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Sucrose phosphate synthase activity and the co-ordination of carbon partitioning during sucrose and amino acid accumulation in desiccation-tolerant leaf material of the C$_4$ resurrection plant *Sporobolus stapfianus* during dehydration

Anne Whittaker[1],*, Tommaso Martinelli[1], Jill M. Farrant[2], Adriana Bochicchio[1] and Concetta Vazzana[1]

[1] *Dipartimento di Scienze Agronomiche e Gestione del Territorio Agroforestale; Università di Firenze, Piazzale delle Cascine 18, I-50144 Firenze, Italy*

[2] *Department of Molecular and Cell Biology, University of Cape Town, Private Bag Rondebosch 7700, South Africa*

Abstract

Both sucrose and amino acids accumulate in desiccation-tolerant leaf material of the C$_4$ resurrection plant, *Sporobolus stapfianus* Gandoger (Poaceae). The present investigation was aimed at examining sucrose phosphate synthase (SPS) activity and various metabolic checkpoints involved in the co-ordination of carbon partitioning between these competing pathways during dehydration. In the initial phase of dehydration, photosynthesis and starch content declined to immeasurable levels, whilst significant increases in hexose sugars, sucrose, and amino acids were associated with concomitant significant increases in SPS and pyruvate kinase (PK) activities, and maximal activity levels of phosphoenolpyruvate carboxylase (PEPCase), NADP-dependent isocitrate dehydrogenase (NADP-ICDH), and NADH-dependent glutamate synthase (NADH-GOGAT). The next phase of dehydration was characterized by changes in metabolism coinciding with net hexose sugar phosphorylation. This phase was characterized by a further significant increase in sucrose accumulation, with increased rates of net sucrose accumulation and maximum rates of SPS activity measured under both saturating and limiting (inhibitory) conditions. SPS protein was also increased. The stronger compet-itive edge of SPS for carbon entering glycolysis during hexose phosphorylation was also demonstrated by the further decrease in respiration and the simultaneous, significant decline in both PEPCase and PK activities. A decreased anabolic demand for 2-oxoglutarate (2OG), which remained constant, was shown by the co-ordinated decrease in GOGAT. It is proposed that the further increase in amino acids in this phase of dehydration may be in part attributable to the breakdown of insoluble proteins.

Key words: Amino acids, carbon partitioning, desiccation tolerance, sucrose, SPS.

Introduction

When exposed to water stress, many C$_3$ and C$_4$ plants accumulate low-molecular mass compounds such as sugars, sugar alcohols, quaternary ammonium compounds, and amino acids (Lunn and Furbank, 1999; Lawlor and Cornic, 2002). Vegetative tissues of resurrection plants are able to survive desiccation (Gaff, 1971), and the acquisition of desiccation tolerance is the result of morphological and physiological adaptive responses to water stress (Oliver *et al.*, 1998). The leaf tissues of resurrection plants are shown to accumulate sucrose as the principal sugar

during dehydration (Ghasempour *et al*., 1998). Many resurrection plant species also accumulate amino acids (Gaff and McGregor, 1979) and proline (Tymms and Gaff, 1978). However, sucrose and amino acid synthesis, respectively, are antagonistic competitors for carbon skeletons and energy in source leaves (Lunn and Furbank, 1999).

Sucrose phosphate synthase (SPS; EC 2.4.1.14) plays a pivotal role in sucrose synthesis, utilizing intermediates derived from glycolysis [fructose-6-phosphate (Fru-6-P) and UDP-glucose (UDPGlc)] as substrates, whereas regulation of phosphoenolpyruvate carboxylase (PEPCase; EC 4.1.1.31) and pyruvate kinase (PK; EC 1.2.1.40) integrates glycolysis and nitrogen metabolism through the provision of glycolytic carbon for amino acid biosynthesis (Lunn and Furbank, 1999; Stitt *et al*., 2002; Foyer *et al*., 2003). In the anaplerotic pathway, PEPCase and PK replenish the tricarboxylic acid (TCA) intermediates withdrawn for the synthesis of organic acid precursors, particularly 2-oxoglutarate (2OG) (Stitt *et al*., 2002; Foyer *et al*., 2003). A more definite role for the involvement of both PEPCase and PK in amino acid biosynthesis was reported (Yanagisawa *et al*., 2004), where metabolic engineering with Dof1 transcription factors was shown to stimulate amino acid production by activating organic acid metabolism via the up-regulation of both PEPCase and PK.

2OG, a principal organic acid at the interface of carbon and nitrogen metabolism (Stitt *et al*., 2002; Foyer *et al*., 2003), is derived primarily from the isocitrate dehydrogenases (Lancien *et al*., 2000). Transfer of fixed nitrogen to 2OG during the net synthesis of glutamate is catalysed by GOGAT, operating in a cycle with glutamine synthetase (GS; Ireland and Lea, 1999). Consequently, a shift in carbon partitioning away from sucrose synthesis towards amino acid biosynthesis has been shown to coincide with increases in PEPCase, PK, and NADP-dependent isocitrate dehydrogenase (NADP-ICDH; EC 1.1.1.42) activities, and a simultaneous decline in SPS activity (Champigny, 1995; Scheible *et al*., 1997; Foyer *et al*., 1998; Morcuende *et al*., 1998). The biosynthesis of major nitrogen-containing compounds including amino acids is derived from the primary donor, glutamate (Ireland and Lea, 1999), which in leaf material is produced primarily by chloroplastic Fd-GOGAT, but may also be produced in the plastids of vascular tissues [NADH-dependent glutamate synthase (NADH-GOGAT; EC 1.4.1.14)] predominantly under stress conditions (Lancien *et al*., 2000).

The accumulation of both amino acids and sucrose during water stress in resurrection plants raises the interesting question of how carbon may be partitioned between these two competing pools during dehydration. To date, there are no metabolic studies on the co-ordination of carbon partitioning using desiccation-tolerant leaf material as a model system.

The objective of the present study was to investigate SPS activity and various metabolic checkpoints involved in co-ordinating the partitioning of carbon skeletons between sucrose and amino biosynthesis in dehydrating leaf material of *Sporobolus stapfianus* Gandoger (Poaceae) during the acquisition of desiccation tolerance. The species *S. stapfianus* is a suitable model system. Increased sucrose (Ghasempour *et al*., 1998; Whittaker *et al*., 2001) and soluble non-protein nitrogen (Gaff and McGregor, 1979) have been reported in dehydrated leaf tissues of this particular resurrection plant, which shows C_4 anatomical morphology (Quartacci *et al*., 1997; Dalla Vecchia *et al*., 1998). Preliminary research on *S. stapfianus* showed a simultaneous accumulation of both pools during the course of dehydration, which is in contrast to the response of the most commonly studied C_4 species, maize and sugarcane, which do not accumulate sucrose in leaf material during water stress (Pelleschi *et al*., 1997; Du *et al*., 1998; Foyer *et al*., 1998). Lack of sucrose accumulation in the latter was attributed to a decline in photosynthetic triose phosphate production and a simultaneous decline in SPS activity. In desiccation-tolerant plants, SPS activity has only been measured in the C_3 species *Craterostigma plantagineum* Hochst, where SPS activity increased during dehydration (Ingram *et al*., 1997). Since the increase was not correlated with an increase in SPS transcript levels, it was proposed that increases in activity may also be a reflection of the activation state of the enzyme (Ingram *et al*., 1997), although no studies were performed to validate this proposal. There are relatively few studies on water stress-induced effects on carbon partitioning, particularly in C_4 plants.

Here a comparison between the carbon competing activities of SPS and PEPCase plus PK for carbon in relation to sucrose and amino acid accumulation during the dehydration of desiccation-tolerant leaf material of *S. stapfianus* is reported.

Materials and methods

Plant material and experimental conditions

Plants were maintained in a greenhouse, and dehydrated as described previously (Whittaker *et al*., 2001). Plants were grown on a soil composition of 40% commercial potting mix, 35% sand, and 25% leaf mould. One month prior to the start of the dehydration experiments, plants were supplied with a single treatment of 4 g l^{-1} of NUTRILEAF (M:P:K 9:15:30; by vol.). Dehydration stress was imposed by withholding water. At various stages during the dehydration course, leaf samples were removed from different plants, sampling being between 11.00 am and 12.30 pm. Only a portion of the leaf blade (15–20 cm) from the two innermost, fully expanded leaves in a tiller were used for the metabolite and enzyme analyses. Only the two innermost leaves were used, since the older, outer leaves of the tiller show a reduced capacity to acquire desiccation tolerance during desiccation stress (Martinelli *et al*., 2007). After discarding the leaf base (~3 cm) and the apical regions (beyond the

15–20 cm point), the leaf sections were immediately frozen in liquid nitrogen and stored at –84 °C. At each sampling, the duplicate leaf samples were removed for the determination of leaf relative water content (RWC). The RWC was calculated according to the formula: RWC = (initial weight–dry weight)/(full turgor weight–dry weight). Full turgor weight was determined following a 24 h incubation of the tissues in sealed flasks containing water. Dry mass was determined after oven drying at 80 °C for 2 d. In order to assess whether desiccation tolerance had been acquired during dehydration, plants were watered after dehydration. There was a 98% recovery of the two innermost, fully expanded leaves within 72 h of rehydration, as was reported previously (Martinelli *et al.*, 2007).

Elemental carbon and nitrogen measurements

Leaf material was completely homogenized to form a fine powder and oven-dried at 80 °C for 2 d. Samples were weighed (2.0–2.5 mg) and sealed in specifically designed tin holders for the determination of carbon and nitrogen content using the Flash EA 1112 NC Analyzer, according to the manufacturer's instructions (CE Instruments, Wigan, UK).

Gas exchange

Photosynthesis was measured on attached leaf samples at different RWCs during dehydration using a Ciras-I infrared gas analyser (closed system) with a Parkinson's Broad Leaf Cuvette (PAR 500 μmol m^{-2} s^{-1}, 26 °C, CO_2 350 ppm, and 223 cm^3 min^{-1} flow rate). Since the leaves of *S. stapfianus* are very narrow (~3–4 mm), approximately seven leaves were inserted alongside each other within the cuvette for each measurement made. Samples were light adapted for 20 min, and measurements of CO_2 assimilation made over a further 20 min period. The reason for the long measurement period was to verify the consistency in photosynthetic rate under the applied conditions since leaves of *S. stapfianus* adapt slowly to changing environmental conditions. Given that leaf area shrinks during dehydration stress, CO_2 assimilation was expressed on a dry mass basis. After each measurement, the leaf blades (inserted in the cuvette) were excised and oven-dried at 80 °C for 48 h in order to determine the dry mass. Respiration was measured in the dark as described in Whittaker *et al.* (2004), with the exception that leaf samples were attached to the parent plant.

Carbon isotope discrimination

Desiccated, finely ground tissue, from hydrated leaf material, was subjected to mass spectrometry (Finnigan Delta S, ThermoQuest, Brema, Germany) to determine the $\delta^{13}C$ isotope composition of the leaf material. Isotope discrimination was then calculated as:

$$\Delta\delta^{13}C\text{‰} = (\delta^{13}C \text{ air} - \delta^{13}C \text{ leaf})/(1 + \delta^{13}C \text{ leaf}).$$

The value $\delta^{13}C$ air of –8‰ used was previously shown to be constant over different treatments (Brugnoli and Farquhar, 2000).

Metabolite extraction and measurement

Both metabolite and enzyme measurements were performed on ground material in which all cell types were broken. Soluble sugars and 2OG were extracted using a modified alkaline extraction procedure based on the method of van Schaftigen (1985), as described previously (Whittaker *et al.*, 2001). The sugars, sucrose, glucose, and fructose were determined enzymatically using the Roche sugar food analysis kit (Bergmeyer and Bernt, 1974). 2OG was determined enzymatically at 340 nm using the glutamate dehydrogenase-catalysed reaction as described in Burlina (1984).

The washed pellet was used for starch extraction. Starch was extracted using a modified extraction procedure based on Beutler

(1984). The pellets were incubated under agitation at 95 °C for 1.5 h in 1.5 ml of 1 M KOH. The extracts then were cooled rapidly to room temperature and the pH adjusted to 4.6 by the addition of 1.45 ml of glacial acetic acid and 1.05 ml of 0.75 M acetate buffer (pH 4.6). After vigorous shaking, the extracts were analysed according to Beutler (1984). The percentage recovery of added starch was 94.2%.

Amino acids and proline were extracted twice in 30 mM NaOH in 60% (v/v) ethanol as described in Whittaker *et al.* (2001). For the determination of total amino acids, the extracts were adjusted to pH 4.6, and 1 ml was added to 0.5 ml of 200 mM citrate (pH 4.6) and 1 ml of ninhydrin solution [1% (w/v) ninhydrin in ethylene glycol monomethyl ether and 0.03% (w/v) ascorbic acid] according to Magné and Larher (1992). After boiling for 15 min, 60% (v/v) ethanol (3 ml) was added to each extract and measurements made at 570 nm using leucine as the amino acid reference standard (Magné and Larher, 1992). For the determination of proline, samples were adjusted to pH 7.0 and added to 2 ml of ninhydrin solution [1% (w/v) ninhydrin in 60:40 (v/v) glacial acetic acid:water], according to Magné and Larher (1992). After heating in a boiling waterbath for 1 h, toluene (5 ml) was added to cooled extracts and the two phases allowed to separate. Proline was measured in the upper phase at 520 nm (Magné and Larher, 1992). Recoveries for exogenously added leucine and proline were 83.5% and 84.0%, respectively.

Inorganic Pi and 2OG were extracted according to Ball and ap Rees (1988). 2OG contents were comparable between the Ball and ap Rees (1988) and modified van Schaftigen (1985) extraction protocols. Pi was measured using the colorimetric method of Joyce and Grisolia (1960). Extracts (50 μl) were added to 0.5 M trichloroacetic acid to attain a final volume of 200 μl. After the addition of 100 μl of colour reagent [4 ml of 16% (w/v) ammonium molybdate in 10 N H_2SO_4 added to 2 g (w/v) of $FeSO_4$ in 36 ml of H_2O], samples were measured at 660 nm (Joyce and Grisolia, 1960). Recovery of Pi was 75%.

Enzyme extraction and measurement

The extraction procedure and desalting technique for leaf samples of *S. stapfianus* have been reported previously (Whittaker *et al.*, 2001). Crude extracts were desalted using 2.5 ml Sephadex G-25 columns (particle size 50–150 μm) equilibrated with extraction buffer. Desalted extracts were maintained at 4 °C until assayed for enzyme activity. With the exception of SPS, all enzyme reactions were measured at 340 nm in cuvettes maintained at 30 °C. The SPS reaction was incubated at 30 °C, and measurements (colorimetric assay) made at 620 nm.

SPS was extracted in 200 mM Tricine (pH 7.8), 4 mM $MgCl_2$, 1 mM EDTA, 5% (v/v) glycerol, 0.1% (v/v) Triton X-100, 5 mM dithiothreitol (DTT) and 1 mM phenylmethylsulphonyl fluoride (PMSF), according to a modification of the method of Foyer *et al.* (1998). Sigma protease inhibitor cocktail was added to each sample extract, according to the manufacturer's instructions. After desalting, SPS activity in each sample extract was measured separately under both saturating substrate contents [50 mM HEPES (pH 7.5), 15 mM $MgCl_2$, 2.5 mM DTT, 10 mM UDPGlc, 10 mM Fru-6-P, and 40 mM glucose-6-phosphate (Glc-6-P)] and limiting substrate plus inhibitor conditions (6 mM UDPGlc, 2 mM Fru-6-P, 6 mM Glc-6-P, and 5 mM Pi), as described by Huber *et al.* (1989). Extracts were incubated for 30 min (within the linear phase of the reaction), and the reaction terminated by the addition of 30% KOH before heating at 100 °C for 20 min. After the addition of anthrone reagent, the A_{620} was measured (Huber *et al.*, 1989). For each extract, a duplicate reaction was set up under saturating conditions, but terminated at time zero to subtract residual sucrose not attributable to SPS activity.

PEPCase was extracted in TRIS-HCl (pH 7.8), 10 mM MgCl$_2$, 5 mM NaF, 20% glycerol, 5 mM DTT, and 1 mM PMSF, according to a modification of the method of Foyer *et al.* (1998). Sigma protease inhibitor cocktail was added to each sample extract, according to the manufacturer's instructions. After desalting, total PEPCase activity was measured at pH 7.5 after the addition of PEP, according to Foyer *et al.* (1998). PK and aldolase were extracted and measured as indicated previously (Moorhead and Plaxton, 1988; Whittaker *et al.*, 2004). NADP-ICDH and NADH-GOGAT were extracted in 100 mM Tricine (pH 7.8), 4 mM MgCl$_2$, 1 mM EDTA, 5% (v/v) glycerol, 0.1% (v/v) Triton X-100, 5 mM DTT, 1 mM PMSF, and Sigma protease inhibitor cocktail. NADP-ICDH and NADH-GOGAT were measured according to Sima *et al.* (2001) and Lea *et al.* (1990), respectively. Total extractable nitrate reductase (NR) was measured under the conditions described by Foyer *et al.* (1998).

Gel electrophoresis and protein blot analysis

Proteins extracted in enzyme samples were separated by SDS–PAGE (Laemmli, 1970), on an 8% polyacrylamide running gel. Equal amounts of protein (10 µg) were loaded in each lane. Proteins were transferred by electrophoresis to nitrocellulose membranes and polypeptide detection was performed using polyclonal antiserum raised against SPS (Global anti-SPS; Agrisera, Vännäs, Sweden; dilution 1:250). After incubation with the secondary antibody, conjugated with peroxidase, immunodetection was performed.

Protein extraction and measurement

Proteins were extracted in 50 mM KH$_2$PO$_4$ (pH 7.5) containing 5 mM MgCl$_2$, 5 mM DTT, 1 mM PMSF, and Sigma protease inhibitor cocktail. Extracts were centrifuged for 15 min at 17 200 *g*, and an aliquot of the supernatant retained for soluble protein measurement. The pellet was washed and centrifuged three times with extraction buffer. Acetone containing 0.07% (w/v) mercapto-ethanol was added to the pellet and samples were stored overnight at –20 °C. Thereafter, the sample pellets were washed a further three times with acetone and dried under vacuum. The insoluble proteins were suspended overnight in 300 µl of resolubilization buffer containing 7 M urea, 2 M thiourea, 4 mM CHAPS, and 20 mM DTT, sonicated in an ultrasonic bath for 1 min, and centrifuged for 15 min at 17 200 *g*. Soluble and insoluble protein content was measured according to the method of Bradford (1976) using bovine serum albumin as a standard.

Statistical analysis

Differences between two separate means were analysed by the one-way analysis of variance (ANOVA) test.

Results

The elemental carbon and nitrogen composition of leaf material at 92, 54, and 14% RWC was analysed. The carbon content remained constant (Table 1). There was a slight but significant (F=6,229; P ≤0.05) decline in nitrogen content in the desiccated leaves, which is reflected by a marginal increase in the C/N ratio (Table 1).

Potential carbon sources to primary metabolism were investigated by measuring trends in photosynthesis and starch content during the course of dehydration. Photosynthesis in the hydrated tissues was equivalent to

Table 1. *Elemental carbon and nitrogen contents at various RWC intervals in dehydrating leaf tissue of* Sporobolus stapfianus

Values are the means ±SD of six replicates. Each replicate is representative of 2–3 separate plants.

RWC (%)	Nitrogen (% DM)	Carbon (% DM)	C/N
92	1.95±0.118	44.8±0.54	23.07
54	1.96±0.173	44.8±0.36	22.90
14	1.75±0.047	44.4±0.55	25.35

326.4 nmol^{-1} g^{-1} dry matter (DM), which if expressed on the more conventional area basis is equivalent to 16.48 µmol m^{-2} s^{-1}. Photosynthesis declined by ~60% within the initial 10% drop in RWC (Fig. 1A), and was immeasurable in the more dehydrated tissues, whilst both sucrose and amino acids accumulated, with maximal levels of both being recorded at 34% RWC. Starch content also decreased immediately after the onset of water stress and was negligible by 65% RWC (Fig. 1B). The isotope discrimination value of fully hydrated leaf material of *S. stapfianus* was δ^{13}C = 13.45±0.412‰. Whereas C$_3$ grasses have a δ^{13}C of approximately –28‰, C$_4$ grasses have an average value of –14‰ (Cerling *et al.*, 1997), hence the present result affirmed a distinct C$_4$-type photosynthesis for *S. stapfianus* in support of the C$_4$ anatomical characteristics previously shown for this species (Quartacci *et al.*, 1997; Dalla Vecchia *et al.*, 1998).

The relationship between sugar and amino acid content, together with associated enzyme activity, was then further investigated over a 10 d dehydration period. The rate of dehydration was determined from the leaf RWC percentage over the 10 d time course (Fig. 2A). There was a gradual decline in leaf RWC from day 5 to day 8 (68% RWC), followed by an accelerated sequential leaf RWC loss of 26% and 33% between days 8–9 and 9–10, respectively (Fig. 2A). Sample harvesting was performed daily between 11.00 am and 12.30 pm in order to measure metabolic variation attributable to dehydration stress and not to circadian rhythm. Amino acid content increased significantly between 84% and 75% RWC (F=9.37; P ≤0.05), and a further significant increase (F=8.74; P ≤0.05) was evident between 68% and 9% RWC (Fig. 2B). Proline content (which was measured separately) was shown to be negligible in desiccation-tolerant younger leaves (0.17–0.40 and 0.0–0.015 µmol^{-1} g^{-1} DM in hydrated and dehydrated tissues, respectively) and therefore not added to the total amino content. Sucrose accumulated throughout dehydration (Fig. 2B). Net sucrose accumulation was significant over the sequential RWC interval periods of 84–75% (F=79.40; P ≤0.01), 75–68% (F=52.17; P ≤0.01), and 68–42% (F=54.17; P ≤0.01), respectively (Fig. 2B). Equimolar levels of
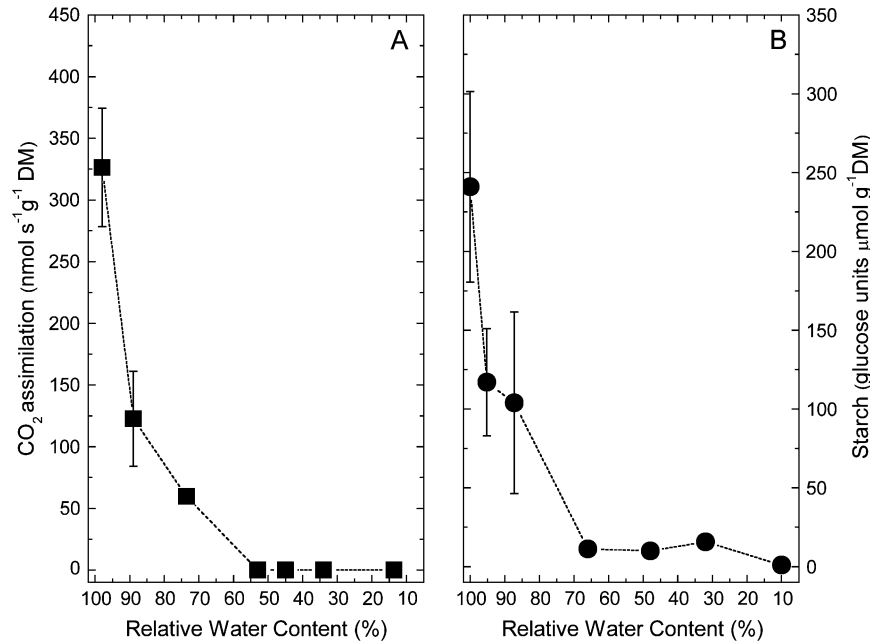
Fig. 1. Effects of dehydration on (A) photosynthetic CO_2 assimilation and (B) starch content in leaf material of *Sporobolus stapfianus*. Values are the means ±SD of three replicates. Each replicate is representative of 2–3 separate plants.

glucose and fructose increased from 84% RWC, peaked at 68% RWC, and then declined (Fig. 2B). The decline in hexose sugar content coincided with the increased drying rate (compare Fig. 2A and B).

SPS activity was then analysed with respect to the rate of net sucrose accumulation (Fig. 3A). The mean rate of sucrose accumulation was calculated for the RWC interval periods of 84–75%, 75–68%, and 68–42%, in which the accumulation was reported to be significant (refer to Fig. 2). Rates of accumulation increased sequentially, attaining a maximum rate in the 68–42% RWC period (Fig. 3A), which in turn coincided with the period demarcating the decline in hexose sugar content (Fig. 2). Total extractable SPS activity, measured under saturating substrate conditions, increased 3-fold between 84% and 68% RWC, and declined after 42% RWC (Fig. 3A). The highest levels of SPS activity corresponded to the maximum rate of net sucrose accumulation. The ratio of maximal catalytic SPS activity to that measured under limiting substrate conditions and in the presence of inhibitor is shown in Fig. 3B. Saturating conditions for substrates in the absence of the inhibitor Pi yield maximum catalytic potential, whereas limiting substrate and activator conditions coupled with Pi de-activate/phosphorylate the enzyme (Huber and Huber, 1992). SPS activity displayed decreasing sensitivity to inhibitory conditions over the course of dehydration (Fig. 3B). SPS activity, when measured under inhibitory conditions, was shown to exceed the corresponding rates of net sucrose accumulation by ~45-, 50-, and 35-fold for the RWC interval periods of 84–75%, 75–68%, and

68–42%, respectively. Although the *in vivo* inhibitory effect of inorganic Pi content on cytosolic SPS activity is not quantifiable due to a lack of knowledge of the intracellular distribution, total *in vivo* Pi content was, however, measured (Fig. 3B). Inorganic Pi increased significantly ($F=59.82$; $P \leqslant 0.01$) from 84% to 75% RWC, and subsequently declined, though levels from 68% and 9% were not shown to be significantly different (Fig. 3B).

Since both desiccation respiration and organic acid production via the anapleurotic pathway compete with sucrose biosynthesis for carbon skeletons entering primary metabolism, the trend in dark respiration along with key enzymes involved in carbon partitioning to 2OG bio-synthesis were examined. Respiration in the hydrated tissues was equivalent to 41.3 nmol s^{-1} g^{-1} DM (Table 2), which if expressed on the more conventional area basis is equivalent to 2.08 μmol m^{-2} s^{-1}, which is equivalent to that measured in the resurrection plants *Eragrostis nindensis* (vander Willigen *et al.*, 2001) and *Xerophyta scabrida* (Tuba *et al.*, 1997). Dark respiration declined by ~35% within the initial 10% drop in RWC (Table 2), and thereafter showed a steady decline over the remaining course of dehydration. 2OG, at the interface of C and N metabolism, is suggested to reflect C/N status (Lancien *et al.*, 2000) and was therefore measured. Levels were unchanged over the course of dehydration (hydrated tissues, 0.83±0.15 μmol g^{-1} DM; 75% RWC, 0.91±0.22 μmol g^{-1} DM; 68% RWC, 0.93±0.08 μmol g^{-1} DM; 42% RWC, 0.89±0.01 μmol g^{-1} DM; and 9% RWC, 0.83±0.34 μmol g^{-1} DM).
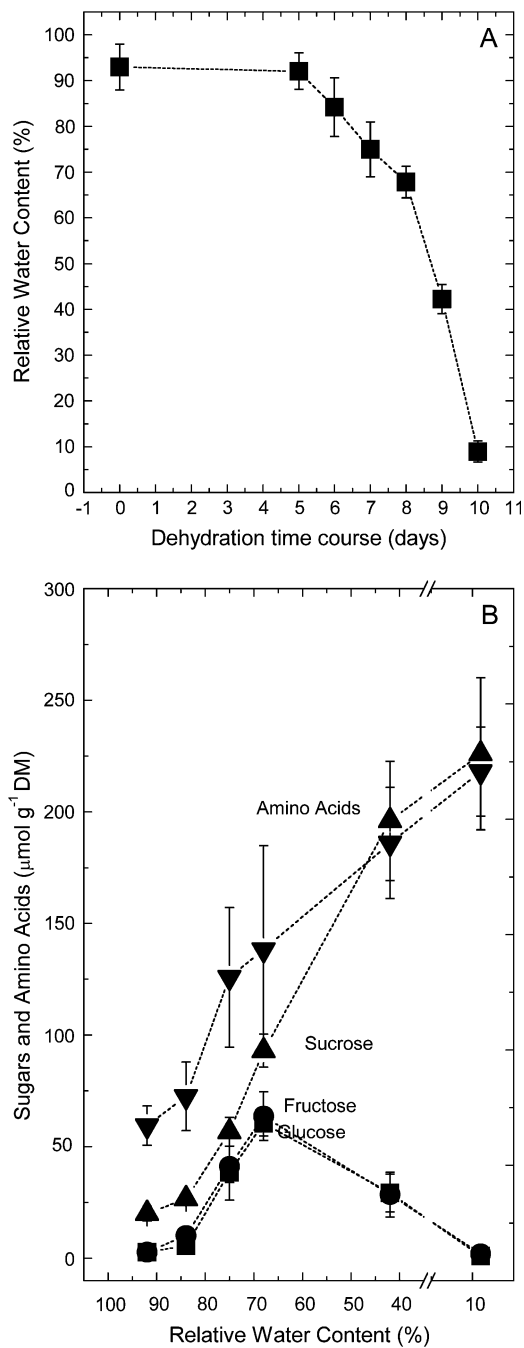
Fig. 2. (A) Relative water content of leaf material in relation to the time. course of dehydration and (B) sucrose (upright triangles), amino acids (inverted triangles), glucose (squares), and fructose (circles) content in leaf material of *Sporobolus stapfianus* at different RWCs over the 10 d dehydration period. Values are the means ±SD of 3–6 replicates. Each replicate is representative of 2–3 separate plants.

PK and PEPCase, regulatory enzymes in partitioning carbon towards anapleurotic organic acid (2OG) synthesis and principal competitors of SPS for glycolytic carbon, were then analysed. PK activity (Fig. 4A), which increased 2-fold from 84% to 68% before declining significantly between 68% and 42% RWC (*F*=8.83;
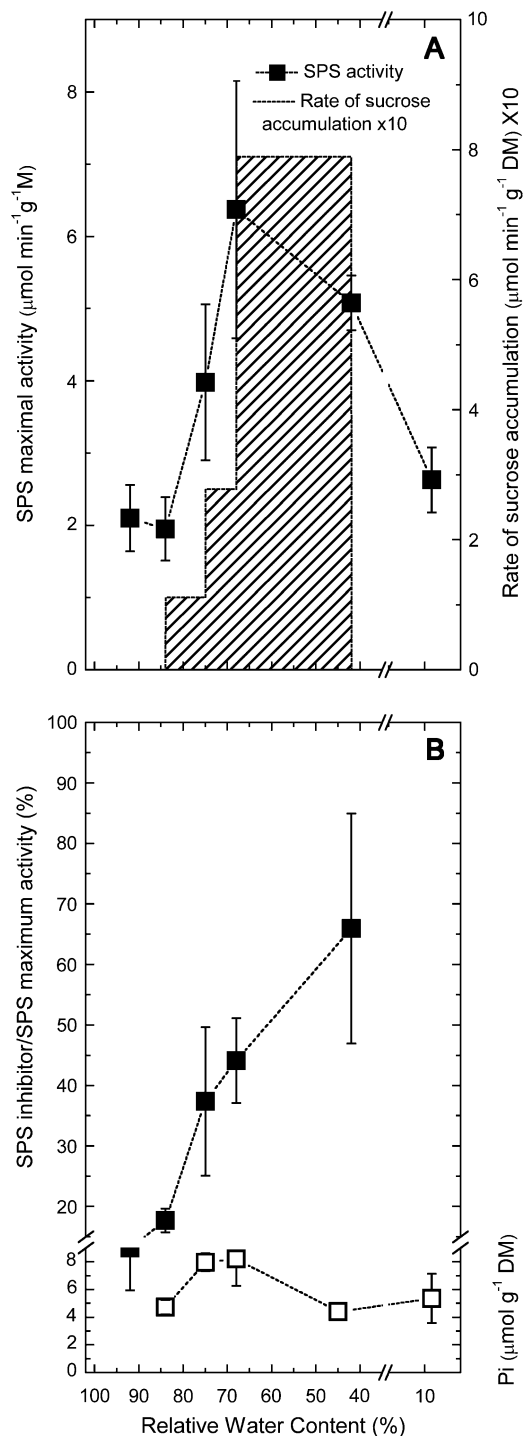

Fig. 3. (A) Average rate of sucrose accumulation (hatched bar) over the RWC interval periods reporting a significant sucrose accumulation in relation to maximal extractable SPS activity (squares) and (B) the ratio (filled squares) of maximal SPS activity to limiting SPS activity (measured under conditions of limiting substrates in the presence of 5 mM Pi) and *in vivo* inorganic Pi content (open squares) in leaf material at different RWCs over a 10 d dehydration period. Values are the means ±SD of 3–5 replicates. Each replicate is representative of 2–3 separate plants.

Table 2. *Desiccation respiration at various RWC intervals in dehydrating leaf tissue of* Sporobolus stapfianus

Values are the means ±SD of three replicates. Each replicate is representative of 2–3 separate plants.

RWC (%)	CO_2 production (nmol s^{-1} g^{-1} DM)
98	41.3±5.24
89	26.0±7.32
74	23.1±3.00
53	20.6±5.40
34	14.3±2.36
28	9.88±2.33
13	7.92±1.96

P ≤0.05) and 42–9% RWC (F=11.48; P ≤0.05), respectively, was positively associated with the transient increase and subsequent removal/phosphorylation of the hexose sugars (Fig. 1). Further upstream in the glycolytic pathway, a similar trend was observed for aldolase, responsible for the production of triose phosphates from the hexose phosphate, Fru-1,6-P_2. Aldolase activity declined 2-fold from 68% to 42% RWC and a further 2-fold from 42% to 9% RWC (results not shown). The decline in PEPCase activity (75% RWC) preceded that of PK and aldolase, with levels decreasing 6-fold over the remaining course of dehydration (Fig. 4B). NADP-ICDH, a key enzyme in the production of 2OG, was measured and its activity was shown to remain consistent up to 68% RWC (Fig. 4C). There was an overall decline in activity between 68% and 9% RWC, although no significant difference was detectable between 68% and 42% RWC. NADH-GOGAT activity, responsible for catalysing the transfer of fixed nitrogen to 2OG in the leaf under stress (Lancien *et al.*, 2000), was then examined. The enzyme declined significantly between 68–42% RWC (F=11.43; P ≤0.05) and 42–9% RWC (F=11.76; P ≤0.05), respectively (Fig. 4D). The Fd-GOGAT was also measured, and was similarly shown to decline in the more dehydrated tissues (Martinelli *et al.*, 2007). Since a major proportion of ammonia assimilation in leaf material is derived from NR activity, the latter was measured. Activities were maximal in hydrated tissues until 80% RWC (80.3±0.77 nmol min^{-1} g^{-1} DM) and completely absent below 68% RWC.

The change in sugar content during dehydration is always characterized by sucrose accumulation together with a transient accumulation of hexose sugars. However, the pattern may be differentially positioned along the course of dehydration between one drying experiment and another (compare Whittaker *et al.*, 2001), as has also been reported for other resurrection plants (Peters *et al.*, 2007). Therefore, it was very important to verify the relationship between increased rates of sucrose accumulation, maximal SPS activities, and decreased glycolytic activities using different batches of plants. For this reason, a separate

drying experiment was performed. The leaf RWC content remained stable over the first 8 d (~80%), before declining rapidly over the remaining 3 d (Fig. 5A). Maximal rates of significant net sucrose accumulation (F=27.97; P ≤0.01) between 77% and 54% RWC coincided with the onset of net hexose sugar removal (Fig. 5A). In turn, the onset of net hexose sugar removal coincided with a period of accelerated % RWC loss (compare Fig. 5A and B). Substantial rates of sucrose accumulation (F=169; P ≤0.05) were also present in the 54–38% RWC period (Fig. 5A). Both PEPCase and PK declined significantly (F=15.53; P ≤0.05 and F=11.65; P ≤0.05, respectively) between 77% and 54% RWC, whereas SPS activity remained unchanged in this period (Fig. 5B). Similar to enzyme activity, SPS protein increased during the initial period of dehydration stress (Fig. 6). Protein levels remained high in the more dehydrated tissues (Fig. 6), despite the decline in total maximal activity after hexose phosphorylation (Figs 3, 5).

An alternative potential source for the increase in amino acids could be via the breakdown of insoluble and/or soluble proteins. Insoluble protein content declined significantly (F=7.33; P ≤0.05) between 68% and 9% RWC (Table 2), whereas there was no significant decline in soluble protein content between 68% and 9% RWC (Table 2).

Discussion

The present study investigated SPS activity and various metabolic checkpoints at the interface of carbon and nitrogen metabolism to examine aspects of carbon partitioning between two major competing pathways, namely sucrose and amino acid synthesis, in dehydrating leaf material of *S. stapfianus*. Since the underlying criterion was the investigation of carbon metabolism in leaves that survive desiccation, studies were performed over separate dehydration experiments using non-senescent, desiccation-tolerant leaf material from different plant batches to identify common characteristic metabolic changes over dehydration. Results suggesting that sucrose accumulation may compete more strongly for carbon entering glycolysis during the period of hexose phosphorylation were provided by the maximal levels of SPS activity and protein together with the potential of the enzyme to withstand inhibitory conditions coinciding with increased rates of sucrose accumulation. The corresponding decline in the potential supply of carbon from glycolysis to the TCA cycle in this study was demonstrated by the decline in PK and PEPCase activity, as well as the further decrease in desiccation respiration. In turn, the decreased anabolic requirement for carbon was demonstrated by the co-ordinated decrease in GOGAT activity.
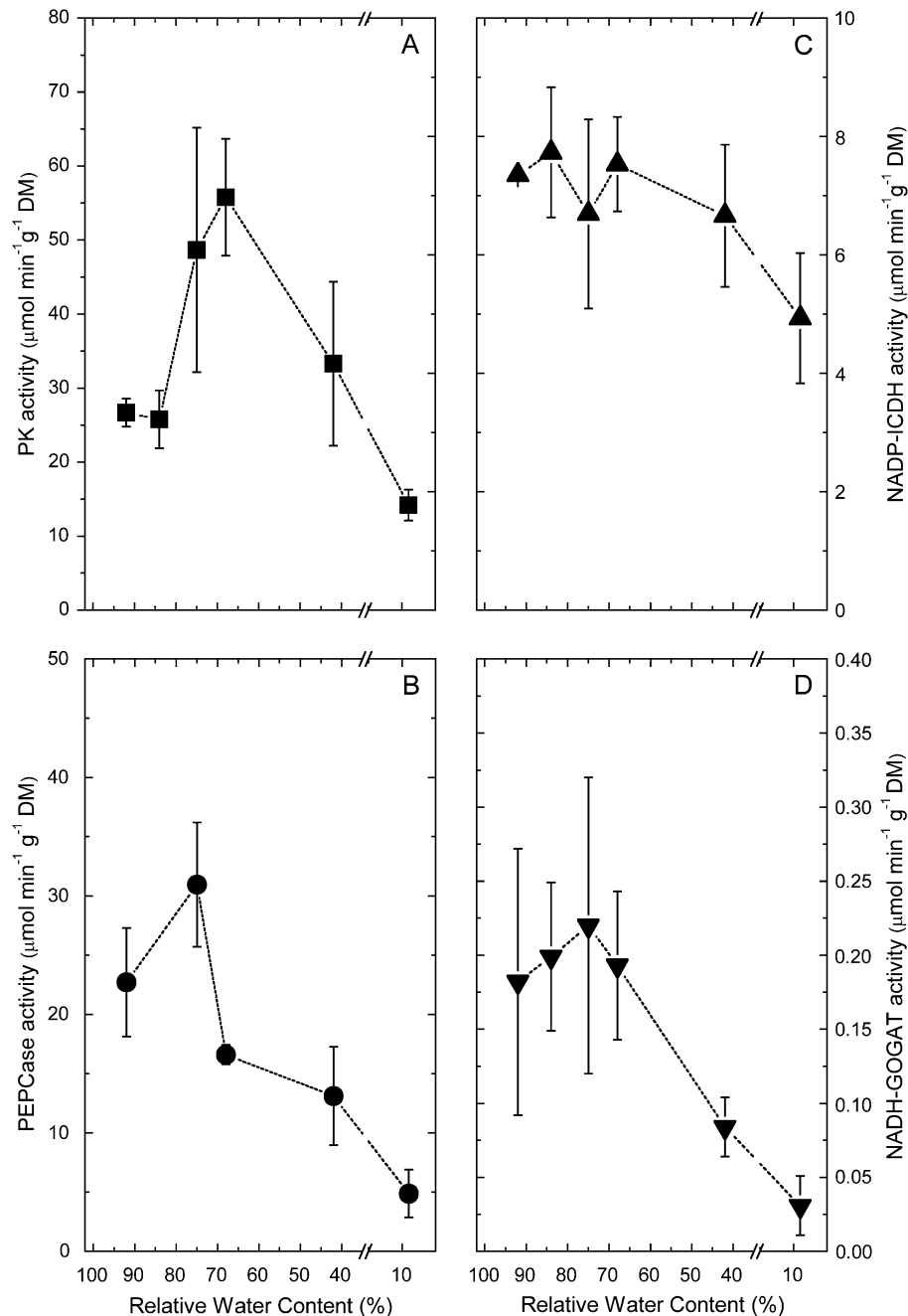
Fig. 4. (A) PK activity, (B) PEPCase activity, (C) NADP-ICDH activity, (D) NADH-GOGAT activity in leaf material at different RWCs over a 10 d dehydration period. Values are the means ±SD of 3–6 replicates. Each replicate is representative of 2–3 separate plants.

In *S. stapfianus* at the onset of dehydration (between ~100% and 70% RWC), PK activity increased and PEPCase activity was maintained in association with the increase in hexose sugars, sucrose, and amino acids. Stimulation of organic acid biosynthesis and subsequent amino acid biosynthesis, as a result of increased carbohydrate availability, has been shown to induce both PEPCase and PK activities (Morcuende *et al.*, 1998; Sima *et al.*, 2001). Given that both photosynthesis and dark respiration decline significantly at the initial stage of dehydra-

tion, the maintenance of high levels of PEPCase together with the increase in PK activity may reflect an increased shift towards an anaplerotic role for both enzymes (i.e. amino acid biosynthesis). Increased levels of PEPCase activity despite a substantial decline in photosynthesis were also reported for drought-stressed maize leaves (Foyer *et al.*, 1998).

Levels of NR and NADH-GOGAT activity from the present study, as well as those of Fd-GOGAT (Martinelli *et al.*, 2007), were maintained during the first part of
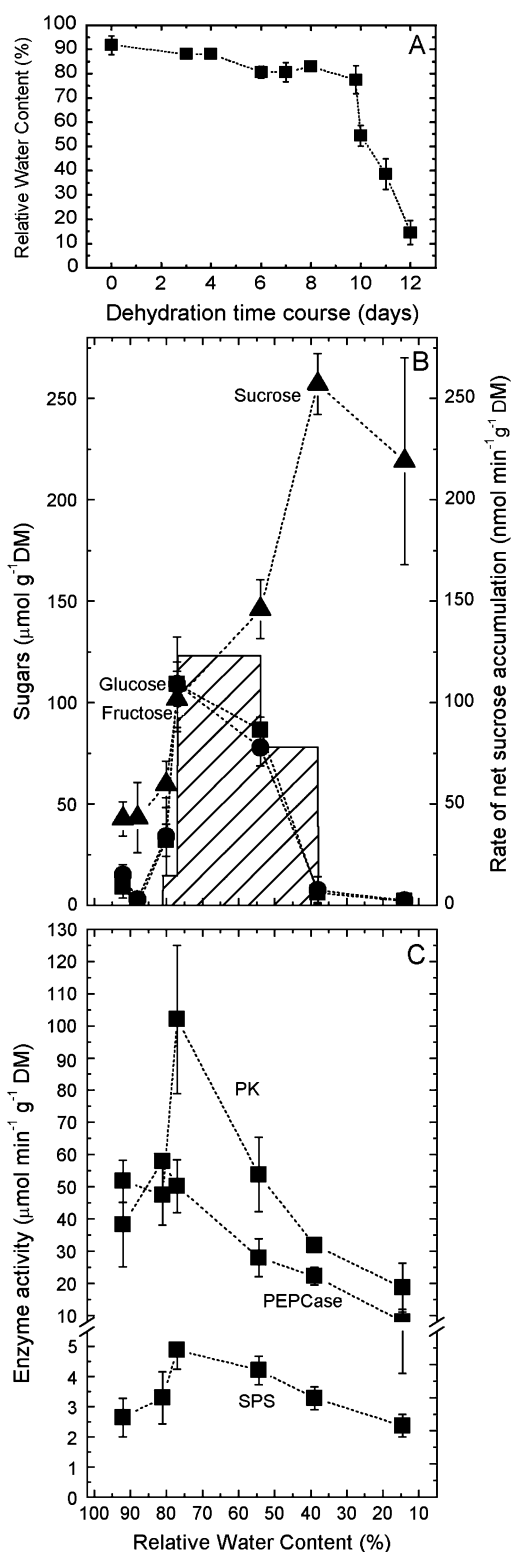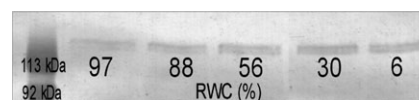
Fig. 6. SPS protein content in *S. stafianus* leaf material at different RWCs over a 12 d dehydration period.

Table 3. *Soluble and insoluble protein content at various RWC intervals in dehydrating leaf tissue of* Sporobolus stapfianus

Values are the means ±SD of 3–6 replicates. Each replicate is representative of 4–6 separate plants.

RWC (%)	Soluble protein (mg g^{-1} DM)	Insoluble protein (mg g^{-1} DM)
92	65.0±5.88	36.1±4.03
75	59.1±9.80	44.8±8.17
68	59.6±18.3	44.3±5.52
42	45.0±8.23	34.4±9.79
9	41.0±6.28	27.3±1.93

dehydration stress, suggesting a co-ordinated provision of nitrogen for the net accumulation of amino acids in this phase. The initial stage of dehydration was also characterized by significant increases in SPS activity and protein, and sucrose accumulation. Collectively, these results indicate that there are sufficient carbon skeletons entering primary metabolism from photosynthesis (despite the significant decline) and starch reserve breakdown to result in the net increase of both amino acids and sucrose. A physiological consequence of imposing dehydration stress to the point of desiccation is the cessation of transport during the course of dehydration. Although the RWC period demarcating the cessation of leaf transport has not been established for *S. stapfianus*, it is possible that transport (albeit reduced) may also supply carbon (and nutrients) to desiccation-tolerant leaves during the initial phase of dehydration. Increases in carbohydrates (hexose sugars but not sucrose) and amino acids in drought-stressed maize were similarly attributed to residual photosynthetic activities and starch turnover (Foyer *et al.*, 1998).

Below 70% RWC, changes in metabolism were suggestive of a declining provision of glycolytic carbon skeletons to net amino acid biosynthesis via GOGAT. Both NADH-GOGAT and Fd-GOGAT activity and protein (Martinelli *et al.*, 2007) significantly decreased below ~65% RWC. As in other studies, the water stress-induced decline and eventual cessation of photosynthetic activity in *S. stapfianus* was paralleled by a similar response in NR activity (Foyer *et al.*, 1998; Lawlor and Cornic, 2002). It is feasible that the cessation of NR activity may be correlated with impeded nitrate import into the leaf during desiccation stress. However, the decline in enzyme activity could not be correlated with a parallel decline in the measurable leaf nitrate levels as based on preliminary

Fig. 5. (A) Relative water content of leaf material in relation to the time. course of dehydration, (B) sucrose (upright triangles), glucose (squares), and fructose (circles) content and rate of sucrose accumulation (hatched bar), and (C) SPS (squares), PK (inverted triangles), and PEPCase (upright triangles) activity in leaf material at different RWCs over the 12 d dehydration period. Values are the means ±SD of 3–6 replicates. Each replicate is representative of 2–3 separate plants.

experiments (control, 0.59 ± 0.17 µmol g^{-1} DM; 65% RWC, 0.39 ± 0.15 µmol g^{-1} DM). Concurrent experiments showed that photorespiratory activity was negligible in *S. stapfianus* (T Martinelli, unpublished data). The cessation of NR activity and minimal photorespiratory activity would result in a reduced nitrogen supply to net glutamate synthesis by GS/GOGAT cycle activity. From the perspective of the leaf C/N balance, it can be predicted that a shortfall of nitrogen must be signalled to carbon metabolism, and vice versa (Foyer *et al.*, 2003). PEPCase and NR are recognized as two of the major metabolic checkpoints co-ordinating primary nitrogen and carbon metabolism (Foyer *et al.*, 2003), and a co-ordination in the regulation of both enzymes has been shown previously (Scheible *et al.*, 1997, 2000). In the present study, the observed decline in PEPCase and PK activity may serve to meet a complementary reduced demand for carbon skeletons (i.e. 2OG) needed for amino acid biosynthesis. Numerous studies have shown that the up- and down-regulation of PEPCase and PK activity in leaf tissue was co-ordinated by nitrate/ammonia assimilation in the chloroplast to balance the supply of carbon skeletons for amino acid biosynthesis (Scheible *et al.*, 1997, 2000; Morcuende *et al.*, 1998; Sima *et al.*, 2001; Foyer *et al.*, 2003). In the present study, the steady 2OG contents during dehydration may reflect a co-ordinated decline in the provision of carbon (2OG) to balance the reduced utilization by GOGAT.

In *S. stapfianus* leaf tissue, the decline in glycolytic and GOGAT activity was associated with increased rates of sucrose accumulation and peak levels of SPS activity. In contrast, loss of leaf SPS activity (measured under saturating conditions) by even mild water stress is reported for most C$_3$ species, as well as the most well-studied C$_4$ species, maize (Vassey and Sharkey, 1989; Zrenner and Stitt, 1991; Pelleschi *et al.*, 1997; Foyer *et al.*, 1998; Lawlor and Cornic, 2002). The fact that hexose phosphate sugars do not accumulate during hexose sugar phosphorylation (Whittaker *et al.*, 2001), and that SPS activity is maximal, indicates a utilization of carbon by SPS to support the increased rates of net sucrose accumulation. Levels of sucrose accumulated in the present investigation were comparable with those previously reported for *S. stapfianus* (Whittaker *et al.*, 2001), and hexokinase activity (results not shown) similarly paralleled SPS activity, in support of previous observations that sucrose accumulation occurs concomitantly with increased synthetic activity (Whittaker *et al.*, 2001). In *S. stapfianus*, a 'stronger competitive edge' of SPS in partitioning carbon towards sucrose during hexose phosphorylation in more dehydrated tissues is also supported by the corresponding loss of PK and PEPCase activity. The antagonistic relationship between SPS and NR plus PEPCase has been well documented in studies where short-term effects of nitrogen supply result in the stimulation of NR and PEPCase, and corresponding deactivation of SPS, thereby inducing a shift in carbon partitioning from sucrose biosynthesis to amino acid biosynthesis (Champigny, 1995, Scheible *et al.*, 1997; Foyer *et al.*, 1998; Lunn and Furbank, 1999).

It would appear that in the C$_4$ resurrection plant, *S. stapfianus*, as in the C$_3$ species *C. plantagineum* (Ingram *et al.*, 1997), SPS functioning has overcome the physiological constraints shown to reduce maximal activity in drought-sensitive plants. Although increased SPS activity was shown to be correlated with increased SPS protein in *C. plantagineum*, the additional role of physiological factors in modulating enzyme activity was proposed (Ingram *et al.*, 1997), but not tested. Those authors suggested that increasing activity of SPS may reflect the activation state of the enzyme rather than just the amount of protein. To this end, the activation state of SPS in *S. stapfianus* was therefore investigated by comparing its activity under maximal *in vitro* conditions and in the presence of Pi which acts to inhibit the enzyme activity via phosphorylation. The present results suggest a decreased sensitivity of SPS to Pi inhibition during dehydration. Direct evidence for this finding will require further verification. The increase in the maximal extractable activity together with the decrease in SPS phosphorylation during dehydration may represent an effective mechanism to ensure carbon flux towards sucrose during declining photosynthetic activity.

The recycling of Pi, a by-product of sucrose synthesis, during photosynthetic activity serves to facilitate photosynthesis and also to prevent the build-up of Pi and, as a consequence, the inactivation of SPS (Paul and Foyer, 2001). In *S. stapfianus*, there is a transient increase in the *in vivo* Pi content and an associated increase in the activation state of the enzyme. Thereafter, the Pi content decreases during the period of hexose sugar phosphorylation and sucrose accumulation. Assuming an equal distribution of Pi in a cell volume (fresh mass basis), the *in vivo* Pi content measured in *S. stapfianus* can be estimated to range from $\sim$1.8 mM to 3.0 mM. However, under conditions of dehydration, this concentration would increase and probably become inhibitory, necessitating decreased sensitivity of the enzyme to the presence of Pi. The most well studied C$_4$ plant, maize, is not shown to accumulate sucrose, and maximal SPS activity declines, presumably to reduce carbon flux to sucrose under declining photosynthetic capacity and export (Pelleschi *et al.*, 1997; Foyer *et al.*, 1998). In the study of Foyer *et al.* (1998), the phosphorylation state of SPS increased with progressive dehydration, which contrasts with a separate study on maize (Pelleschi *et al.*, 1997), where the decline in SPS measured under saturating and limiting (Pi) conditions was similar. Recent work (Lee *et al.*, 2005) has shown that Pi had no effect on the activity of SPS isoforms in rice suspension cells, corroborating emerging

knowledge that mechanisms of SPS regulation between different species are very diverse (Huber *et al.*, 1989; Vassey and Sharkey, 1989; Zrenner and Stitt, 1991; Huber and Huber, 1992; Pelleschi *et al.*, 1997; Foyer *et al.*, 1998; Lawlor and Cornic, 2002).

Of interest is that amino acids accumulated in the more dehydrated tissues. Rates of carbon and nitrogen assimilation and turnover determine the C/N ratio (mostly carbohydrate/protein amino acids). Leaf C/N ratios between hydrated and dehydrated tissue reflect an overall co-ordination in the C+N status. Despite the decline in activity of the principal carbon and nitrogen enzyme reactions in the more dehydrated tissues, it is not possible to estimate the exact contribution of these enzymes to the net accumulation of amino acids. That desiccation respiration persisted in this species suggests that TCA cycle activity is operational, which is suggestive of a certain level of glycolytic activity. The persistence in respiratory activity termed desiccation respiration has been documented previously (Tuba *et al.*, 1997). The latter study showed that since *X. scabrida* is a poikilo-

chlorophyllous desiccation-tolerant species (complete degradation of thylakoids and chlorophyll), respiration persists throughout dehydration in order to supply energy for the controlled breakdown of the chloroplasts. *Sporobolus stapfianus* is shown to display intermediate behaviour between poikilochlorophyllous and homiochlorophyllous (retain thylakoids with no loss in chlorophlyll) species (Quartacci *et al.*, 1997; Dalla Vecchia *et al.*, 1998). Interestingly, lipid degradation in *S. stapfianus* during dehydration (Quartacci *et al.*, 1997) may, in turn, furnish an alternative carbon/energy source to metabolism in dehydrating tissues, though this remains to be investigated. Nonetheless, an alternative possibility to the increase in amino acids in the more dehydrated tissues may be derived from the breakdown of proteins. Previous research indicated a reduced insoluble protein content in dehydrated leaf material of *S. stapfianus* (Gaff and McGregor, 1979). Since the comparisons in the latter study were made between hydrated and dehydrated material, it was not apparent during which phase of dehydration the insoluble protein content may have
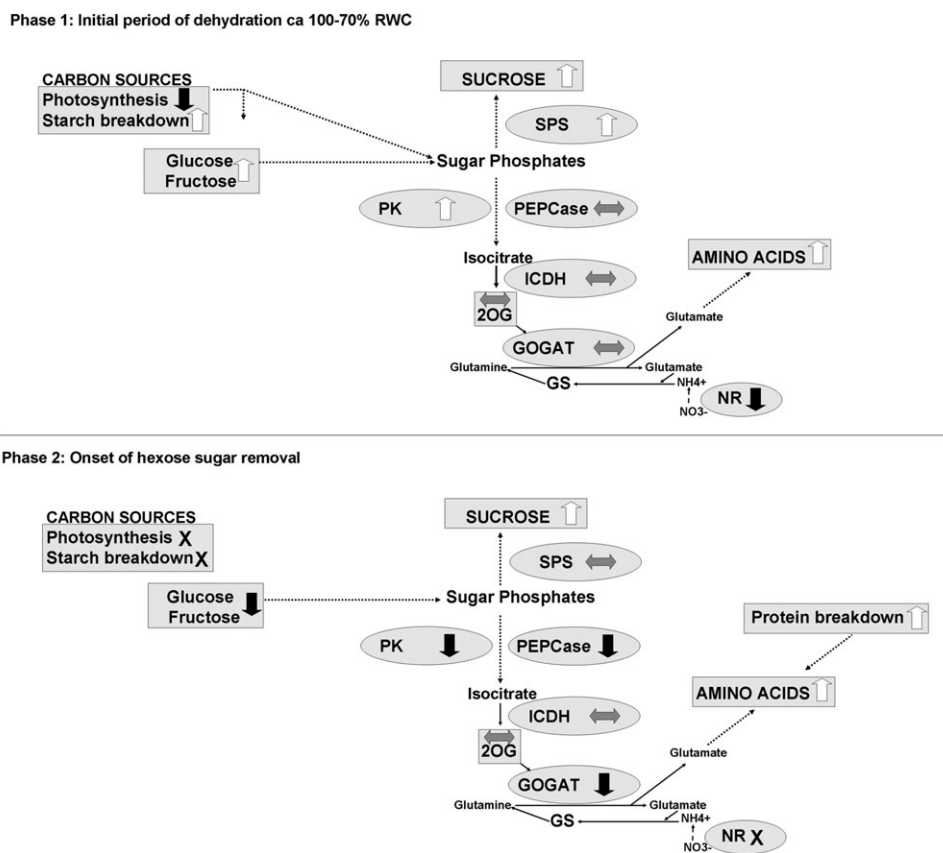

Fig. 7. Schematic diagram of the metabolic pathways involved in sucrose and amino acid biosynthesis to depict proposed carbon partitioning based on measured metabolite levels and enzyme activities in phase 1 (~100–70% RWC) and phase 2 (onset of hexose sugar removal, accelerated rates of sucrose accumulation, and loss of leaf RWC) of desiccation-tolerant leaf material of *S. stapfianus* during dehydration. Upward pointing arrow, increase in enzyme activity or metabolite content; downward pointing arrow, decrease in enzyme content and metabolite content; and double-headed arrow, no change in enzyme activity and metabolite content. Solid arrows are indicative of a single reaction step and dotted arrows indicate multiple enzyme-catalysed reaction steps.

contributed to the increase in amino acids. A decline in the insoluble protein content between 68% and 9% RWC was evident in leaf tissue of *S. stapfianus*. The tendency towards the hydrolysis of insoluble protein rather than soluble protein during dehydration was previously reported by Gaff and McGregor (1979).

Conclusions

During the initial phase of dehydration (~100–70% RWC), simultaneous increases in hexose sugars, sucrose, and amino acids were sustained by carbon sources including photosynthesis and starch breakdown (Fig. 7). This phase was associated with increases in SPS and PK activity and maximal levels of PEPCase, ICDH, and GOGAT activity, suggesting carbon flux from the sugar phosphate pool in the direction of both sucrose and amino acid biosynthesis (Fig. 7). The next phase of dehydration coincided with the net phosphorylation of hexose sugars, at which point starch was completely degraded and photosynthesis was negligible. The onset of hexose phosphate removal was also associated with the onset of an accelerated decline in the leaf RWC. This second phase of dehydration was associated with increased rates of sucrose accumulation and maximal levels of SPS protein, as well as activity under maximal conditions. SPS showed a decreased sensitivity to Pi during dehydration, which may suggest a decreased phosphorylation. However, direct experimental evidence is required to verify this finding. This work points to an increased competitive capacity of SPS in carbon utilization as both catabolic respiration and potential anapleurotic activity declined, as evidenced from the decrease in PK, PEPCase, and GOGAT activities (Fig. 7). It is, therefore, proposed that the increase in amino acids in this phase be derived from insoluble protein breakdown and subsequent amino acid transformations.

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