

RESEARCH PAPER

Sedoheptulose accumulation under CO₂ enrichment in leaves of *Kalanchoë pinnata*: a novel mechanism to enhance C and P homeostasis?

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Abstract

In contrast to the well-documented roles of its mono- and bisphosphate esters, the occurrence of free sedoheptulose in plant tissues remains a matter of conjecture. The present work sought to determine the origin of sedoheptulose formation *in planta*, as well as its physiological importance. Elevated CO₂ and sucrose induction experiments were used to study sedoheptulose metabolism in the Crassulacean acid metabolism (CAM) plants *Kalanchoë pinnata* and *Sedum spectabile*. Experimental evidence suggested that sedoheptulose is produced from the oxidative pentose phosphate pathway intermediate sedoheptulose-7-phosphate, by a sedoheptulose-7-phosphate phosphatase. Carbon flux through this pathway was stimulated by increased triose-phosphate levels (elevated CO₂, compromised sink availability, and sucrose incubation of source leaves) and attenuated by ADP and inorganic phosphate (P_i). The accumulation of free sedoheptulose is proposed to act as a mechanism contributing to both C and P homeostasis by serving as an alternative carbon store under elevated CO₂ or a compromised sink capacity to avoid sucrose accumulation, depletion of inorganic phosphate, and suppression of photosynthesis. It remains to be established whether this acclimation-avoiding mechanism is confined to CAM plants, which might be especially vulnerable to P_i imbalances, or whether some C₃ and C₄ plants also dispose of the genetic capacity to induce and accelerate sedoheptulose synthesis upon CO₂ elevation.

Key words: C homeostasis, Crassulacean acid metabolism, elevated CO₂, P homeostasis, photosynthetic acclimation, sedoheptulose, sucrose.

Introduction

During the last 100 years, human activities have strongly contributed to the rising CO₂ levels in the atmosphere. The impact of this change and its putative correlation with global

warming has been extensively debated (Rogelj *et al.*, 2010). It is clear that the increasing CO₂ concentrations will also affect the production of future crops and algae, but the effect might

Abbreviations: Ac, acetate; CAM, Crassulacean acid metabolism; COSY, correlation spectroscopy; DCMU, (3,4-dichlorophenyl)-1,1-dimethylurea; HMBC, heteronuclear multiple bond correlation; HPAEC-PAD, high-performance anion-exchange chromatography with pulsed amperometric detection; HSQC, heteronuclear single quantum correlation; NMR, nuclear magnetic resonance; oxPPP, oxidative pentose phosphate pathway; PEP, phosphoenolpyruvate; P_i, inorganic phosphate; PPFD, photosynthetic photon flux density.

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differ greatly depending on the species, nutritional status, and other environmental factors (Xu *et al.*, 2010). In general, elevation of atmospheric CO₂ concentration enhances photosynthesis and stimulates growth and productivity in most plant species covering different photosynthetic pathways, i.e. C₃, C₄, and Crassulacean acid metabolism (CAM) (Poorter and Navas, 2002; Ainsworth and Long, 2005; Ceusters and Borland, 2011). However, the initial enhancement is often found to diminish during long-term exposure to elevated CO₂ by excessive starch and sugar accumulation causing feedback inhibition of photosynthesis (Sage *et al.*, 1989; Stitt, 1991). As this acclimation process is especially noted in annual C₃ plants whilst some C₃ trees and perennial CAM plants do not suffer from acclimation, the availability of sink capacity has been attributed as an important determinant for the long-term responses of plants to elevated CO₂ (Nobel and Israel, 1994; Ceulemans *et al.*, 1995).

In contrast to the universal presence of its mono- and bisphosphate esters in the plant kingdom, free sedoheptulose only accumulates to a high extent in some members of the Crassulaceae family such as *Sedum spectabile* (La Forge and Hudson, 1917; Hegnauer, 1964). However, its presence, albeit at lower levels, in a range of other families including Apiaceae, Aquifoliaceae, Euphorbiaceae, Lamiaceae, Primulaceae, and Saxifragaceae suggests a broader range of occurrence (Tolbert *et al.*, 1957; Okuda and Mori, 1974; Häflinger *et al.*, 1999; Soria *et al.*, 2009). The only other free C7 sugar described in plants is mannoheptulose (Tsfay *et al.*, 2012). Nordall *et al.* (1956) postulated that the presence of free sedoheptulose in amounts exceeding the steady-state concentration of its phosphate might be attributed to weak activity of sedoheptulokinase (EC 2.7.1.14), specifically producing sedoheptulose-7-phosphate from sedoheptulose. However, except for some studies involving sedoheptulokinase deficiency in humans, this enzymatic activity has been poorly studied (Kardon *et al.*, 2008), and sedoheptulokinase activity has never been reported in plants. Alternatively, starting from sedoheptulose-7-phosphate, two routes might be considered in plant tissues that potentially yield the free heptose sugar, as both the Calvin–Benson cycle and the oxidative pentose phosphate pathway (oxPPP) contain sedoheptulose-7-phosphate as an intermediary reagent. Although the basic features of the oxPPP are well established (Kruger and von Schaewen, 2003), details on how exactly the pathway influences other processes are subject to further investigation. In addition to its putative origin, the role of free sedoheptulose in plants remains a matter of debate, except for serving as a precursor for the polyol volemitol in the horticultural hybrid polyanthus (Häflinger *et al.*, 1999). A possible function as a carbohydrate reserve in CAM (such as starch or soluble sugars), essential to catalyse nocturnal CO₂ uptake, was ruled out earlier by Kull (1965, 1967) based on the absence of clear changing diel patterns of sedoheptulose. Therefore, only speculation exists about its physiological roles in plant tissues (Kardon *et al.*, 2008).

To gain more insight into the occurrence of free sedoheptulose and its physiological function in plant tissues, elevated CO₂ and sucrose induction experiments were used to study

sedoheptulose metabolism in *K. pinnata* and *S. spectabile*, two closely related members of the Crassulaceae family performing CAM. The results indicated that sedoheptulose formation *in planta* might occur by a sedoheptulose-7-phosphate phosphatase acting on the oxPPP intermediate sedoheptulose-7-phosphate. Furthermore, experimental evidence suggested that sedoheptulose accumulation might confer a novel mechanism to contribute to C and P homeostasis in CAM plants. Other possible complimentary functions are also discussed.

Materials and methods

Plant material and sampling

The closely related *Kalanchoë pinnata* and *Sedum spectabile* species belong both to the Crassulaceae and perform CAM (Brulfert *et al.*, 1988; Lüttge *et al.*, 1991). Plants were originated from leaf cuttings and grown under controlled greenhouse conditions (Leuven, Belgium). The elevated CO₂ experiment was performed with *K. pinnata* plants that were divided equally between two greenhouse compartments (Leuven, Belgium). Control plants were grown under ambient atmospheric CO₂ concentrations (~380 μmol mol⁻¹). The CO₂-treated plants were exposed to ~700 μmol mol⁻¹, as described previously (Ceusters *et al.*, 2008). All other environmental conditions in both compartments were identical. During the day, a minimum temperature of 21 °C was maintained, whilst at night a minimum of 19.5 °C was achieved. Between 6:00 and 22:00h, additional artificial lighting was provided [photosynthetic photon flux density (PPFD)=30 μmol m⁻² s⁻¹] and the average integrated daily PPFD was ~7 mol photons m⁻² d⁻¹. Plants were watered twice weekly with a conventional nutrient solution, as described by Londers *et al.* (2009).

After 12 weeks, young fully developed leaves were cut in the afternoon (15:00h) from plants (n=5) under both CO₂ regimens and immediately frozen in liquid nitrogen to arrest any enzyme activity.

Purification of sedoheptulose and confirmation by nuclear magnetic resonance (NMR)

About 50 g of leaf material (cut into pieces of 1 cm²) was combined with 100 ml of Milli-Q water, boiled for 15 min, and homogenized with a Waring blender. The homogenate was centrifuged at 20 000g for 20 min. The supernatant was put on a mixed-bed Dowex column [50 ml resin of acetate⁻ (Ac⁻) and 50 ml of H⁺ Dowex; Acros Organics, Morris Plains, NJ, USA] and further concentrated with a Rotavap to 15 ml, which was loaded in three 5 ml aliquots onto a Ca-Dowex column (50 × 300 mm) and fractionated. Sedoheptulose-containing fractions were checked with analytical high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) and combined. Excess glucose was removed by the addition of glucose oxidase (500 U). The resulting glucuronic acid was removed by loading the mixture onto 50 ml Ac⁻ Dowex. The column flowthrough was concentrated to 4 ml with a Rotovap and subjected in ten 400 μl aliquots to preparative HPAEC-PAD as described previously (Vanhaecke *et al.*, 2006). Manually collected fractions were neutralized, combined, and loaded onto an active charcoal column (25 × 150 mm, 12-20 mesh; Sigma). The column was subsequently washed with 500 ml Milli-Q water. Sedoheptulose was eluted with 20 % ethanol, which was subsequently removed with a Rotovap. Finally, the remaining volume was lyophilized (LSL Secfroid) and pure sedoheptulose (10 mg) was dissolved in 0.6 ml D₂O for NMR analysis. Spectra were recorded at 22 °C on a Bruker Avance II 600 equipped with a 5 mm TCI HCN Z gradient cryoprobe. Bruker Topspin 2.1 software

was used to process all spectra. The two-dimensional pulse programs of ¹H-¹H correlation spectroscopy (COSY), heteronuclear single quantum correlation (HSQC), heteronuclear multiple bond correlation (HMBC), and nuclear Overhauser effect spectroscopy experiments were carried out as described previously (Vanhaecke *et al.*, 2008).

Induction with sucrose

K. pinnata leaves were cut in pieces of 0.5 cm³ and for the (3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) experiment they were first incubated overnight in 100 ml of 20 μM DCMU (or water as a control) in Petri dishes (17 cm diameter), before transferring them for 2 d in 100 ml of 200 mM sucrose (or water) with or without DCMU in the dark or in continuous light (PPFD=100 μmol m⁻² s⁻¹) at 22 °C. Sucrose induction was also performed by placing flower-decapitated *S. spectabile* stem parts (with leaves included) in a beaker with 200 mM sucrose for 2 d under continuous light. Subsequently, sugar concentrations and sedoheptulose-7-phosphate phosphatase activities were determined in the leaves.

Sugar extraction and quantification

Leaves were boiled in 2 volumes of water for 15 min and further homogenized with a mortar and pestle. The extract was centrifuged at 13 200 rpm for 5 min. Subsequently, 200 μl of the supernatant was added to a mixed-bed Dowex column (300 μl Dowex H⁺ and 300 μl Dowex Ac⁻; both 100-200 mesh; Acros Organics). The column was eluted six times with 200 μl ddH₂O (Vergauwen *et al.*, 2000). From the supernatant, an aliquot was analysed using HPAEC-PAD, as described previously (Vergauwen *et al.*, 2000). Peak quantification and identification was performed using the external standards method (Vergauwen *et al.*, 2000). No losses in sedoheptulose occurred during the applied extraction procedures, as verified by adding a known amount of sedoheptulose to an extract from a non-sedoheptulose producing species followed by quantification of the amount found after the purification procedure (not shown).

Enzyme extraction and enzyme activity measurements

Standardized procedures were followed (Eisenthal and Danson, 1992). Leaves were extracted in 2 volumes of extraction buffer (20 mM KMES, pH 6.0, containing 2 mM mercaptoethanol, 2 mM MgCl₂, 10 mM NaHSO₃, 0.1 % Polyclar and 1 mM PMSF). The extract was dialysed overnight against 500 volumes of 20 mM KMES buffer. After dialysis, the enzyme was centrifuged for 5 min at 16 000g. For the sedoheptulose-7-phosphate phosphatase assay, an enzyme aliquot was combined with 2 mM sedoheptulose-7-phosphate, 4 mM MgCl₂, 50 mM KMES (pH 6.0) (final concentrations), with (or without) the extra addition of 4 mM ADP or 4 mM potassium phosphate (pH 6.0) and incubated at 30 °C. After 0, 3, and 20 h of incubation, an aliquot of 10 μl was removed and added to 50 μl of 20 μM mannitol (internal standard) with 0.04% sodium azide and heated to 90 °C for 5 min. Identical concentrations and treatments were used in the sedoheptulose kinase assay, except that sedoheptulose and ATP (4 mM) were used as substrates. All reaction products were analysed by HPAEC-PAD, as described above.

Chloroplast isolation

S. spectabile chloroplasts were isolated using the method of Kley *et al.* (2010) with minor modifications. A layer of 3 ml of 50 % Percoll (GE Healthcare) was covered with 7 ml of chloroplasts and centrifuged in a Sigma 203 centrifuge (13020 swing-out rotor) at 2800 rpm for 10 min. After washing in 10 ml Clp buffer (Kley *et al.*, 2010), the chloroplasts were spun down at 2500 rpm for 5 min.

Data analysis

Data were analysed using the statistical software package SAS Enterprise Guide 4.0. Before carrying out statistical tests, the normality of the datasets was checked by means of the Kolmogorov–Smirnov statistic ($P > 0.05$). Means were compared using a two-sample *t*-test ($\alpha = 0.05$).

Results

Detection of sedoheptulose in *K. pinnata* by NMR

The studied sugar adopted three conformations in a D₂O solution. The ¹³C signals of the three anomeric carbon atoms could easily be recognized by their typical chemical shift close to 100 ppm (Fig. 1). Starting from these quaternary carbons, assignment in the individual spin systems was possible using a combination of two-dimensional HMBC, HSQC–total correlation spectroscopy, HSQC, and COSY spectra. The observed ratio of three conformations and the obtained ¹³C data corresponded to those published for D-sedoheptulose-7-phosphate (Charmantray *et al.*, 2009), despite minor differences that are expected from its phosphorylation state. Chemical shift assignments and ³J_{HH} couplings within each ring are listed in Table 1. The obtained NMR spectra could be differentiated clearly from those obtained for mannoheptulose (Waschke *et al.*, 2011).

Sedoheptulose accumulation under elevated CO₂ in *K. pinnata* in vivo

In the leaves of *K. pinnata*, CO₂ elevation caused the levels of glucose and fructose to increase ($P < 0.05$), whilst sucrose was maintained at a similar level compared with control plants ($P > 0.05$; Table 2). Initially, free sedoheptulose was present only at values representing 20–30 % in comparison with hexoses and sucrose, but after 12 weeks of CO₂ enhancement, sedoheptulose had increased by 500 % ($P < 0.05$) and became the dominant sugar in the leaves of *K. pinnata*. No other major carbohydrate accumulations were detected by HPAEC-PAD.

Artificial induction of sedoheptulose accumulation in isolated leaves of *K. pinnata*

To gain more insight into the strong accumulation of sedoheptulose noted under CO₂ enrichment, additional experiments were set up under ambient CO₂ involving the elimination of sinks by isolating source leaf pieces and incubating them in water or 200 mM sucrose under continuous illumination for 4 d.

Incubation of source leaves in a water solution showed a steady small but significant increase ($P < 0.05$) in hexose sugars over a time course of 4 d (Fig. 2A, B). After 1 d of incubation, sucrose levels decreased threefold ($P < 0.05$) and remained invariable thereafter for the considered time period (Fig. 2C). Sedoheptulose showed a tenfold increase ($P < 0.05$) after 3 d of incubation (Fig. 2D). Feeding the leaves with 200 mM sucrose solution resulted in a massive accumulation of 20 and 40 times the original concentration for glucose and fructose, respectively (Fig. 2A, B). Glucose build-up only

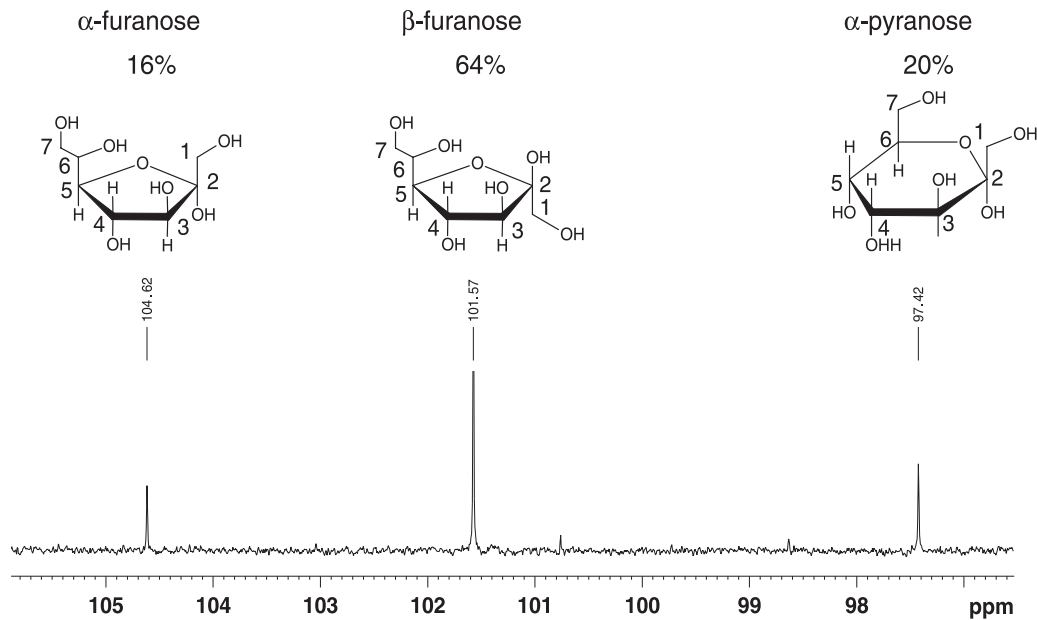


Fig. 1. Region of the ^{13}C -spectrum in D_2O with signals of anomeric C2 carbons. Chemical structures of the three observed conformations are depicted above their anomeric signal. Relative amounts of the different conformations were calculated from resolved signals in a one-dimensional proton spectrum.

Table 1. Chemical shift assignments for the three forms of sedoheptulose that appear in D_2O and $^3\text{J}_{\text{HH}}$ couplings within each ring.

| | α -Furanose | β -Furanose | α -Pyranose |
|-------------------|--------------------|-------------------|--------------------|
| C1 | 62.7 | 62.4 | 64.0 |
| C2 | 104.6 | 101.6 | 97.4 |
| C3 | 81.7 | 75.6 | 68.2 |
| C4 | 76.4 | 75.4 | 70.1 |
| C5 | 81.6 | 80.1 | 63.4 |
| C6 | 71.6 | 72.6 | 68.7 |
| C7 | 62.2 | 62.2 | 61.2 |
| H11/H12 | 3.51 | 3.41 | 3.54 |
| H3 | 3.94 | 3.94 | 3.80 |
| H4 | 4.03 | 4.15 | 3.92 |
| H5 | 3.85 | 3.61 | 3.69 |
| H6 | 3.73 | 3.69 | 3.88 |
| H71/H72 | 3.59 / 3.47 | 3.61 / 3.46 | 3.74 / 3.62 |
| $^3\text{J}_{34}$ | 4.6 | 7.4 | 3.6 |
| $^3\text{J}_{45}$ | 5.8 | 7.4 | 4.4 |
| $^3\text{J}_{56}$ | ND | ND | 11.0 |

occurred for 1 d, whilst fructose enhancement took about 2 d before reaching a maximum steady-state level ($P>0.05$). The accumulation of sucrose was much more limited compared with the hexoses, and showed only a threefold increase over 3 d (Fig. 2C), suggesting that the sucrose concentration is strictly controlled in *K. pinnata*. After the first day of incubation in a 200 mM sucrose solution, sedoheptulose showed a small increase ($P<0.05$), similar to that in leaves incubated in a water solution ($P>0.05$) (Fig. 2D). However, over the next 2 d, sedoheptulose increased at a much higher rate, resulting in a 30-fold accumulation after 3 d.

Table 2. Concentrations of glucose, fructose, sucrose, and sedoheptulose (mM) in young, fully expanded leaves of *K. pinnata* after 12 weeks at ambient ($380 \mu\text{mol mol}^{-1}$) or elevated ($700 \mu\text{mol mol}^{-1}$) CO_2 . The data are means \pm standard error for five leaves, each from a separate plant and were compared by a two-sample t -test ($\alpha=0.05$).

| CO_2 ($\mu\text{mol mol}^{-1}$) | Glucose | Fructose | Sucrose | Sedoheptulose |
|--|---------------|---------------|---------------|---------------|
| 380 | 1.6 ± 0.2 | 2.1 ± 0.4 | 1.7 ± 0.2 | 0.5 ± 0.1 |
| 700 | 2.2 ± 0.2 | 3.0 ± 0.4 | 1.7 ± 0.1 | 2.5 ± 0.5 |
| P value | <0.05 | <0.05 | >0.05 | <0.05 |

Inhibition of photosynthesis reduces sedoheptulose formation

Leaf pieces of *K. pinnata* were incubated overnight in the presence and absence of DCMU, an inhibitor of photosynthesis. Thereafter, they were kept under continuous light and in the dark for 2 d, respectively (\pm DCMU), both in the presence or absence of 200 mM sucrose (Fig. 3). Without additional sucrose, only the treatment with light and without DCMU yielded a significant amount of sedoheptulose, whilst only negligible amounts were detected in the light plus DCMU condition and in the dark. All sucrose-treated leaf pieces accumulated high levels of glucose and fructose, and showed an increased level of sucrose and sedoheptulose. However, in the light without DCMU, the sedoheptulose:sucrose ratio was substantially higher than in the three other conditions (Fig. 3). These results suggested that photosynthesis stimulates sedoheptulose formation. Nevertheless, synthesis also appeared to be possible in the dark, although to a lower extent. This was further corroborated using etiolated

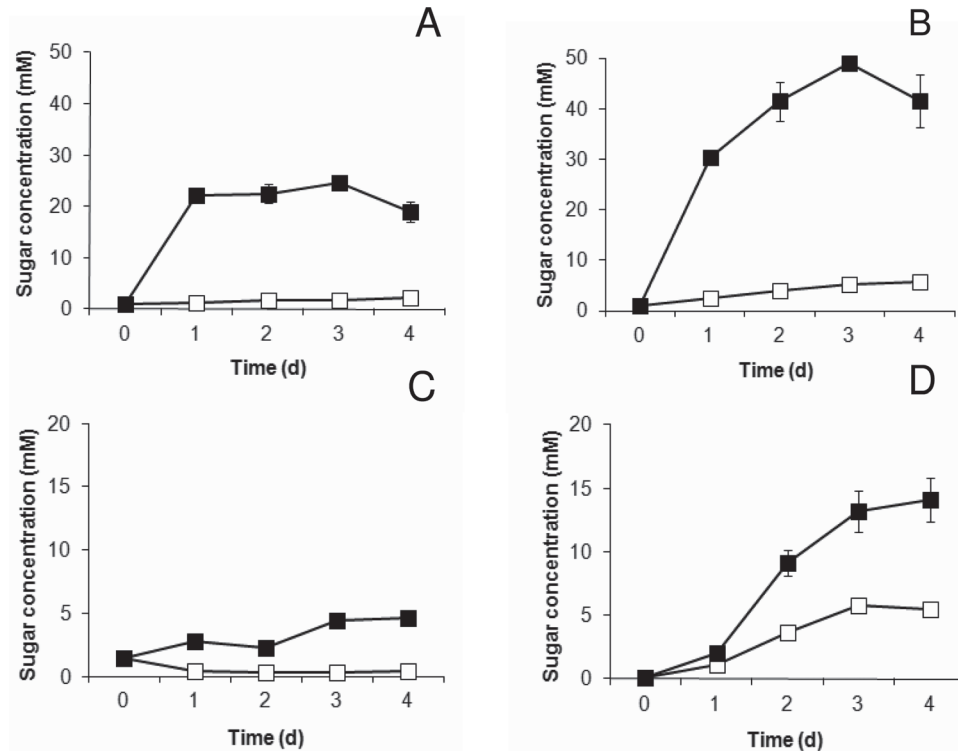


Fig. 2. Changes in glucose (A), fructose (B), sucrose (C), and sedoheptulose (D) concentrations (mM) in young, fully expanded, isolated source leaves of *K. pinnata* incubated in water (open squares) or in a sucrose solution (filled squares, 200mM).

K. pinnata material (not shown). When green stem parts (with leaves) of *S. spectabile* were induced with 200mM sucrose, sedoheptulose concentrations in the leaves increased from 6.7mM (control) to 15.5mM.

Sedoheptulose is produced from sedoheptulose-7-phosphate in vitro

Because it proved practically impossible to obtain substantial amounts of proteins from *K. pinnata*, the close relative species *S. spectabile*, known to accumulate high levels of sedoheptulose under standard conditions in its organs (La Forge and Hudson, 1917; Fig. S1 at JXB online), was used for protein extraction. Both species belong to the Crassulaceae and are situated in the same subfamily, Sedoideae (Mort et al., 2001). Desalted protein extracts of sucrose-induced leaves were incubated with 2mM sedoheptulose-7-phosphate. Fig. 4A shows the time-dependent formation of sedoheptulose from sedoheptulose-7-phosphate. No sedoheptulose kinase activity (the reverse reaction) could be detected by combining sedoheptulose and ATP (not shown), strongly suggesting that the sedoheptulose concentration *in planta* might be controlled by the sedoheptulose-7-phosphate supply and the sedoheptulose-7-phosphate phosphatase activity. Strikingly, the addition of both ADP and inorganic phosphate (P_i) at a concentration of 4mM attenuated the sedoheptulose-7-phosphate phosphatase activity (Fig. 4A). This was not observed for the non-specific phosphatases present in enzyme extracts of the C₃ plant wheat (Fig. 4B), suggesting the presence of a rather specific sedoheptulose-7-phosphate phosphatase in

S. spectabile that can be regulated by ADP and P_i. Moreover, sedoheptulose-7-phosphate phosphatase activities increased to 490 nmol mg⁻¹ of protein min⁻¹ in the leaves of sucrose-induced stems, compared with 25 nmol mg⁻¹ of protein min⁻¹ in control leaves (Table S1 at JXB online). Unfortunately, our continued efforts to purify this enzyme remained unsuccessful due to enzyme instability or removal of an essential co-factor.

Sedoheptulose formation probably originates in the cytosol via the oxPPP and is phloem transportable

To determine the putative subcellular location of sedoheptulose synthesis in *S. spectabile*, chloroplasts were isolated and lysed and the supernatant was analysed. The amount of sedoheptulose was negligible (Fig. S1), arguing for the actual synthesis of sedoheptulose in the cytosol via the oxPPP, as the Calvin-Benson cycle solely acts inside the chloroplasts. However, the absence of sedoheptulose in freshly prepared chloroplasts should be confirmed by non-aqueous fractionation methods.

Compared with leaves under full light (maximal activities up to 1100 nmol mg⁻¹ of protein min⁻¹ were recorded), much lower sedoheptulose-7-phosphate phosphatase activities (up to 33 nmol mg⁻¹ of protein min⁻¹) could be detected in *S. spectabile* roots, despite the fact that roots also accumulate sedoheptulose, although to a lower extent than leaves (Nordall et al., 1956; Fig. S1). This implied that sedoheptulose is produced mainly in the leaves and transported to the roots through the phloem, as suggested previously by Liu et al. (2002). Typically, the honeydew of aphids still partly

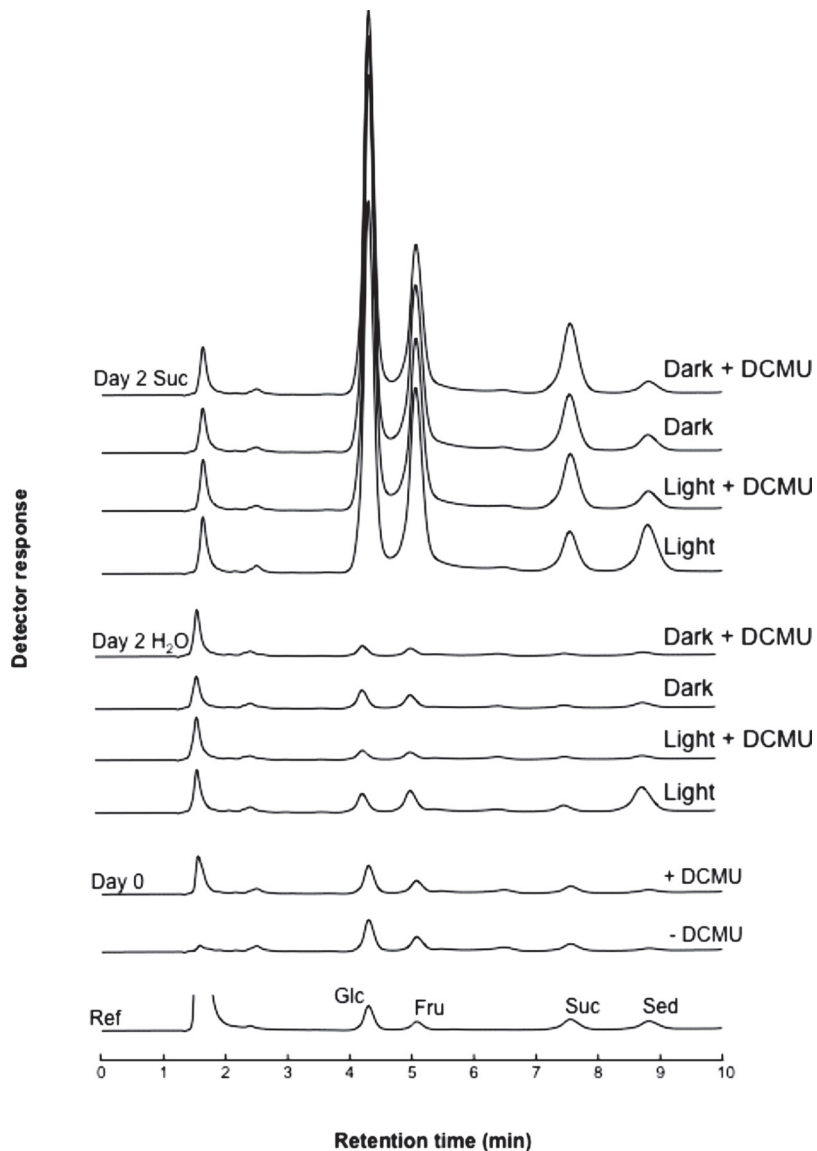


Fig. 3. Typical chromatograms showing glucose (Glc), fructose (Fru), sucrose (Suc), and sedoheptulose (Sed) in *K. pinnata* leaf pieces incubated with or without the photosynthesis inhibitor DCMU (day 0 + and – DCMU), before transferring them to 100 ml water or 200 mM sucrose solution for 2 d in the dark or in continuous light (PPFD $100 \mu\text{mol m}^{-2} \text{s}^{-1}$) at 22°C . The reference chromatogram ($10 \mu\text{M}$ of Glc, Fru, Suc, and Sed) is displayed at the bottom.

contains phloem-derived sugars, although substantial sugar pattern modulation occurs by the action of glycosyl transferases in the aphid gut (e.g. producing melezitose from sucrose; Vantaux *et al.*, 2011). Intriguingly, next to melezitose as a major sugar, honeydew from *S. spectabile*-resident aphids contained substantial amounts of sedoheptulose (Fig. S2 at JXB online), providing further evidence for the phloem transport of sedoheptulose in *S. spectabile*.

Discussion

In contrast to the well-documented roles of the phosphate esters of sedoheptulose, either in the regenerative part of the Calvin cycle or in the oxPPP, the occurrence of substantial amounts and the physiological role of free sedoheptulose in a range of CAM plants has remained enigmatic, despite the

fact that sedoheptulose was discovered almost a century ago in *S. spectabile* (La Forge and Hudson, 1917). Here, we have shed light on the putative importance of sedoheptulose anabolism in the CAM plants *K. pinnata* and *S. spectabile*.

We showed that sedoheptulose, whose identity was confirmed by NMR (Fig. 1, Table 1), was produced from sedoheptulose-7-phosphate *in vitro*, probably by the activity of a specific sedoheptulose-7-phosphate phosphatase (Fig. 4) operating in the leaves. Increased sedoheptulose-7-phosphate phosphatase activities were noticed concomitantly with elevated sedoheptulose concentrations in the leaves of sucrose-induced *S. spectabile* stems, suggesting that this enzyme is involved in the biosynthesis of sedoheptulose *in planta*. Sedoheptulose-7-phosphate constitutes an intermediary product in two pathways in plants: the Calvin-Benson cycle and the oxPPP. In contrast to the first, which is restricted to a

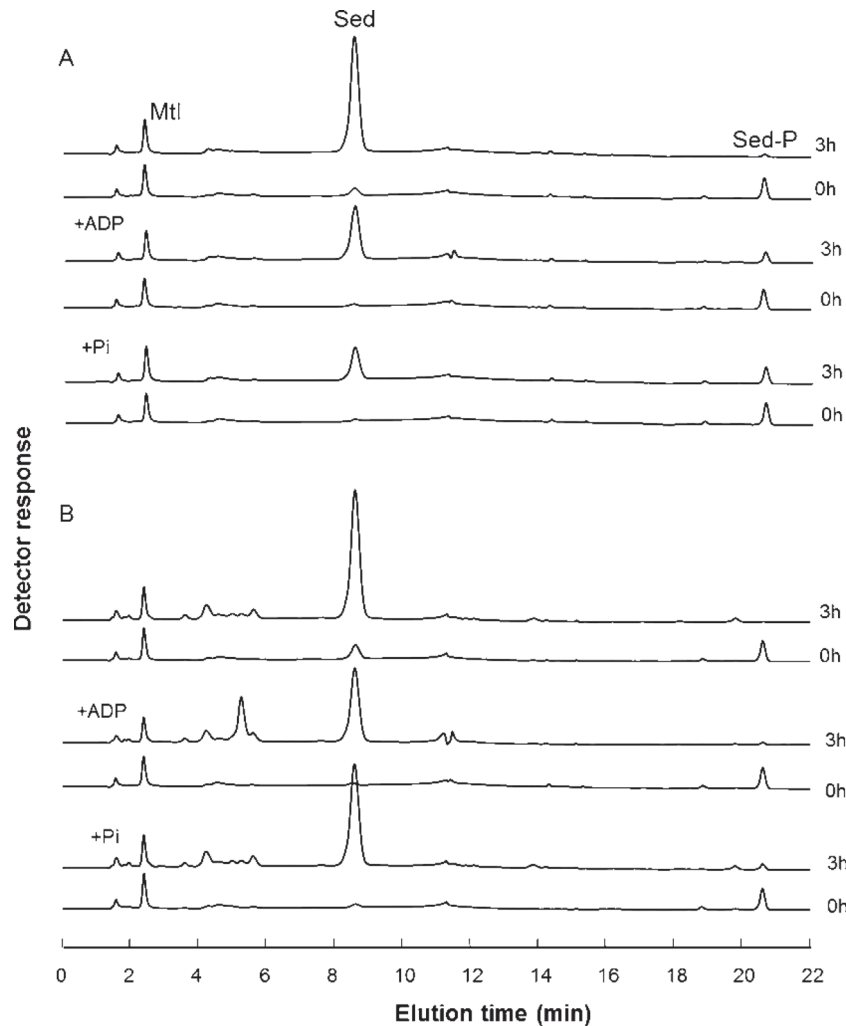


Fig. 4. Typical chromatograms showing the time-dependent (0 and 3 h) formation of sedoheptulose from 2 mM sedoheptulose-7-phosphate. Desalted protein extracts of sugar-induced *S. spectabile* (A) and wheat (B) leaves were compared. The attenuation of sedoheptulose formation by ADP and P_i is much more pronounced in (A). Mannitol (Mtl) served as an internal standard.

plastid origin, the oxPPP can be completed in the cytosol alone or involve compartmentalization between cytosol and chloroplast, depending on species, tissue, developmental stage, and environmental conditions (Kruger and von Schaewen, 2003). The absence of sedoheptulose in chloroplasts suggested an oxPPP-mediated synthesis of sedoheptulose in the cytosol. However, by converting sedoheptulose-7-phosphate to sedoheptulose, significant amounts of carbohydrate may be withdrawn from the actual oxPPP. This observation prompted further investigation into its physiological significance.

Under control conditions, free sedoheptulose concentrations were only of minor importance in *K. pinnata*, but doubling of the atmospheric CO₂ concentration caused sedoheptulose to increase by 500 % whilst sucrose was not influenced in source leaves (Table 2). Strict metabolic control of the amount of sucrose under CO₂ elevation has been noted previously in other CAM plants, which show no downward acclimation of photosynthesis in response to elevated CO₂, but the precise underlying mechanisms have not been elucidated (Wang and Nobel, 1996; Ceusters and Borland,

2011). It is well known that, as well as reducing expression of genes implicated in photosynthesis, accumulated sucrose might reduce the rate of its own synthesis. This might cause sugar phosphates to accumulate and inorganic phosphate (P_i) pools to be depleted in the cytosol and in chloroplasts, ultimately inhibiting photophosphorylation and leading to long-term acclimation of photosynthesis under elevated CO₂ in a range of plants (Stitt, 1991; Sheen, 1994). In different CAM plants, a lack of long-term acclimation has been attributed mainly to increased rates of phloem sucrose transport to supply growing sinks and avoiding sucrose accumulation in the source leaves (Wang and Nobel, 1996). In addition to this mechanism, we propose in our model (Fig. 5) that oxPPP-mediated sedoheptulose formation in the cytosol in *K. pinnata* might function as an excess carbon escape valve to deal with a surplus of triose phosphates originating from the chloroplasts (Calvin-Benson cycle), and as such avoiding sucrose accumulation under CO₂ enrichment when sink capacities might become saturated. To test this hypothesis, source-sink transport was compromised by

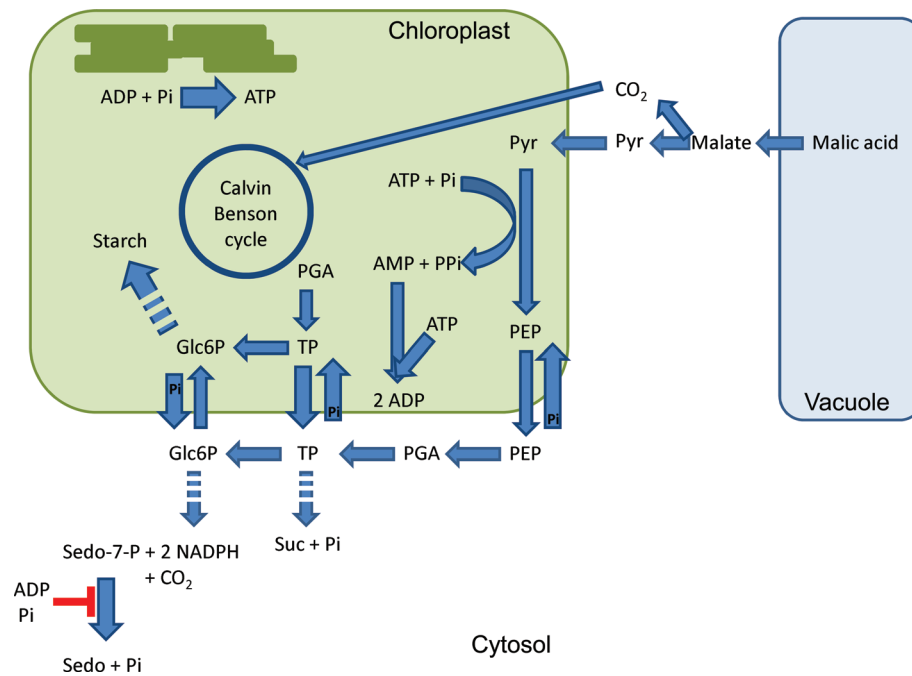


Fig. 5. Hypothetical model of daytime metabolite fluxes in starch-storing CAM species such as *Mesembryanthemum crystallinum*, *K. pinnata* and *S. spectabile* (based on Silvera *et al.*, 2010; Holtum *et al.*, 2005). Dotted arrows represent multiple steps. Free sedoheptulose formation under elevated CO_2 in *K. pinnata* is indicated and originates from the action of a sedoheptulose-7-phosphate phosphatase acting on the oxPPP intermediate sedoheptulose-7-phosphate. A negative feedback control by ADP and P_i is proposed, contributing to both C and P homeostasis. Glc6P, glucose-6-phosphate; PGA, 3-phosphoglycerate; PEP, phosphoenolpyruvate; Pyr, pyruvate; Sedo, sedoheptulose; Sedo-7-P, sedoheptulose-7-phosphate; Suc, sucrose; TP, triose phosphate. (This figure is available in colour at JXB online.)

detaching *K. pinnata* leaves and incubating them in solutions containing extra sucrose (200 mM) under full light (Fig. 2). The sucrose concentration inside the leaves rose slightly from 1.5 to about 5 mM, representing only a threefold increase, indicating that there is a strict metabolic control over the sucrose concentration inside the leaves but without the possibility of diverting excess sucrose to sinks. Initially, significant amounts were converted to glucose and fructose (Fig. 2), but after 2 d, the levels of these sugars were also saturated and a massive 30-fold increase in sedoheptulose level occurred (Fig. 2). These results demonstrated that sedoheptulose synthesis can act as an alternative or additional mechanism to deal with excess carbohydrate in the leaf or even at the cell level, whilst elevated increased phloem transport of sucrose requires coordination between different plant organs. In this context, sedoheptulose formation offers a higher degree of flexibility in comparison with sucrose. Both sugars are phloem transportable, but when this possibility is restricted, significant accumulation of sedoheptulose can take place inside source leaves without suppressing photosynthesis. Moreover, CAM plants are particularly well established to store large amounts of sugars in the typically large central vacuoles, which dominate about 95% of the cell's volume (Steudle *et al.*, 1980; Kenyon *et al.*, 1985). Of course, the drawback of carbon through sedoheptulose formation *in vivo* should only be active when triose phosphates are accumulating excessively, i.e. when sink capacity is limited and triose phosphates are synthesized at high rates under elevated CO_2 .

Our findings that incubating leaf pieces in a water solution without extra sucrose in the dark or under photosynthesis-inhibiting conditions (DCMU) resulted in only marginal sedoheptulose formation (Fig. 3) confirm this view, and significant sedoheptulose accumulation was restored when leaf pieces were incubated in a water solution in the light.

Diverting sedoheptulose from triose phosphates via glucose-6-phosphate in the oxPPP also involves the liberation of P_i and reducing power (NADPH) (Fig. 5). NADPH might be utilized in biosynthetic processes, for the production of antioxidants (e.g. ascorbate, glutathione), or, after conversion to NADH, oxidized in the mitochondrial electron transport chain. An elevated amount of reducing power could also favour CAM operation under elevated CO_2 because it energizes the conversion of oxaloacetic acid to malate during nocturnal CO_2 uptake. However, previous measurements from Winter *et al.* (1997) showed that *K. pinnata* only showed an elevated daytime uptake of CO_2 , whilst nocturnal sequestration and consequently malate formation remained unaffected upon CO_2 treatment. As already mentioned above, the availability of P_i is crucial to drive photosynthesis, and in this respect, CAM plants can be particularly vulnerable to P_i imbalances as they generally utilize more ATP and as such more P_i to convert carbon, originating from CO_2 , into sugars during photosynthesis (Iglesias *et al.*, 1993; Winter and Smith, 1996). Moreover, pyruvate phosphatase dikinase also requires both ATP and P_i to convert pyruvate to phosphoenolpyruvate (PEP) during the daytime (Fig. 5), and as such these reactions might compete

with direct Rubisco assimilation for P_i during the transition phases II and IV, leading to P_i limitation in the chloroplast. It is clear that the production and transport of PEP are crucial for the functioning of CAM plants to provide consistent carbohydrate storage each day to ensure the continuation of nocturnal carboxylation via PEP carboxylase (Ceusters *et al.*, 2010; Borland *et al.*, 2011). As such, the formation of sedoheptulose is not only beneficial with regard to carbon allocation under elevated CO₂ but also contributes to P_i homeostasis. Of course, this operation is only useful when chloroplast and cytosolic P_i and ADP are low, and this is completely in line with our finding that P_i and ADP exerted a negative feedback control on sedoheptulose-7-phosphate phosphatase activity (Figs 4 and 5). Besides the proposed functions of sedoheptulose in CO₂-enriched *K. pinnata*, the free heptose sugar might also serve a role in cellular antioxidant mechanisms. CAM habitats are generally characterized by high solar radiations, e.g. exposed terrestrial or epiphytic habitats where photosynthetic active radiation is not limiting photosynthesis but over-energization of the photosynthetic apparatus in CAM plants might constitute a real threat (Lüttge 2002). Moreover, it has been proposed that sugar(like) compounds, when accumulating at higher concentrations, might act as reactive oxygen species scavengers (Van den Ende and Valluru, 2009; Van den Ende *et al.*, 2011). Typically, resurrection species maintain higher soluble sugar levels compared with closely related non-resurrection species (Djilianov *et al.*, 2011).

In conclusion, we propose that sedoheptulose formation *in planta* occurs via the oxPPP intermediary sedoheptulose-7-phosphate, probably by a dedicated cytosolic sedoheptulose-7-phosphate phosphatase, although the exact subcellular localization is a subject for further investigation. Furthermore, it is proposed that sedoheptulose formation contributes to both C and P homeostasis in *K. pinnata* by acting as an alternative carbon store under elevated CO₂ to avoid sucrose accumulation and depletion of P_i. Our experimental evidence (elevated CO₂, external sugar, and light) strongly suggest a stimulated flux of C through this pathway, mediated by increased triose-phosphate levels but attenuated by ADP and P_i. It remains to be established whether this mechanism is confined to CAM plants, which might be especially vulnerable to P_i imbalances, or whether some C₃ and C₄ plants also dispose of the genetic capacity to induce and accelerate sedoheptulose synthesis upon CO₂ elevation.

Supplementary data

Supplementary data are available at *JXB* online.

Fig. S1. Typical chromatograms showing the sugar composition of different organs and isolated chloroplasts of *S. spectabile*.

Fig. S2. Typical chromatogram showing the sugar composition of honeydew from *S. spectabile*-resident aphids.

Table S1. Sedoheptulose-7-phosphate (S7P) phosphatase activities and sedoheptulose concentrations in leaves derived from sucrose-induced (200 mM, 2d) stem fragments of *S. spectabile* compared with an untreated control.

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