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Published on: 01 Aug 1993 - Journal of Experimental Botany (Oxford University Press)

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DOI: <https://doi.org/10.1093/jxb/44.8.1351>

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Journal Article

Published Version

Originally published at:

Keller, F; Ludlow, M M (1993). Carbohydrate Metabolism In Drought-stressed Leaves Of Pigeonpea (cajanus Cajan). *Journal of Experimental Botany*, 44(8):1351-1359.

DOI: <https://doi.org/10.1093/jxb/44.8.1351>

Carbohydrate Metabolism in Drought-Stressed Leaves of Pigeonpea (*Cajanus cajan*)

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Received 18 March 1993; Accepted 18 May 1993

ABSTRACT

Pigeonpea is a tropical grain-legume, which is highly dehydration tolerant. The effect of drought stress on the carbohydrate metabolism in mature pigeonpea leaves was investigated by withholding water from plants grown in very large pots (50 kg of soil). The most striking feature of drought-stressed plants was the pronounced accumulation of D-pinitol (1D-3-methyl-*chiro*-inositol), which increased from 14 to 85 mg g⁻¹ dry weight during a 27 d stress period. Concomitantly, the levels of starch, sucrose and the pinitol precursors *myo*-inositol and ononitol all decreased rapidly to zero or near-zero in response to drought. The levels of glucose and fructose increased moderately. Drought stress induced a pronounced increase of the activities of enzymes hydrolysing soluble starch (amylases) and sucrose (invertase and sucrose synthase). The two anabolic enzymes sucrose phosphate synthase (sucrose synthetic pathway) and *myo*-inositol methyl transferase (pinitol synthetic pathway) also showed an increase of activity during stress. These results indicate that pinitol accumulated in pigeonpea leaves, because the carbon flux was diverted from starch and sucrose into polyols.

Key words: Drought, polyols, pinitol, sucrose, starch, pigeonpea.

INTRODUCTION

Pigeonpea is a tropical grain-legume with high tolerance of dehydration (Ford, 1984; Singal *et al.*, 1985; Flower and Ludlow, 1986; Lopez *et al.*, 1987; Nandwal *et al.*, 1991). The lethal leaf water potential (Ψ_L) and relative water content (*RWC*) are -6.3 MPa and 32%, respectively (Flower and Ludlow, 1986). Although the exact reason for pigeonpea's dehydration tolerance is not known, there is strong evidence that it is due to its capacity to adjust osmotically as a result of solute accumulation (Ford, 1984; Flower and Ludlow, 1986). The main solutes to accumulate in drought-stressed pigeonpea leaves are the cyclitol D-pinitol (1D-3-methyl-*chiro*-inositol) and the amino acid proline (Ford, 1984). Both of these compounds are typical 'compatible solutes', i.e. non-toxic compounds which accumulate in the cytoplasm in response to low water potential (Brown and Simpson, 1972). By so doing, pinitol and proline might protect proteins and membranes from the deleterious effects of

dehydration and, ultimately, from denaturation (Schobert, 1977; Crowe *et al.*, 1988; Smirnow and Cumbes, 1989; Sommer *et al.*, 1990). Besides being compatible solutes, pinitol and proline also act as osmolytes and, finally, they may serve as storage compounds for carbon and nitrogen, respectively.

For the present study, pinitol was chosen as the main focus. The biosynthetic pathway of pinitol proceeds, in legumes, in the sequence of triose-P → fructose-6-P → glucose-6-P → *myo*-inositol-1-P → *myo*-inositol → D-ononitol → D-pinitol (Fig. 1; Dittrich and Brandl, 1987). Pinitol formation, therefore, is closely related to the primary photosynthetic carbon metabolism, directly through hexose-P and indirectly through (transitory) starch and sucrose (Fig. 1). Accumulation of pinitol in response to low water potential caused by drought stress and salt stress is a widespread phenomenon. Apart from pigeonpea, other tropical legumes were found to accumu-

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Abbreviations: INV, invertase; SS, sucrose synthase; SPS, sucrose phosphate synthase; AMY, soluble starch hydrolysing activity; IMT, *myo*-inositol *O*-methyl transferase; PAD, pulsed amperometric detection; SAM, *S*-adenosyl-L-methionine; Ψ_L , leaf water potential; Ψ_T , osmotic potential; *RWC*, relative water content.

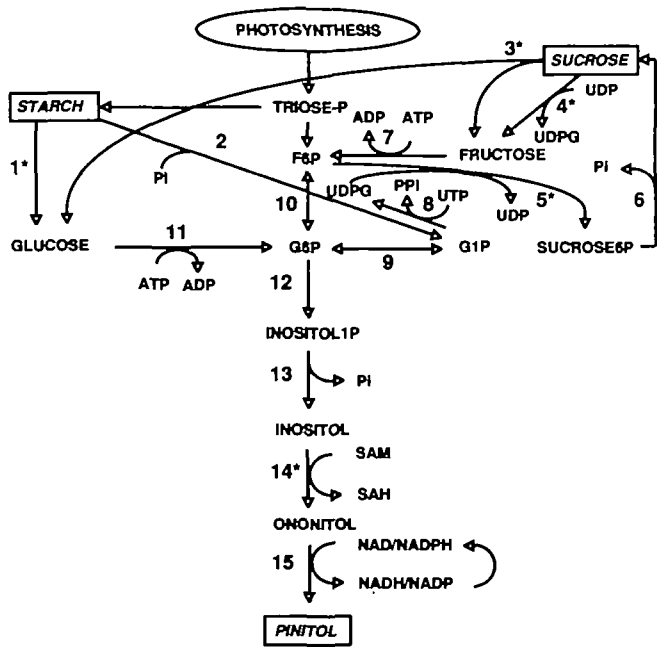


FIG. 1. Simplified scheme depicting the partitioning of carbon between starch, sucrose and pinitol in pigeonpea leaves. Enzymes: 1, total amylolytic activity; 2, starch phosphorylase; 3, invertase; 4, sucrose synthase; 5, sucrose phosphate synthase; 6, sucrose phosphate phosphatase; 7, fructokinase; 8, UDPG-pyrophosphorylase; 9, glucose phosphate mutase; 10, glucose phosphate isomerase; 11, glucokinase; 12, *myo*-inositol 1-phosphate synthase; 13, *myo*-inositol phosphate phosphatase; 14, *myo*-inositol *O*-methyl transferase; 15, pinitol epimerase. Metabolites: G1P, glucose 1-phosphate; G6P, glucose 6-phosphate; F6P, fructose 6-phosphate; SAM, *S*-adenosyl-L-methionine; SAH, *S*-adenosyl-L-homocysteine. Enzymes marked with an asterisk were measured in this study.

late pinitol as a result of drought stress (Ford, 1984). Pinitol levels were also observed to be positively correlated with salt tolerance of plants such as maritime pine (*Pinus pinaster*) (Nguyen and Lamant, 1988), mangroves (Popp, 1984), dhaincha (*Sesbania bispinosa*) (Gorham *et al.*, 1988), *Honkenya peploides* (Gorham *et al.*, 1981), and common ice plant (*Mesembryanthemum crystallinum*) (Paul and Cockburn, 1989; Adams *et al.*, 1992; Vernon and Bohnert, 1992a, b).

No information is currently available on the biochemical mechanisms regulating pinitol accumulation and mobilization due to drought stress and rewatering, respectively. Pinitol accumulation due to salt stress, however, has recently been shown, in the facultative halophyte *Mesembryanthemum crystallinum*, to be accompanied by the increased expression of a gene encoding a *myo*-inositol *O*-methyl transferase, which catalyses the first step in the biosynthetic pathway of pinitol (Vernon and Bohnert, 1992a, b). This salt-dependent up-regulation of a key enzyme of pinitol formation by transcriptional induction is a good indication that pinitol metabolism might indeed be important in the regulation of stress tolerance in plants.

The aim of this study was to obtain some insight into the biochemical mechanisms responsible for drought-

induced pinitol accumulation. We speculated that pinitol accumulation could mainly occur at the expense of starch and sucrose. Therefore, we investigated the effect of drought on the activities of some enzymes of starch, sucrose and pinitol metabolism (of the catabolic enzymes AMY, INV and SS and of the anabolic enzymes SPS and IMT) and on the levels of the main non-structural carbohydrates. We present evidence that the build-up of drought tolerance in pigeonpea is paralleled by a diversion of fixed carbon away from starch and sucrose into pinitol.

MATERIALS AND METHODS

Plant culture and leaf sampling

Pigeonpea (*Cajanus cajan* [L.] Millsp. cv. Royes) plants were grown in large plastic-lined pots (1 × 0.25 m) containing 50 kg of air-dried soil. The following nutrients were dissolved in water and added to each pot: 1.2 g CaH₄(PO₄)₂·H₂O, 0.75 g K₂SO₄, 0.07 g CuSO₄, 0.07 g ZnSO₄, and 0.004 g (NH₄)₆Mo₇O₂₄·4H₂O. Seedlings were thinned to one per pot and the soil water potential was kept at 9.8 kPa. The plants were grown in a controlled environment chamber that maintained the following conditions: 14 h photoperiod, 500 μmol m⁻² s⁻¹ photon irradiance, 30/25 °C day/night temperature and 60/90% day/night RH.

Drought stress was imposed by withholding water from the soil when the plants were 6-weeks-old. There were no nutrient deficiencies because of the added nutrients, which were mixed with the soil to ensure that the roots had access to them even when surface layers of the soil dried. Some plants were rewatered after 27 d of drought stress and their soil water potential was then kept again at 9.8 kPa.

At each sampling, the three youngest fully-expanded leaves of two similar branches of two plants each were harvested just prior to the commencement of the photoperiod, and leaf water relations were measured. Samples for enzyme assays and chemical analyses were frozen in liquid N₂ and stored at -40 °C.

Leaf water relations

Ψ_L , Ψ_{II} and *RWC* were measured as described by Flower and Ludlow (1986). Briefly, Ψ_L was measured with a pressure chamber, and *RWC* was determined gravimetrically. Ψ_{II} was measured on discs cut from leaves in a 16-channel Wescor (Logan, UT) dewpoint hygrometer, calibrated with sodium chloride solutions.

Enzyme extraction

The frozen leaf tissue (without petiole) was ground in a mortar containing an ice-cold medium consisting of 50 mol m⁻³ Hepes-NaOH (pH 7.5), 10 mol m⁻³ MgCl₂, 1 mol m⁻³ EDTA, 2.5 mol m⁻³ DTT, 0.05% (w/v) Triton X-100 and 0.1% (w/v) BSA (6 and 8 cm³ medium g⁻¹ fw for well-watered and stressed leaves, respectively). The homogenate was squeezed through two layers of Miracloth and centrifuged at 13 000 g for 1 min. The supernatant was immediately desalted by centrifugal filtration (Helmerhorst and Stokes, 1980) on a Sephadex G-25 column equilibrated with the appropriate assay buffer (see below).

Enzyme assays

All enzyme assays were carried out at 35 °C, except that IMT was assayed at 23 °C. In preliminary experiments with leaf

extracts of well-watered and drought-stressed plants, it was ascertained that all the assays were linear with time and amount of enzyme. Recombination experiments were carried out by mixing extracts of leaves with high and low enzyme activities, respectively. Comparison of the activities of the mixed samples with those of the sum of the measurements made on the separate components showed that the values for the mixed samples were always within 95% and 105% of the predicted values.

INV (EC 3.2.1.26): Acid and neutral INV activity was assayed in a reaction mixture (200 mm³) containing assay buffer A [McIlvaine, pH 5.0 or 7.6, 0.1% (w/v) BSA], 50 mol m⁻³ sucrose and 100 mm³ desalted sample. After 0 and 15 to 30 min, the reaction was terminated by addition of 50 mm³ 500 mol m⁻³ K₂HPO₄ and immediate boiling for 2 min. Glucose formed was determined using the glucose oxidase-Perid kit from Boehringer (Mannheim, Germany).

SS (EC 2.4.1.13): SS activity was assayed in the synthetic direction in a reaction mixture (90 mm³) containing assay buffer B [50 mol m⁻³ Hepes-NaOH (pH 7.5), 10 mol m⁻³ MgCl₂, 1 mol m⁻³ EDTA, 2.5 mol m⁻³ DTT and 0.1% (w/v) BSA], supplemented with 10 mol m⁻³ UDP-Glc and 10 mol m⁻³ Fru, and 45 mm³ desalted sample. After 0 and 10 to 20 min, the reaction was terminated by addition of 90 mm³ 1 N NaOH and immediate boiling for 10 min. Sucrose formed was determined by the resorcinol-HCl method of Roe (1934).

SPS (EC 2.4.1.14): SPS was assayed like SS, except that fructose was replaced by 10 mol m⁻³ Fru-6-P and 50 mol m⁻³ Glc-6-P.

AMY (mainly EC 3.2.1.1, α -amylase; some EC 3.2.1.2, β -amylase and EC 3.2.1.41, debranching enzyme): Soluble starch hydrolysing activity was assayed in a reaction mixture (145 mm³) containing assay buffer C [50 mol m⁻³ succinic acid-NaOH (pH 6.0), 1 mol m⁻³ CaCl₂ (to activate α -amylase; Li *et al.*, 1992), 0.1% (w/v) BSA], supplemented with 2% (w/v) Zulkowski soluble starch (Merck), and 45 mm³ desalted sample. After 0 and 15 min, the reaction was terminated by addition of 1 cm³ dinitrosalicylic acid reagent, boiling for 10 min, and the reducing sugars released were determined at 570 nm (Chaplin, 1986).

IMT: IMT activity was assayed by measurement of [¹⁴C]ononitol (1D-4-O-methyl-*myo*-inositol) from *myo*-inositol and [¹⁴CH₃]SAM. A simple radioactive IMT assay was developed based, in principle, on a combination of published methods (Koller and Hoffmann-Ostenhof, 1976; Miura and Chiang, 1985). The assay mixture (90 mm³) contained assay buffer D (buffer B at pH 7.0), 0 or 20 mol m⁻³ *myo*-inositol, 2 mol m⁻³ [¹⁴CH₃]SAM (3.7 kBq; 2.1 GBq mmol⁻¹; Amersham) and 45 mm³ desalted sample. After 5 h, the reaction was stopped by addition of 400 mm³ ethanol and 500 mm³ of an aqueous 1:1 slurry (v/v) of the cation exchange resin IRA 120 (16–50 mesh; NH₄⁺-form; Biorad). Unreacted SAM was removed by continuous gyratory shaking for 20 min. After centrifugation at 13 000 g for 1 min, 500 mm³ of the supernatant was combined with 3 cm³ Ultima-Gold (Canberra Packard, Zürich, Switzerland) and the radioactivity was counted in a Tri-Carb Scintillation counter (Canberra Packard). The *myo*-inositol-dependent counts were used for calculation of the IMT activity. The identity of [¹⁴C]ononitol as the assay product in the desalted supernatant was confirmed by radio-HPLC as described (Keller and Matile, 1989) using a Benson-Pb and Benson-Ca column, respectively (300 × 7.8 mm; Benson Polymeric, Reno, Nevada).

Extraction and analysis of carbohydrates and proline

Frozen leaf tissue was lyophilized and ground to a fine powder in a Waring blender. Powdered sample (50 mg) was extracted

with 5 cm³ 80% (v/v) acetonitrile in an ultrasonic bath for 10 min and centrifuged at 1000 g for 10 min. The pellet was re-extracted two additional times with 5 cm³ H₂O as above. The three supernatants were combined, brought to a volume of 15 cm³ with H₂O and passed through a 0.45 μ m membrane filter.

Soluble carbohydrates were analysed in 20 mm³ aliquots of filtered extract by HPLC-PAD. The chromatographic system consisted of a Waters WISP 710B autosampler (Waters-Millipore, Wallisellen, Switzerland), a Sykam S1000 pump (Stagroma, Wallisellen, Switzerland), a Benson-Pb column (300 × 7.8 mm), a Jones 7960 column heater (Ercatech, Berne, Switzerland) maintained at 77 °C, an ESA Coulochem II electrochemical detector (E1 = 200 mV, E2 = 700 mV, E3 = -900 mV, T1 = 500 ms, T2 = 100 ms, T3 = 100 ms, AD = 300 ms; Stagroma) and a GynkoSoft chromatography data system (Henggeler Analytic, Riehen, Switzerland). Distilled H₂O was used as the solvent at a flow rate of 0.6 cm³ min⁻¹. Post-column addition of NaOH (250 mol m⁻³; 0.2 cm³ min⁻¹) was performed pneumatically with helium at 2–3 atm. Quantitation was accomplished using peak area calculations related to regression curves of standards. Pinitol was a kind gift from Professor Amadò (Food Science, ETH, Zürich). Ononitol was isolated from *Ononis spinosa* leaves. The effectiveness of the simple extraction procedure chosen was ascertained by comparison with (i) the same procedure but extractions carried out by boiling instead of ultrasonication and (ii) the same procedure but four instead of two H₂O extractions.

Starch was determined in the leaf residue remaining after the soluble carbohydrates had been extracted. The pellet was re-extracted once more with 5 cm³ H₂O. Starch was then gelatinized in 2 cm³ H₂O in the autoclave for 2 h. An aliquot (250 mm³) was digested after addition of 250 mm³ 100 mol m⁻³ Na-acetate buffer (pH 4.8), 14 mm³ amyloglucosidase (2 U, from *Aspergillus niger*) and 3 mm³ α -amylase (4 U, from *Bacillus subtilis*) (both enzymes from Boehringer) at 37 °C for 3 h. The reaction was terminated by boiling for 10 min and the released glucose was determined as described for INV.

Proline was determined in the filtered extracts by the acidic ninhydrin procedure of Bates *et al.* (1973).

RESULTS

Leaf water relations

Withholding water from potted 6-week-old pigeonpea plants caused a gradual decrease of Ψ_L , Ψ_{II} and *RWC* of the youngest fully expanded leaves reaching values of -4.8 MPa, -5.0 MPa and 53%, respectively, after 27 d without watering (Fig. 2). Upon rewatering, the leaf water relations recovered rapidly to their initial values. Such a response is typical for a dehydration tolerant plant (Ludlow and Muchow, 1990) and is similar to that observed in earlier studies with pigeonpea (Ford, 1984; Flower and Ludlow, 1986).

Carbohydrate and proline levels

HPLC analyses of the neutral fractions of leaf extracts of well-watered and 27 d drought-stressed pigeonpea plants are shown in Fig. 3B. The most striking features are the pronounced increase of pinitol and the complete disappearance of sucrose during drought stress.

Detailed quantitative estimations of the carbohydrate levels in leaves of well-watered plants harvested at pre-

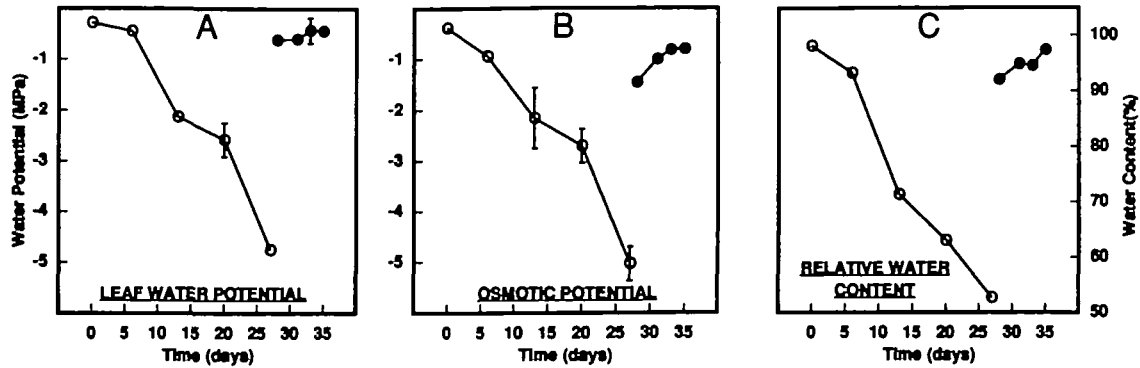


FIG. 2. Effect of drought stress on leaf water relations in pigeonpea leaves. Leaves were harvested immediately prior to the photoperiod. (A) Leaf water potential; (B) osmotic potential; (C) relative water content. Values are means \pm s.e. of four replicates. (O) Stressed plants; (●) rewatered plants (after 27 d of drought stress).

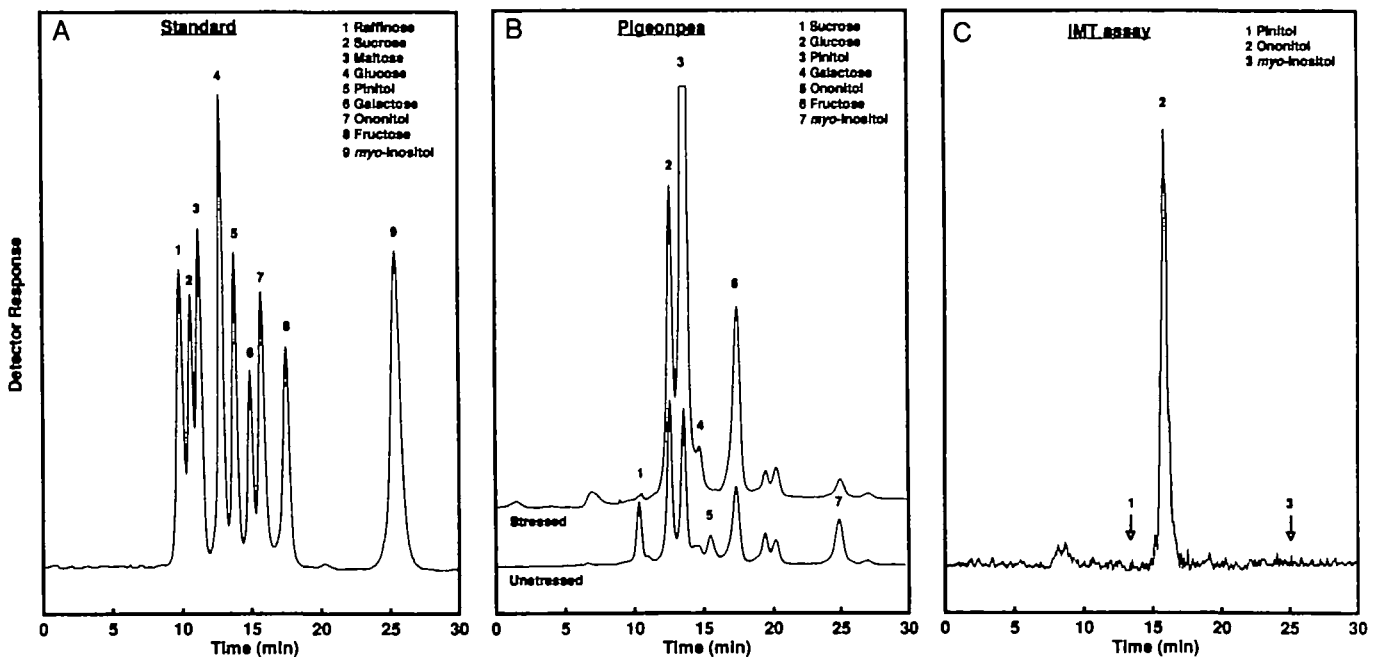


FIG. 3. HPLC chromatograms of carbohydrates found in pigeonpea leaves and identification of the neutral product of the IMT assay. (A) Standard; (B) comparison of leaf extracts of 27 d-drought-stressed and unstressed pigeonpea plants; (C) neutral product of the IMT assay. The separation was performed on a Benson-Pb column (300×7.8 mm) kept at 77°C and eluted with water at $0.6\text{ cm}^3\text{ min}^{-1}$. Detection was by pulsed amperometric detector after post-column addition of NaOH (A, B) or by radio-detector after post-column addition of scintillant (C). For details see 'Materials and Methods'.

dawn revealed that starch was predominant (71 mg g^{-1} dwt; Fig. 4A), followed by pinitol (14 mg g^{-1} dwt; Fig. 4E), sucrose (9.2 mg g^{-1} dwt; Fig. 4B), glucose (6.6 mg g^{-1} dwt; Fig. 4C), fructose (6.0 mg g^{-1} dwt; Fig. 4D), *myo*-inositol (2.6 mg g^{-1} dwt; Fig. 4F), and ononitol (1.6 mg g^{-1} dwt; Fig. 4G). These carbohydrate levels are similar to those found in other dehydration tolerant legumes such as soybean (Ford, 1984; Huber *et al.*, 1984; Huber, 1989; Bensari *et al.*, 1990). During drought stress, starch, sucrose, *myo*-inositol and ononitol disappeared almost completely, whereas glucose, fructose and especially pinitol increased to various degrees (1.4-, 2.0- and 5.8-fold, respectively) (Fig. 4A–G). After 27 d of drought

stress, pinitol accounted for 76% of the total non-structural carbohydrates found in the leaves, as compared with 12% in well-watered leaves (Fig. 4H).

Rewatering caused the levels of starch, sucrose and *myo*-inositol to recover quickly to their original values (Fig. 4A, B, F), whereas the levels of ononitol (Fig. 4G) recovered only moderately. The hexoses levelled off at their values after full drought stress (Fig. 4C, D) and pinitol (Fig. 4E) recovered to a medium value after 8 d of rewatering.

For comparison, the effect of drought on proline levels was also investigated (Fig. 4I). Proline, the second putative compatible solute of stressed pigeonpea leaves (Ford,

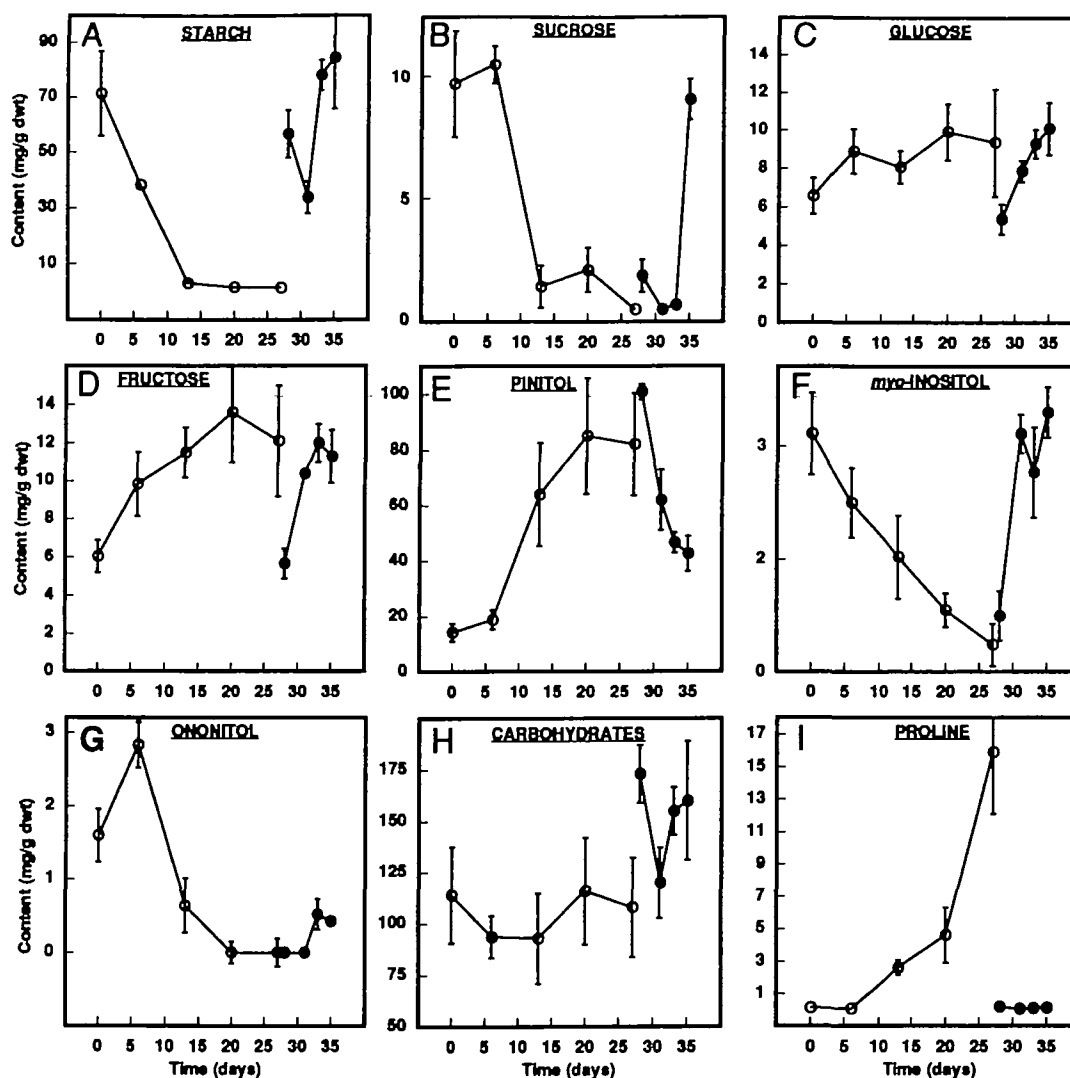


FIG. 4. Effect of drought stress on the carbohydrate and proline contents in pigeonpea leaves. Leaves were harvested immediately prior to the photoperiod. (A) Starch; (B) sucrose; (C) glucose; (D) fructose; (E) pinitol; (F) *myo*-inositol; (G) ononitol; (H) total non-structural carbohydrates; (I) proline. Values are means \pm s.e. of four replicates. (O) Stressed plants; (●) rewatered plants (after 27 d of drought stress).

1984), increased about 100-fold during stress and decreased rapidly to its low pre-stress level upon rewatering.

Enzyme activities

Drought stress induced a pronounced increase of activities of enzymes hydrolysing starch (AMY; Fig. 5A) and sucrose (INV and SS; Fig. 5B, C). AMY activity was fairly high throughout the experiment, increased 1.5-fold during the first 2 weeks of drought stress and levelled off afterwards. The exact biochemical mechanisms by which starch is degraded in pigeonpea leaves are unclear. The assay used in this study picks up the non-phosphorolytic, soluble starch degrading activity, the main one most probably being that of α -amylase (besides some β -amylase and debranching enzyme; Beck and Ziegler, 1989; Ziegler, 1990; Li *et al.*, 1992). Acid INV activity was also high

throughout the experiment. However, drought stress still induced a 2-fold increase of activity during the first 2 weeks. Neutral INV was low and did not show any change throughout the experiment. SS showed the most pronounced increase of activity of the hydrolytic enzymes tested. During the entire drought stress of 27 d, a linear increase of SS activity of up to 10-fold was observed.

The two anabolic enzymes tested were SPS and IMT. They also showed an increase of activity with drought stress of 2- and 1.5-fold, respectively. SPS activity, measured under V_{max} conditions (non-selective: saturating hexose-phosphates, no Pi; Huber and Huber, 1992), increased steadily during the first 2 weeks of drought stress. IMT activity increased only during the first 6 d and decreased steadily afterwards.

The measurement of IMT activity needed special attention since only a time-consuming assay was found in the

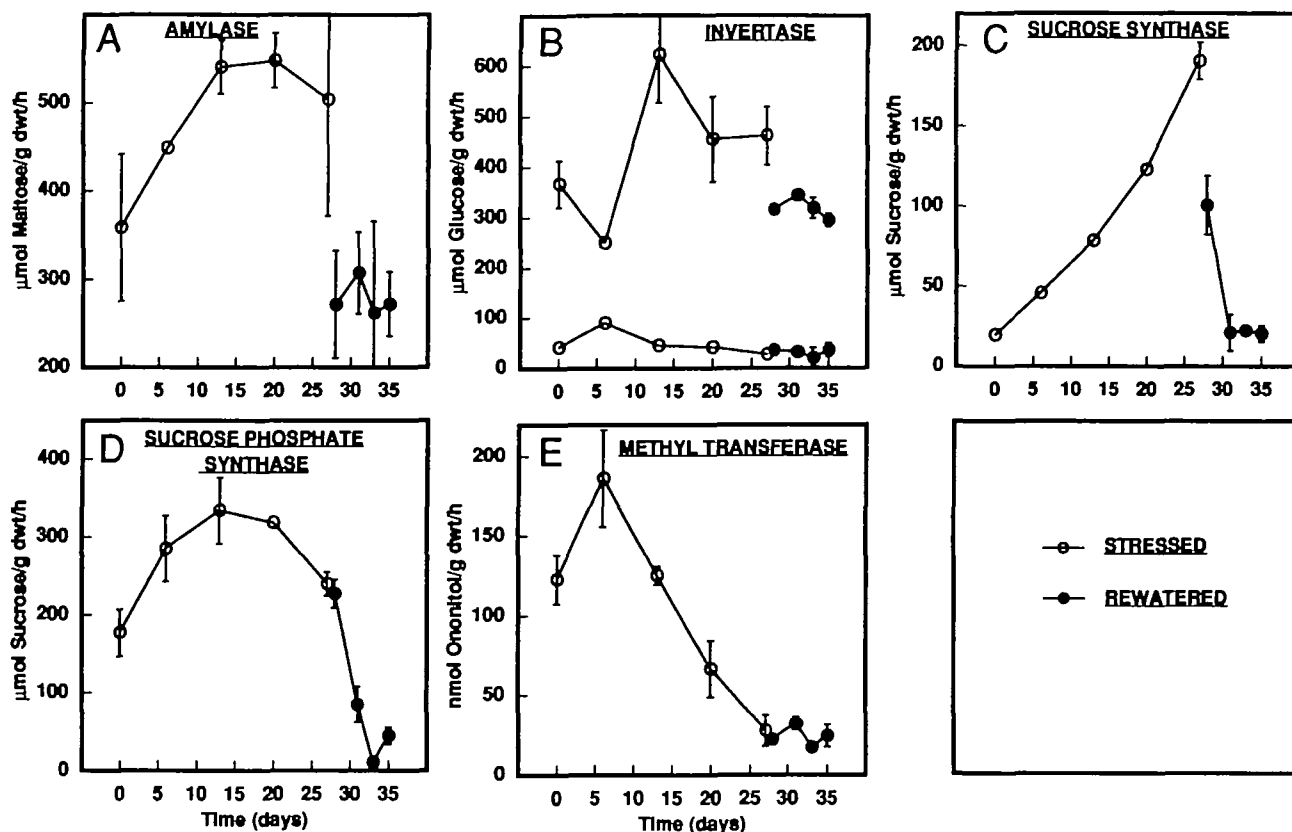


FIG. 5. Effect of drought stress on the activities of some enzymes of the carbohydrate metabolism in pigeonpea leaves. Leaves were harvested immediately prior to the photoperiod. (A) Soluble starch hydrolysing activity; (B) invertase; (C) sucrose synthase; (D) sucrose phosphate synthase; (E) *myo*-inositol *O*-methyl transferase. Values are means \pm s.e. of three replicates. (○) Stressed plants; (●) rewatered plants (after 27 d of drought stress).

literature (Koller and Hoffmann-Ostenhof, 1976). IMT activity was assayed by measurement of [14 C]ononitol (1D-4-*O*-methyl-*myo*-inositol) formed from *myo*-inositol and [14 CH $_3$]SAM. The simplified assay is based on the measurement of *myo*-inositol-dependent counts after removal of unreacted [14 CH $_3$]SAM by incubation with a cation exchange resin (Miura and Chiang, 1985). The identity of [14 C]ononitol as the assay product was confirmed by radio-HPLC on a Pb-loaded cation exchange column which has a high selectivity for polyols (Fig. 3A, C). The validity of the new IMT assay was further confirmed by demonstrating that it was linear with time for up to 6 h and with amount of enzyme used (data not shown). After completion of the experiments, an alternative IMT assay was published (Vernon and Bohnert, 1992a), which is similar to the one used in this study with the exception of the measurement of the product, [14 C]ononitol, which was performed by radio-HPLC, and, therefore, is also quite time-consuming. Furthermore, the HPLC column used by Vernon and Bohnert (1992a), a Ca-loaded cation-exchange resin, does not separate ononitol from pinitol (Adams *et al.*, 1992 and unpublished observation), whereas the Pb-loaded column used for the

identification of the IMT product in this study does (Fig. 3A).

Rewatering of the stressed plants caused the activities of AMY, INV and SS to be quickly restored to their original levels, whereas those of SPS and IMT decreased further (SPS) or stayed low (IMT) during 8 d of rewatering (Fig. 5).

DISCUSSION

The slow drought stress imposed on 6-week-old pigeonpea plants grown in large pots caused a gradual decrease of Ψ_L , Ψ_{II} and *RWC* of the youngest fully expanded leaves. After 27 d without watering, the values for Ψ_L , Ψ_{II} and *RWC* were -4.8 MPa, -5.0 MPa and 53%, respectively (Fig. 2). In earlier experiments, it was shown that these leaves lose turgor at a Ψ_L of -2.5 MPa and cease net photosynthesis at -3.5 MPa. However, they did not die until Ψ_L and *RWC* had reached values of -6.3 MPa and 32%, respectively (Flower, 1985; Flower and Ludlow, 1986). The highest level of drought stress chosen for this experiment was, therefore, clearly below lethal and a positive net photosynthetic activity was maintained for at least 3 weeks of stress.

The main focus of this study was to investigate the effect of drought on the metabolism of non-structural carbohydrates. The total content of non-structural carbohydrates found in pigeonpea leaves remained constant during drought stress (Fig. 4H). The relative composition of the non-structural carbohydrates, however, changed dramatically. After 27 d of stress, the levels of pinitol increased 6-fold (from 14 to 85 mg g⁻¹ dwt; Fig. 4E), whereas the levels of starch (Fig. 4A), sucrose (Fig. 4B) and the pinitol precursors *myo*-inositol (Fig. 4F) and ononitol (Fig. 4G) decreased to zero or near-zero. Glucose and fructose levels increased moderately (Figs 4C, D). Decrease of starch levels with drought stress has been observed in leaves of many different plant species such as soybean (Huber *et al.*, 1984; Bensari *et al.*, 1990), sugar beet (Fox and Geiger, 1986; Harn and Daie, 1992), spinach (Zrenner and Stitt, 1991), resurrection plants (Gaff, 1989), lupin, eucalyptus, sunflower, and grapevine (Quick *et al.*, 1992). Decreased starch levels are often the result of both decreased starch formation and increased starch degradation. Decrease of sucrose levels with drought stress has also been observed (in four tropical legumes; Ford, 1984), but it seems to be the exception rather than the rule. A decrease in the levels of *myo*-inositol and ononitol with drought stress is not surprising as they are the direct precursor molecules of pinitol. Their pool sizes are relatively small as compared with pinitol suggesting rapid throughput and efficient anabolic enzymes.

In well-watered plants, starch and sucrose accounted for 71% of the total non-structural carbohydrates (63% and 8%, respectively), whereas pinitol accounted for 12%. After drought stress, pinitol accounted for 76% of the non-structural carbohydrates, whereas starch and sucrose were almost absent. This change of relative composition of the non-structural carbohydrates indicates, but does not prove, that pinitol accumulation during drought stress might mainly occur at the expense of starch and sucrose. Estimations, on a molar basis, provide further evidence for a positive correlation between the decrease in starch and sucrose and the increase in pinitol. During the 27 d stress period, starch and sucrose decreased by 350 μ mol hexose units g⁻¹ dwt (70 mg g⁻¹ dwt) and 58 μ mol hexose units g⁻¹ dwt (10 mg g⁻¹ dwt), respectively. This total decrease of 408 μ mol hexose units g⁻¹ dwt corresponds quite well to the increase in pinitol which amounted to 366 μ mol hexose units g⁻¹ dwt (71 mg g⁻¹ dwt).

Production of pinitol and other osmotica from stored carbohydrates such as starch and sucrose, rather than from current photosynthate, is consistent with observed patterns of osmotic adjustment in pigeonpea leaves during drought stress (Flower, 1985). Leaf photosynthesis ceases at a leaf water potential of -3.5 MPa. However, osmotic adjustment is only half its maximum value at this water potential. Thus osmotic adjustment that occurs between

leaf water potentials of -3.5 and -6.3 MPa must be based on solutes produced from carbon that was fixed at higher (less negative) water potentials.

To test the suggestion further that pinitol accumulation might mainly occur at the expense of starch and sucrose the activities of enzymes hydrolysing starch (AMY) and sucrose (INV and SS) and of the enzyme methylating *myo*-inositol (IMT) were measured. Drought stress induced a pronounced increase of the activities of the hydrolytic enzymes AMY, INV and SS (Fig. 5A-C). The net result of such an increase of activities would be an increased supply of hexoses and hexose-phosphates, which could be further utilized for pinitol formation (Fig. 1). As the pool of free hexoses increased only moderately (Fig. 4C, D) and the activity of one enzyme of the pinitol synthetic pathway (IMT) showed an initial temporary increase of activity (Fig. 5E) the suggestion is strengthened that pinitol accumulation might indeed proceed by deviation of the carbon flux away from starch and sucrose into cyclitols (via hexoses).

Sucrose metabolism seems to be dynamic and responsive to drought stress in pigeonpea leaves. In well-watered plants, sucrose levels are quite apparent (10 mg g⁻¹ dwt; Fig. 4B) despite high sucrolytic activities (mainly acid INV; Fig. 5B). This is most probably due to differential compartmentation. Sucrose synthesis (by SPS) and storage occurs in the cytosol whereas any surplus sucrose would be hydrolysed by acid INV in the vacuole giving rise to free glucose and fructose. In drought-stressed plants, sucrose is absent in leaves despite the increase of SPS activity. The main reason for this might be the increased hydrolytic SS activity in the cytosol (besides increased acid INV activity in the vacuole). A futile cycling of concurrent sucrose synthesis and degradation cannot be ruled out, particularly in stressed leaves. Futile cycling of sucrose has been described for a number of different plant systems such as suspension cultures of *Chenopodium rubrum* (Dancer *et al.*, 1990) and sugar cane (Wendler *et al.*, 1991), leaves of several 'high-invertase-type' species (Huber, 1989), banana fruits (Hubbard *et al.*, 1990) and cotyledons of *Ricinus communis* (Geigenberger and Stitt, 1991). Its function has been suggested to allow an increased sensitivity of metabolic regulation (Hue, 1980).

As expected from this coarse, survey-type study several important questions have to remain unanswered and will need special attention in a subsequent study. The main efforts will be directed towards the unambiguous determination of the key-enzyme(s) responsible for stress-induced pinitol accumulation. Potential candidates for such enzymes include IMT, the enzymes of starch metabolism and the enzymes leading to the synthesis of the ononitol precursor *myo*-inositol (Fig. 1). For this purpose, we will need to (i) characterize IMT biochemically and reinvestigate its change of activity during drought,

(ii) measure the enzyme activities of *myo*-inositol synthesis, i.e. *myo*-inositol 1-phosphate synthase and *myo*-inositol 1-phosphate phosphatase (Loewus, 1990), (iii) resolve the relative contributions of all the possible starch degrading enzymes, i.e. α - and β -amylase, debranching enzyme and starch phosphorylase (Beck and Ziegler, 1989; Ziegler, 1990; Li *et al.*, 1992), and (iv) consider the main enzyme responsible for starch formation in chloroplasts, ADP glucose phosphorylase (Preiss, 1991), to assess if the starch depletion observed with drought stress is mainly due to decreased formation or increased degradation of starch.

Finally, it has to be kept in mind that the observed pinitol accumulation is only one of the possible mechanisms responsible for pigeonpea's drought tolerance. This study indicates that proline accumulation (from 0 to 16 mg g⁻¹ dwt) might be equally important, creating additional questions such as how is proline formation stress-regulated and, on a broader scale, how is it linked to the carbon metabolism (Champigny and Foyer, 1992).

In conclusion, this study provides clear evidence that drought stress, in pigeonpea, induces the accumulation of pinitol (besides proline). It indicates that pinitol accumulation might be caused by deviation of the carbon flux away from starch and sucrose.

ACKNOWLEDGEMENTS

We would like to thank Helen Greutert and Frank Lameree for their competent technical assistance and Steve Huber, Philippe Matile and Marianne Popp for their critical readings of the manuscript. F.K. was a recipient of a travel grant of the Ciba-Geigy Foundation. This work was supported by the Swiss National Foundation.

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