. The extraction procedure was performed at 4 °C. The extraction was performed with a solution of 50 mM HEPES (pH 7.5), 2 mM MgCl₂, 0.5 mM EDTA, 2 mM dithiothreitol, and 0.1% (v/v) Triton X-100. Cold extraction buffer was added to the ground leaf tissue in a mass to volume relationship of 4 mg DW ml⁻¹. After extraction, 2 ml of each crude extract was desalted using 2.5 ml Sephadex G-25 columns (particle size 50–150 μm) equilibrated with cold extraction buffer. The activities of glycolate oxidase (GOX; EC 1.1.3.15), hydroxypyruvate reductase I (HPR-1; EC 1.1.1.29), hydroxypyruvate reductase II (HPR-2; EC 1.1.1.81), glyoxylate substrate, HPR-2 hydroxypyruvate substrate, and glyoxylate reductase (GR-1; EC 1.1.1.79) were analysed according to Igamberdiev and Kleczkowski (2000). However, for the HPR-2 hydroxypyruvate substrate assay, the substrate concentration in the reaction mixture was increased to 0.4 mM, whereas for the HPR-2 glyoxylate substrate assay, the substrate concentration was increased to 2.4 mM. In the GR-1 assay, the substrate concentration was decreased to 0.2 mM. These adjustments were necessary to optimize enzyme activity in *S. stapfianus*. The activity of glycerate kinase (GK; EC 2.7.1.31) was analysed according to Kleczkowski and Randall (1985). All the enzyme assays were performed at 30 °C.

Soluble proteins were measured according to Bradford (1976) using bovine serum albumin as a standard.

Amino acid analysis

Amino acids and ethanolamine were determined after extraction in a 2% (w/v) solution of 5-sulphosalicylic acid (30 mg DW tissue ml⁻¹ extraction buffer). The individual amino acid composition was determined by ion-exchange chromatography using the Amino Tac JLC-500/V amino acid analyser (ninhydrin coloration), according to the instructions of the manufacturer [JEOL (Europe), Croissy sur Seine, France]. Total amino acid content was calculated by summing the 20 protein amino acids.

CO₂/H₂O gas exchange and fluorescence measurements

Gas exchange and fluorescence measurements were performed simultaneously on the same leaves at 97% and 85% RWC. Further dehydration resulted in a too severe depletion of photosynthesis, which did not permit accurate measurements under different CO₂ and O₂ conditions. Groups of 6–9 leaves (previously selected

according to leaf age, OL and YL) were used. Leaves were enclosed side by side in a gas exchange cuvette. A 2.5 cm portion of each leaf was analysed. The gas exchange system allowed the control of the following parameters in the cuvette: O₂ and CO₂ concentrations, incident light intensity, air temperature, and relative humidity. Leaves were exposed to a 300 ml min⁻¹ flow of mixed N₂, O₂, and CO₂, changing the CO₂ concentration from ambient (380 ppm) to zero, and the O₂ concentration from 20% to 2% with mass flow controllers. All measurements were made by maintaining leaf temperature at 35 °C and exposing leaves at a photosynthetic flux density of 1000 μmol photons m⁻² s⁻¹. The gas flow into the cuvette was humidified by bubbling it in water and subsequently condensing part of the humidity in a water bath at a lower temperature. A low relative humidity was set (30%) to achieve experimental conditions consistent with the growth conditions. CO₂ and H₂O gas exchange was measured with a differential infrared gas analyser (Li 6262; Li-Cor, Lincoln, NE, USA) and photosynthesis was calculated using the formulations of von Caemmerer and Farquhar (1981). All gas exchange measurements were performed 30 min after reaching a steady-state CO₂ and H₂O exchange. Fluorescence measurements were carried out simultaneously with gas exchange measurements with a polyfurcated optic fibre of a modulated fluorimeter (PAM 101, Walz, Effeltrich, Germany) inserted in the leaf cuvette. A complete description of the gas exchange fluorescence system is given in earlier work (Loreto *et al.*, 1995; Laisk and Loreto, 1996). Leaf fluorescence was measured in the dark and under actinic light (1000 μmol photons m⁻² s⁻¹) and saturating light pulses (≥10 000 μmol photons m⁻² s⁻¹ for 2 s), to calculate the quantum yield of photosystem II ($\Delta F/F_m'$) (Genty *et al.*, 1989). This parameter was then multiplied by the absorbed light intensity and by a coefficient to take into account the electron transport partitioning between the two photosystems, as shown by Laisk and Loreto (1996), to calculate the J_f under photorespiratory (20% O₂) and non-photorespiratory (2% O₂) conditions, and the alternative electron transport rate (J_{f0}) when both photosynthesis and photorespiration were suppressed (2% O₂, no CO₂).

Statistical analysis

Differences between two separate means were analysed by the ANOVA test. Values expressed as a percentage were transformed (arcsine $\sqrt{\text{percentage}}$) before the statistical analysis.

Results

Total soluble protein, Rubisco, and phosphoenolpyruvate carboxylase protein content

At the beginning of water stress, the total soluble protein content was similar in YL and OL (Fig. 2). Between 97% and 88% RWC, the decrease observed in YL (~20%) was smaller than in OL (~50%). This 30% difference between OL and YL was maintained throughout the dehydration stress.

In C₄ plants, both photosynthesis and photorespiration depend on the co-ordinated activity of Rubisco in the bundle sheath and of PEPC in mesophyll cells, respectively. The Rubisco large subunit (LSU) protein was localized at ~50 kDa, and showed the same molecular weight as that of the *A. thaliana* Rubisco LSU protein. During the entire stress period, the desiccation-sensitive

OL contained higher Rubisco contents than the desiccation-tolerant YL (Fig. 3). In both OL and YL, a decrease in Rubisco content was observed during water stress.

PEPC content also decreased during the dehydration stress in both YL and OL. In contrast to Rubisco, PEPC content was not different between YL and OL during dehydration stress (Fig. 3).

Activity of enzymes involved in photorespiratory metabolism

To evaluate if photorespiratory flux may be up- or down-regulated during dehydration stress, the activities of key enzymes (Fig. 1) involved in photorespiratory metabolism were measured.

GOX is the first peroxisomal enzyme involved in the photorespiratory pathway. There was no significant

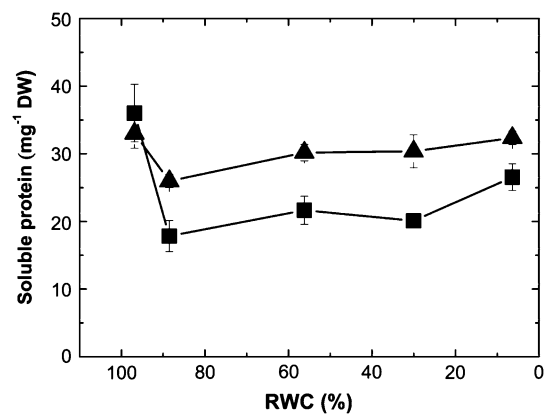


Fig. 2. Soluble protein content in YL (filled triangles) and OL (filled squares) during dehydration stress. Each point is the mean of three samples \pm SE. Error bars are shown when larger than the symbols.

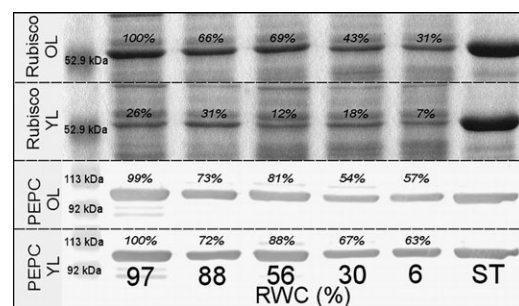


Fig. 3. Coomassie blue-stained gels showing Rubisco LSU content (~50 kDa) and PEPC western blot during desiccation stress in OL and YL; ST, positive standard performed with *A. thaliana* protein extract (Rubisco) or with a mixture of different leaves (dry and fresh leaves) of *S. stappianus* (PEPC). An equal amount of total soluble proteins (10 μg) was loaded per lane. Each protein extract was obtained from three pooled leaf samples. Numbers in italics represent the protein content expressed as a percentage of the highest value obtained, i.e. Rubisco content of OL at 97% RWC and PEPC content of YL at 97% RWC. Proteins were quantified using the ImageJ 1.34s application. In the first line of each gel, a low range pre-stained SDS-PAGE standard (from Bio-Rad) was loaded as a molecular weight marker.

difference in GOX activity between OL and YL during dehydration stress (Fig. 4A). GOX activity was maintained until 88% RWC, and an ~40% decrease was observed between 88% and 56% RWC. After this point, the activity remained unchanged until the end of dehydration stress.

HPR-1 is the last peroxisomal enzyme directly involved in the photorespiratory cycle. At the beginning of water stress there were no differences in HPR-1 activity between OL and YL, and the activity was maintained until 88% RWC (Fig. 4B). Thereafter, an ~45% decrease was observed between 88% and 56% RWC, and an additional 20% decrease between 56% and 30% RWC.

GK is the chloroplastic enzyme that allows the reintroduction of photorespiratory carbon into the Calvin cycle. GK activity was similar (curve trend and activity level) for OL and YL (Fig. 4C). GK activity was maintained until 88% RWC; thereafter an ~45% decrease was observed between 88% and 56% RWC.

Under conditions leading to increased synthesis of photorespiratory glyoxylate, the cytosolic enzymes HPR-2 and GR-1 can operate as auxiliary enzymes facilitating

glyoxylate detoxification (Fig. 1; Igamberdiev and Kleczkowski, 1997). The changes in auxiliary HPR-2 and GR-1 enzyme activities during dehydration stress in *S. stapfianus* were therefore analysed. No major differences in the GR-1 and HPR-2 activities were detected between YL and OL. GR-1 activity steadily decreased during dehydration stress from 88% RWC (Fig. 4D), whereas only a slight decrease in HPR-2 activity was observed (Fig. 4E). Conversely, HPR-2 can also catalyse the reaction from hydroxypyruvate to glycerate in the cytosol (Fig. 1). HPR-2 could be important for the recovery of hydroxypyruvate leakage from the peroxisome (Igamberdiev and Kleczkowski, 2000). The activity of HPR-2 was similar, irrespective of whether hydroxypyruvate or glyoxylate was used as a substrate (levels and curve trend, Fig. 4E, F).

Glycine, serine, aspartate, alanine, and ethanolamine determination and total free amino acid contents

Glycine, serine, alanine, and aspartate have been described as biological markers for photorespiration (Novitskaya *et al.*, 2002; Foyer *et al.*, 2003) and were

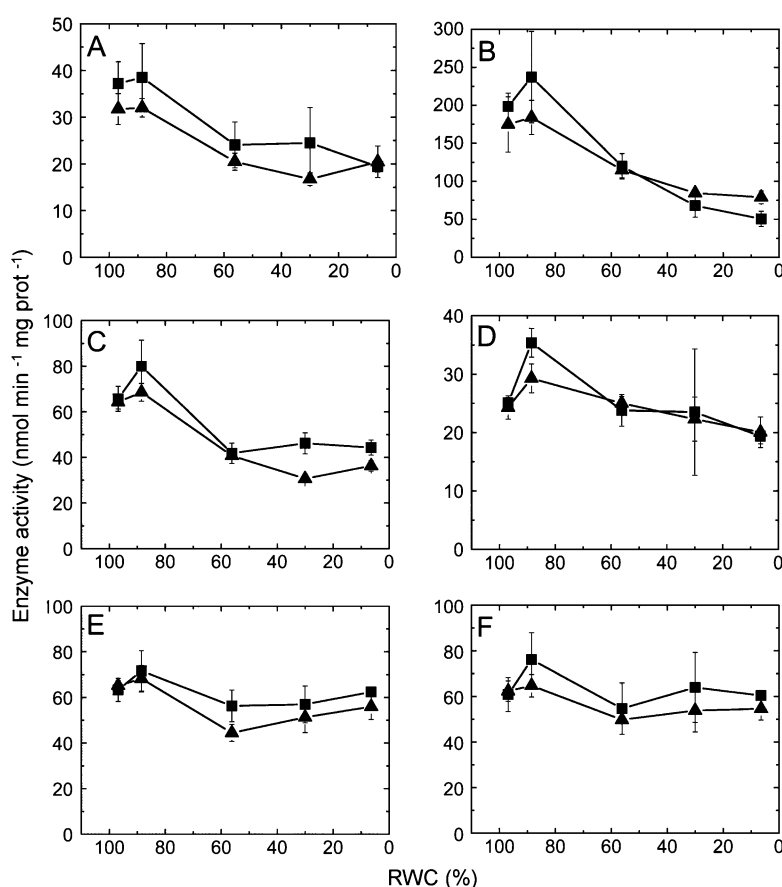


Fig. 4. Activities of different photorespiratory enzymes in YL (filled triangles) and OL (filled squares) during dehydration stress. (A) GOX; (B) HPR-1; (C) GK; (D) GR-1; (E) HPR-2, glyoxylate substrate; (F) HPR-2, hydroxypyruvate substrate. Each point is the mean of three samples \pm SE. Error bars are shown when larger than the symbols.

therefore analysed. The analysis was initiated with total free amino acid determination. Total free amino acid contents were similar in OL and YL regardless of the extent of dehydration stress (Fig. 5A). Unstressed leaves showed high free amino acid contents ($\sim 70 \mu\text{mol g}^{-1}$ DW). At 88% RWC, an $\sim 50\%$ decrease was observed in both OL and YL. Afterwards, further dehydration led to a large increase in leaf amino acid content. Between 88% and 6% RWC, a significant ($P \leq 0.05$) increase in total amino acid content is measured for both OL and YL (Fig. 5A).

Thereafter, alanine, aspartate, glycine, and serine were analysed individually and the results expressed as a percentage of the total amino acid content. Alanine decreased in both OL and YL between 97% and 30% RWC, and then significantly increased between 30% and 6% RWC to reach $\sim 10\%$ of the total amino acid content (Fig. 5B). Aspartate was slightly but not significantly higher in YL than in OL, and decreased during dehydration stress. At 6% RWC, the difference in content between OL and YL

was significant (Fig. 5C). Glycine was maintained throughout the dehydration stress and did not show any significant difference between OL and YL, except between 97% and 88% RWC when a significant ($P \leq 0.05$) increase of the relative glycine content was observed in OL (Fig. 5D). In OL, relative glycine content increased from $\sim 1\%$ at 97% RWC to $\sim 3.5\%$ at 88% RWC, which corresponded to $0.56 \pm 0.07 \mu\text{mol g}^{-1}$ DW (\pm SE) and $1.22 \pm 0.37 \mu\text{mol g}^{-1}$ DW, respectively. Relative serine content was higher in OL compared with YL in unstressed tissue as well as during the initial period of water stress (97% and 88% RWC, Fig. 5E). In unstressed leaves, serine content was $\sim 4.5\%$ in OL and $\sim 2.5\%$ in YL ($3.00 \pm 0.80 \mu\text{mol g}^{-1}$ DW and $1.95 \pm 0.27 \mu\text{mol g}^{-1}$ DW, respectively). At 88% RWC, serine content constituted $\sim 7\%$ of the total leaf amino acids in OL and $\sim 3\%$ in YL (Fig. 5E). This corresponded to $2.56 \pm 0.58 \mu\text{mol g}^{-1}$ DW (\pm SE) and $1.09 \pm 0.18 \mu\text{mol g}^{-1}$ DW, respectively. With further dehydration, serine content was not different from the levels measured in YL.

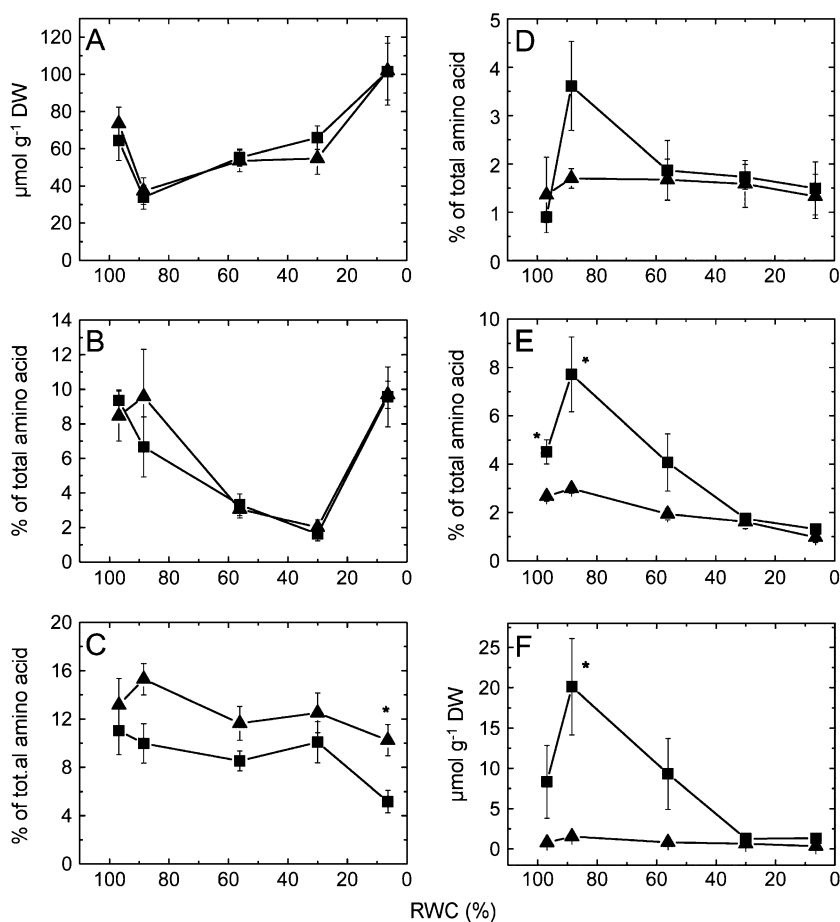


Fig. 5. Total and individual amino acid content in YL (filled triangles) and OL (filled squares) during dehydration stress. (A) Total amino acid content; (B) alanine; (C) aspartate; (D) glycine; (E) serine; (F) ethanolamine content. Values of individual amino acids are expressed as a percentage of total leaf amino acids. Ethanolamine is expressed in $\mu\text{mol g}^{-1}$ DW because it has not been included in the calculation of total amino acid content. Each point is the mean of three samples \pm SE. Error bars are shown when larger than the symbols. An asterisk indicates a significant ($P \leq 0.05$) difference between OL and YL.

Ethanolamine is a direct product of serine decarboxylation and, therefore, ethanolamine content was measured concurrently with individual amino acids. Ethanolamine is significantly higher in OL at 88% RWC, reaching $20 \mu\text{mol g}^{-1}$ DW. This value is 10-fold higher than that measured in YL (Fig. 5F), indicating a significant flux through serine.

Gas exchange and fluorescence measurements

To measure photorespiration *in vivo*, simultaneous measurements of the electron transport rate through chlorophyll fluorescence (J_f) and Pn in photorespiratory (20% O_2) and non-photorespiratory (2% O_2) conditions, at different external CO_2 concentrations (C_e), were performed in OL and YL (Fig. 6). Unstressed OL showed higher photosynthetic activity than unstressed YL under 20% O_2 , 380 ppm

Ce (Fig. 6A, B). In both unstressed YL and OL, the relationship between J_f and Pn did not show significant differences when the O_2 concentration in the cuvette was decreased from 20% to 2% at all the different C_e tested, and no stimulation of photosynthesis at low oxygen was observed (Fig. 6A, B).

At 85% RWC (Fig. 6C, D), OL showed a significant decrease of photosynthetic activity ($\sim 50\%$ at 380 ppm CO_2 ; compare Fig. 6B and D), whereas YL appeared to be less affected by the decrease in RWC (Fig. 6A, C). As in the case of unstressed leaves, photosynthesis was not stimulated at low O_2 . In both unstressed YL and OL, and in partially stressed YL, Pn was compensated by processes releasing CO_2 (respiration and, possibly, photorespiration) at a C_e of ~ 20 ppm (the second data set from the bottom of all panels). However, in partially stressed

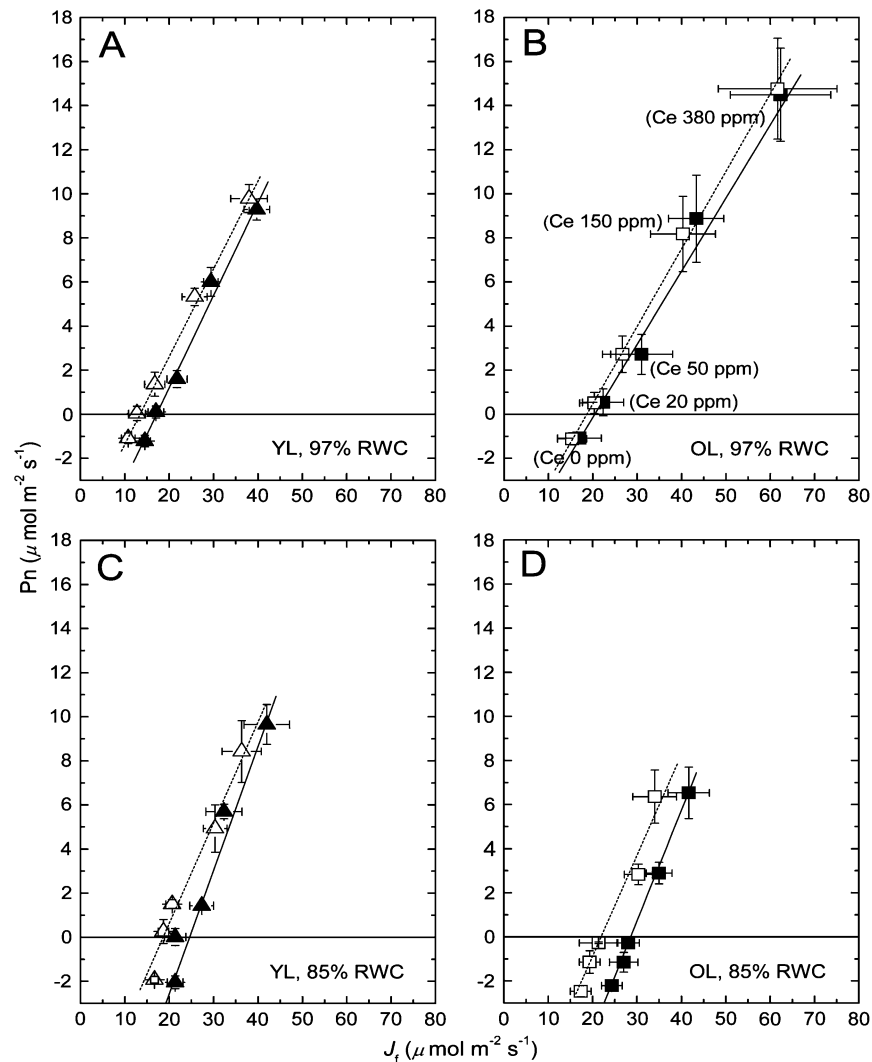


Fig. 6. Photosynthetic assimilation (Pn) as a function of linear electron transport through PSII (J_f). (A) Unstressed (97% RWC) YL; (B) unstressed OL; (C) partially stressed (85% RWC) YL; (D) partially stressed OL. Both gas exchange and fluorescence measurements were performed in 20% O_2 (filled symbols), in 2% O_2 (open symbols), and at different external CO_2 concentrations (C_e , as described in B). A linear fit is shown for each data set. Each point is the mean of three samples \pm SE. Error bars are shown when larger than the symbols.

OL, this compensation point increased up to >50 ppm (the third data set from the bottom of Fig. 6D).

In partially stressed OL, J_f was slightly reduced, when reducing O_2 to 2%, in comparison with that observed at 20%, while P_n was comparable at the two O_2 concentrations (Fig. 6D). This response was observed at all Ce tested and was even more evident at very low CO_2 levels, when P_n was totally inhibited. When pooling together data at different Ce in these plants, J_f values measured at 20% O_2 were significantly higher ($P \leq 0.05$) than those measured at 2% O_2 .

The residual linear electron transport (J_{fo} , the linear electron transport trough PSII measured under 2% O_2 and no CO_2) was 10.7 ± 1.5 (SE) $\mu\text{mol m}^{-2} \text{s}^{-1}$ in unstressed YL, and significantly increased to $16.7 \pm 0.8 \mu\text{mol m}^{-2} \text{s}^{-1}$ in YL leaves at 85% RWC (Fig. 6A, C). On the other hand, J_{fo} did not change significantly in OL exposed to dehydration with respect to unstressed leaves (Fig. 6B, D).

At 97% RWC, dark respiration (DR) was $-1.04 \pm 0.3 \mu\text{mol m}^{-2} \text{s}^{-1}$ and $-1.12 \pm 0.22 \mu\text{mol m}^{-2} \text{s}^{-1}$ in YL and OL, respectively. DR increased during dehydration stress, and at 85% RWC DR was $-2.46 \pm 0.40 \mu\text{mol m}^{-2} \text{s}^{-1}$ and $-2.62 \pm 0.45 \mu\text{mol m}^{-2} \text{s}^{-1}$ in YL and OL, respectively. At both 97% and 85% RWC, DR was not significantly different between YL and OL.

Discussion

In *S. stapfianus*, the majority of OL do not acquire desiccation tolerance during dehydration stress, whereas YL are desiccation tolerant (Martinelli et al., 2007). To address the role of photorespiration as a protective mechanism during dehydration, *in vitro* enzymatic activities involved in the photorespiratory pathway were analysed in OL and YL. Additionally, the contents of the key amino acids known to be biological markers for enhanced photorespiratory flux were determined (Foyer et al., 2003) during the entire dehydration period. In addition, *in vivo* photorespiratory activity was assessed by simultaneous measurements of gas exchange and chlorophyll fluorescence under different O_2 concentrations (Di Marco et al., 1994) during the first phase of dehydration, when metabolic changes were more evident.

In hydrated tissues (97% RWC), the amino acid, soluble protein, metabolite, and enzyme contents and/or activities showed similar results in both YL and OL (with the exception of serine and Rubisco contents, which were higher in OL). The subsequent changes in the above-mentioned metabolic activities, which arose during the course of dehydration stress between OL and YL, may explain the differential tolerance behaviour of OL and YL. Previous studies performed on other (non-resurrection) species have analysed the trends of photorespiratory enzymes under water stress conditions. The two enzymes,

glycine decarboxylase and serine:glyoxylate aminotransferase, involved in the photorespiratory pathway were found to be unaffected by drought stress (Wingler et al., 2000). However, an up-regulation of HPR-1 was found in water-stressed barley leaves (Wingler et al., 1999), thereby suggesting a direct involvement of this enzyme in the stress protection response mechanism. The present results did not show any up-regulation of photorespiratory enzymes during dehydration stress (Fig. 4A–C) in *S. stapfianus*. On the contrary, there was a concomitant decrease of all the enzyme activities involved in the photorespiratory cycle between 88% and 56% RWC, suggesting that the entire pathway is down-regulated at these points of dehydration stress. Under conditions that favour the synthesis of photorespiratory glyoxylate, GR-1 and HPR-2 can operate as auxiliary cytosolic enzymes necessary for glyoxylate detoxification (Igamberdiev and Kleczkowski, 1997). The present study shows that the *in vitro* activities of GR-1 and HPR-2 slightly decreased during dehydration stress in both YL and OL (Fig 4D–F). Since the activities of all the above-mentioned enzyme activities declined during dehydration stress and there were no significant differences between desiccation-sensitive OL and desiccation-tolerant YL, it is concluded that photorespiratory enzymes are not directly involved in the protection mechanisms responsible for desiccation tolerance in YL of *S. stapfianus*.

Although photorespiratory enzyme activities were not different between YL and OL, the analysis of the key amino acids, known to be involved in photorespiratory metabolism, showed major differences between YL and OL during the early phases of dehydration. In particular, the amino acid analysis performed in the present study showed a significant increase of glycine content (as a percentage of total amino acid content) and higher serine content at 88% RWC in OL only. A decrease in alanine content was observed in both OL and YL between 97% and 30% RWC, and OL exhibited lower aspartate levels than YL at the end of dehydration stress. In C_3 plants, serine and glycine are positively correlated with photorespiratory flux, while alanine and aspartate are negatively correlated (Novitskaya et al., 2002; Foyer et al., 2003). Due to the limited number of studies on photorespiratory activity in C_4 plants, a complete set of metabolites useful for recognizing photorespiration has not been described. Despite that, previous research has shown that an increase in serine and glycine is always positively correlated with increased photorespiratory activity in C_4 plants (Lawlor and Fock, 1978; Farineau et al., 1984; De Veau and Burris, 1989). The increase in glycine (as a percentage of total amino acid content) and the higher serine content in OL suggest a higher photorespiratory flux in OL than in YL during the early phases of dehydration (at a RWC of ~88%) when photorespiratory enzyme activities were still high. The increases of

serine and glycine content subsequent to enhanced photorespiratory flux is always associated with an increase in the glycine to serine ratio (Lawlor and Fock, 1978; Farineau *et al.*, 1984; De Veau and Burris, 1989). In contrast to those findings, the present study showed higher levels of serine during the stress period in *S. stapfianus* (Fig. 5D, E). Wingler *et al.* (1999) showed that barley mutants with reduced serine:glyoxylate aminotransferase activity show no increase in the glycine to serine ratio under photorespiratory conditions. High serine levels in *S. stapfianus* can be explained by the low HPR-1 activity measured. Under enhanced photorespiratory conditions, low HPR-1 activity could cause accumulation of hydroxypyruvate and subsequently higher serine in OL, resulting in an altered glycine to serine ratio. While the measured activities of GOX, GK, HPR-2, and GR-1 were in agreement with previously reported results (Usuda and Edwards, 1980; Kleczkowski and Randall, 1986; Givan and Kleczkowski, 1992; Ueno *et al.*, 2005), the measurable HPR-1 activity was not and appeared to be very low. Ueno *et al.* (2005) measured HPR-1 activity in 10 species with C_4 -PCK metabolism. The activity measured in unstressed leaves of *S. stapfianus* was six times lower than the average activity reported for these 10 species. These considerations can in part explain the higher serine levels and the lack of increase in the glycine to serine ratio.

Of interest to serine metabolism was the 10-fold higher ethanolamine content in OL than in YL at 88% RWC (up to $20 \mu\text{mol g}^{-1} \text{DW}$; Fig. 5F). Since ethanolamine is a direct product of serine decarboxylation, the increase in ethanolamine content then suggests that serine biosynthesis is enhanced at the beginning of the stress period in desiccation-sensitive OL, and it is proposed that this is driven by an increase in photorespiratory activity between 97% and 88% RWC, as supported by the fluorescence and gas exchange measurements.

Ethanolamine is an intermediate in the choline biosynthetic pathway. Choline is a precursor of quaternary ammonium compounds in plants. The accumulation of such metabolites has been widely described to be involved in salt and drought stress protection in many plant species (Rhodens and Hanson, 1993). The present results show that ethanolamine accumulated only in desiccation-sensitive OL and that this stimulation of ethanolamine synthesis disappeared at 30% RWC, suggesting an involvement of quaternary ammonium compounds in the desiccation tolerance/sensitivity mechanism. To date, the involvement of quaternary ammonium compounds in the resurrection plant stress response has never been investigated.

Rubisco, the first enzyme involved in the photorespiration mechanism, was observed to be higher in OL than in YL during dehydration stress. This might support the hypothesis of potentially higher photorespiratory activity in OL compared with YL, especially since the photosyn-

thetic rates of these two leaf types were already similar at 85% RWC. In contrast to Rubisco, PEPC protein content did not show differences between YL and OL. Even though *in vitro*, the Rubisco and PEPC activity and activation state have not been measured, there is at least a 2-fold difference in the Rubisco content between OL and YL, suggesting that the PEPC to Rubisco ratio is lower in OL than in YL of *S. stapfianus*. In OL during dehydration stress, Rubisco protein content decreases more rapidly in comparison with PEPC. This results in a slight increase in the PEPC to Rubisco ratio in OL during dehydration stress. Despite this, the ratio is always lower in OL compared with YL. A decreasing PEPC to Rubisco ratio was shown to affect the C_4 cycle efficiency during leaf ageing in *Zea mays* (Crespo *et al.*, 1979) and might be a further indication of a higher photorespiratory activity in OL of *S. stapfianus* during the early phases of dehydration stress. Previous work has reported higher photorespiratory activity in older leaves of C_4 plants. In *Z. mays* and *Amaranthus edulis*, due to an increased permeability of the bundle sheath cells of older leaves (and subsequent exposure of Rubisco to ambient O_2), a higher photorespiratory activity was found (Dai *et al.*, 1995; Kiirats *et al.*, 2002).

An *in vivo* analysis was carried out of whether a measurable rate of photorespiration actually occurs in OL under the early stress conditions in which a stimulation of glycine, serine, and ethanolamine was observed and in which the PEPC/Rubisco ratio was low. Measurements at low O_2 to suppress photorespiration substrate showed no differences in electron transport rate with respect to measurements at ambient O_2 in YL at 85% RWC as well as in unstressed YL and OL. This indicates that oxygenation of Rubisco, which is driven by a remarkable rate of electron transport (Di Marco *et al.*, 1994; Laisk and Loreto, 1996), is generally absent or extremely low in *S. stapfianus*. In contrast, OL measured under early dehydration stress (85% RWC) showed a lower J_f , at comparable P_n , when measured at 2% O_2 rather than at 20% O_2 . This difference was small but was observed throughout the entire range of CO_2 at which it was tested. Comparison between the best fits of the J_f/P_n relationships at different CO_2 under 2% and 20% O_2 confirmed that indeed a certain amount of J_f , which may be attributed to photorespiration requirements, is suppressed under low O_2 in OL undergoing desiccation stress. Differences between measurements at low and ambient O_2 could also be caused by an increase of alternative electron sinks (other than photosynthesis and photorespiration), which is common in drought-stressed leaves (Loreto *et al.*, 1995). However, a small stimulation of alternative electron sinks was only observed in YL at 85% RWC and was therefore unlikely to be contributing to the difference between J_f/P_n rates at 2% and 20% which were attributed to photorespiration in OL at 85% RWC.

The present *in vivo* experiments also showed an ~2-fold increase of the CO₂ at which photosynthesis was compensated by CO₂-releasing processes (compensation point) in OL in the early phase of dehydration with respect to YL. During water stress, the increase in the CO₂ compensation point of photosynthesis is often observed, and may depend on an increase in CO₂ evolution through either photorespiration or DR while Pn is drought and CO₂ limited (Lawlor and Cornic, 2002). When compared with hydrated tissue, an increase in DR was observed in both YL and OL at 85% RWC. However, the rates of DR were not different in YL and OL at 85% RWC, and, therefore, this result cannot explain the different compensation point observed in the two leaf types. Given the measured increase of J_f under photorespiratory conditions (20% O₂), it is proposed that the increase in the compensation point in OL at 85% RWC was contributed by higher photorespiration. Why CO₂ evolution through photorespiration in OL at 85% RWC was not associated with a decrease in Pn at 20% O₂ is not clear. This may be due either to an increase in respiration in the light, which is often different from respiration in the dark (Pinelli and Loreto, 2003), or to increased bundle sheath permeability during water stress in OL. CO₂ concentration in the bundle sheath is derived mainly from the decarboxylation of C₄ acids which is not dependent on O₂ concentration. Thus, a leakage of CO₂ from bundle sheath cells would affect the CO₂ compensation point and the rate of photosynthesis under both photorespiratory and non-photorespiratory conditions.

Conclusions

In summary, numerous experiments indicate that photorespiration occurs in leaves of the C₄ resurrection plant *S. stapfianus* when exposed to early stages of dehydration. The occurrence of photorespiration is shown to be limited to early stages of dehydration in OL. Since OL are more sensitive than YL to dehydration stress, it is concluded that photorespiration is not involved in the acquisition of desiccation tolerance by scavenging excess electrons. In desiccation-sensitive OL, photorespiration may be responsible for the high levels of serine and ethanolamine accumulation, which suggests an incomplete photorespiratory cycle probably due to low HPR-1 activity. The role of serine and ethanolamine accumulation in the quaternary ammonium compound biosynthetic pathway remains to be determined.

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