Evidence for carbon flux shortage and strong carbon/nitrogen interactions in pea nodules at early stages of water stress

Loli Gálvez, Esther M. González and Cesar Arrese-Igor*

Departamento de Ciencias del Medio Natural, Universidad Pública de Navarra, Campus de Arrosadia, E-31006 Pamplona, Spain

Received 11 February 2005; Accepted 17 June 2005

Abstract

Symbiotic N₂ fixation in legume nodules declines under a wide range of environmental stresses. A high correlation between N₂ fixation decline and sucrose synthase (SS; EC 2.4.1.13) activity down-regulation has been reported, although it has still to be elucidated whether a causal relationship between SS activity down-regulation and N₂ fixation decline can be established. In order to study the likely C/N interactions within nodules and the effects on N₂ fixation, pea plants (Pisum sativum L. cv. Sugar snap) were subjected to progressive water stress by withholding irrigation. Under these conditions, nodule SS activity declined concomitantly with apparent nitrogenase activity. The levels of UDP-glucose, glucose-1-phosphate, glucose-6-phosphate, and fructose-6-phosphate decreased in water-stressed nodules compared with unstressed nodules. Drought also had a marked effect on nodule concentrations of malate, succinate, and α-ketoglutarate. Moreover, a general decline in nodule adenylate content was detected. NADP⁺-dependent isocitrate dehydrogenase (ICDH; EC 1.1.1.42) was the only enzyme whose activity increased as a result of water deficit, compensating for a possible C/N imbalance and/or supplying NADPH in circumstances that the pentose phosphate pathway was impaired, as suggested by the decline in glucose-6-phosphate dehydrogenase (G6PDH; EC 1.1.1.49) activity. The overall results show the occurrence of strong C/N interactions in nodules subjected to water stress and support a likely limitation of carbon flux that might be involved in the decline of N₂ fixation under drought.

Key words: Drought, isocitrate dehydrogenase, nitrogen fixation, nodule metabolism, pentose phosphate pathway, Pisum sativum L., sucrose synthase, sugar-phosphates.

Introduction

N₂ fixation depends on an adequate combination of carbon, nitrogen, and oxygen fluxes within nodules. Photosynthate in the form of sucrose is the ultimate source of carbon required for both N₂ fixation and ammonia assimilation (Gordon et al., 1998). Once in the nodule, sucrose can be cleaved by either sucrose synthase (SS; EC 2.4.1.13) or alkaline invertase (AI; EC 3.2.1.26). Studies with rug4 mutants of pea (Pisum sativum L.) showed that SS is essential for nodule functioning (Gordon et al., 1999). Hexose-phosphates derived from sucrose cleavage are metabolized through the glycolytic pathway to render phosphoenolpyruvate, which is converted to malate via the combined action of phosphoenolpyruvate carboxylase (PEPC; EC 4.1.1.31) and malate dehydrogenase (MDH; EC 1.1.1.82). Multiple evidence supports that C₄-dicarboxylates are the main products of sucrose breakdown supplied to bacteria to fuel N₂ fixation (Lodwig and Poole, 2003). Malate is the most abundant organic acid in nodules and it is involved in many critical functions. It provides a significant portion of carbon skeletons for the assimilation of fixed nitrogen (Rosendahl et al., 1990), it is the preferred substrate for bacteroid respiration (Lodwig and Poole, 2003) and it may also be involved in the regulation of oxygen diffusion through an osmoelectrical mechanism (Gálvez et al., 2000). The fact that ineffective nodules have strikingly reduced levels of malate...
compared with effective nodules is evidence of the pivotal role that malate plays in nodules (Schulze, 2004).

It has been widely reported that biological N₂ fixation in legume nodules declines under drought and other environmental stresses (Zahran, 1999). SS has been shown to be the first nodule enzyme activity that declines under water stress (González et al., 1995) and other environmental constraints (Gordon et al., 1997), and a strong correlation between nodule SS activity and N₂ fixation under a variety of stresses has been reported (Gordon et al., 1997; Arrese-Igor et al., 1999). Under drought, an accumulation of sucrose takes place in nodules as a result of SS down-regulation (González et al., 1995, 1998). It has been hypothesized that this down-regulation of the glycolytic pathway might provoke a shortage of substrates for bacteroid respiration, and, as a consequence, a transient accumulation of oxygen in the infected region would occur leading to an increase in the resistance of the oxygen diffusion barrier in order to avoid nitrogenase damage. Both the depletion of respiratory substrates and the concomitant closure of the oxygen diffusion barrier would cause the observed decline in N₂ fixation (González et al., 2001). However, it still remains to be ascertained if the reduced SS activity is responsible for the subsequent decrease in N₂ fixation rate or whether the control process takes place in the opposite sense. If SS activity decline in response to environmental stresses precedes that of N₂ fixation, a subsequent reduction of carbon flux and, hence, a depletion of available dicarboxylic acids for bacteroids would be expected. On the contrary, if N₂ fixation impairment precedes that of SS, an accumulation of organic acids would take place.

Moreover, if water stress provokes SS activity down-regulation, carbon metabolism would be altered, and other metabolic pathways related to carbon metabolism in nodules could, in turn, be affected. The role of the oxidative pentose phosphate pathway (OPPP) for carbon supply to bacteroids would become particularly relevant under these conditions. In non-photosynthetic tissues, the OPPP has been proposed as the main site of production of the required reducing power (Thom et al., 1998). Glucose-6-phosphate dehydrogenase (G6PDH; EC 1.1.1.49) activity, the first and rate-limiting enzyme of the pathway, and 6-phosphogluconate dehydrogenase (6PGDH; EC 1.1.1.44) activity constitute the oxidative part of the pathway and produce NADPH in their respective reactions. Furthermore, the OPPP together with NADP⁺-isocitrate dehydrogenase activity (ICDH; EC 1.1.1.42) and the malic enzyme (EC 1.1.1.40) are considered to be the main sources of NADPH and substrates for energy-yielding metabolism of bacteroids, in addition to carbon skeletons for ammonia assimilation (Chopra et al., 2002). Thus, an appropriate operation of these NADPH-generating enzymes is considered to be physiologically important to ensure an adequate supply of NADPH and carbon intermediates. Under water stress conditions, an imbalance between production and scavenging of reactive oxygen species leads to oxidative stress (Moran et al., 1994). Therefore, reducing power in the form of NADPH will be required by the antioxidant ascorbate/glutathione pathway, which implies that both OPPP and ICDH activity might be of crucial relevance. Due to its function as a carbon skeleton supplier, ICDH activity in water-stressed pea nodules may provide an indication of the C/N balance within nodules under these conditions.

The aim of this study was to ascertain whether a carbon flux limitation is taking place in pea nodules subjected to mild water deficit. This has been achieved by monitoring nodule enzyme activities related to carbon metabolism, together with the levels of sugar-phosphates and organic acids in nodules subjected to drought stress covering a range of intensity from very mild to severe.

Materials and methods

Growth conditions

Pea plants (Pisum sativum L. cv. Sugar snap) were inoculated with Rhizobium leguminosarum bv. viciae strain NLV 8 which is hup⁺, according to Southern-blot analysis of EcoRI digested total DNA using a hup-specific DNA probe prepared by dioxigenin labelling of cosmid pAL618 containing the entire hup gen cluster from Rhizobium leguminosarum bv. viciae strain UPM791 (Matamoros et al., 1999). Plants were grown in 1.0 I pots, with a 1:1 (v:v) mixture of vermiculite-perlite as substrate in a controlled environmental chamber (22/18 °C day/night temperature, 70% relative humidity, 500 μmol m⁻² s⁻¹ (PPF), and 15 h photoperiod). Two plants per pot were sown; one of them was removed after 10 d. Plants were watered manually three times a week with a nutrient solution lacking nitrogen (Rigaud and Puppo, 1975).

Experimental procedures and water relations

Experiments were carried out when plants were 4 weeks-old. At that time, plants were separated randomly into two sets: control and water-stressed plants. Control plants were supplied daily with nutrient solution to field capacity whereas water stress was imposed to the other group by withholding water/nutrients. For every independent series, four droughted plants and their corresponding controls were harvested at days 4, 7, 9, and 12 after the onset of drought in order to obtain very mild, mild, moderately, and severely water-stressed plants. Leaf water potential was measured in the first youngest fully-expanded leaf 2 h after the beginning of the photo-period using a pressure chamber (Soil Moisture Equipment, Santa Barbara, CA, USA) as described by Scholander et al. (1965). Nodule water potential was determined by a psychrometer Wescor HR-33T (Wescor Inc. 5500, Logan, UT, USA). Nodules were harvested, frozen in liquid N₂ and stored at −80 °C for further analysis. Roots and shoots were separated and dried for 48 h at 70 °C for dry weight determinations.

Gas exchange measurements

Net photosynthesis was measured in the second fully-expanded leaf with a portable IRGA (LI-6200, Li-Cor, Lincoln, NE, USA). For apparent nitrogenase activity (ANA) determinations, H₂ evolution of intact plants with root systems sealed in the growth pots and housed inside a chamber, was measured in an open flow-through system under N₂:O₂ (79%:21%) according to Witty and Minchin (1998).
using an electrochemical H2 sensor (Quibit System Inc., Canada). The H2 sensor was calibrated with high purity gases (Praxair, Madrid, Spain) using a gas mixer (Air Liquide, Madrid, Spain) flowing at the same rate as the sampling system (500 ml min⁻¹).

**Extraction and assay of enzymes**

Nodules were homogenized in a mortar and pestle with 50 mol m⁻³ MOPS, 20% PVPP, 10 mol m⁻³ DTT, 10 mol m⁻³ 2-mercaptoethanol, 1 mol m⁻³ EDTA, 20 mol m⁻³ KCl, and 5 mol m⁻³ MgCl₂, pH 7 at 0–2 °C (5 cm² g⁻¹ fresh weight). The homogenate was centrifuged for 30 min at 20 000 g at 4 °C. Samples (50 mm³) of the supernatant were retained for plant fraction protein (Bradford, 1976) and for the assays of phosphoenolpyruvate carboxylase (PEPC) (EC 4.1.1.31), NADP-dependent glucose-6-phosphate dehydrogenase (G6PDH) (EC 1.1.1.49), and NADP-dependent 6-phosphogluconate dehydrogenase (6PGDH) (EC 1.1.1.44). One cm³ aliquots were desalted by low speed centrifugation (180 g, 1 min) through 5 cm³ columns of Bio Gel P6DG (Bio-Rad) equilibrated with 50 mol m⁻³ MOPS pH 7, 20 mol m⁻³ KCl, and 5 mol m⁻³ MgCl₂. The desalted extract was used to determine leghaemoglobin (Appleby and Bergersen, 1980) and the following enzyme activities: sucrose synthase (SS) (EC 2.4.1.13), alkaline invertase (AI) (EC 3.2.1.26), UDP-glucose pyrophosphorylase (UDPGPP) (EC 2.7.7.9), malate dehydrogenase (MDH) (EC 1.1.1.37), glutamine synthetase (GS) (EC 6.3.1.2), glutamate synthase (GOGAT) (EC 1.4.1.14), aspartate aminotransferase (AAT) (EC 2.6.1.1), NADP-dependent isocitrate dehydrogenase (ICDH) (EC 1.1.1.42), pyruvate decarboxylase (PDC) (EC 1.2.4.1), and alcohol dehydrogenase (ADH) (EC 1.1.1.1). PEPC, SS, AI, GS, and AAT were assayed according to González et al. (1995). UDPGPP and MDH were assayed according to Gordon and Kessler (1990), GOGAT as described by Groat and Vance (1981), G6PDH and 6PGDH according to Copeland et al. (1989), ICDH according to Ferri et al. (2000), and PDC and ADH according to John and Greenway (1976).

**Isoenzymes of NADP-dependent dehydrogenases activities**

The same amount of nodule soluble crude protein (see above) of control and water-stressed plants was loaded on each well of the precast gradient (10–15%) native polyacrylamide gels. Electrophoresis was performed at 4 °C in a Phast System apparatus (Amersham Biosciences, Upsala, Sweden). After electrophoresis, gels were stained for ICDH activity according to the method described by Canino et al. (1996). Gels were incubated at 30 °C for 30–60 min in the dark with 60 mol m⁻³ TRIS-NaOH pH 8.5, 10 mol m⁻³ citrate, 2 mol dm⁻³ LiOH, 6 mol m⁻³ boric acid, 4 mol m⁻³ isocitrate, 0.2 mol m⁻³ NADP⁺, 15 mol m⁻³ MgCl₂, 80 mol m⁻³ MnCl₂, 0.13 mol m⁻³ PMS, and 0.5 mol m⁻³ MTI. Gels were stained for GDPDH activity according to Hong and Copeland (1991) incubating them in the dark with 60 mol m⁻³ TRIS-PH 8.5, 25% glycerol, 1 mol m⁻³ glucose-6-phosphate, 0.2 mol m⁻³ NADP⁺, 15 mol m⁻³ MgCl₂, 0.13 mol m⁻³ PMS, and 0.5 mol m⁻³ MTI. Gels were stained for 6PGDH activity according to Hong and Copeland (1992) by incubating them for 30–60 min in the dark with 60 mol m⁻³ TRIS-PH 8.5, 25% glycerol, 2 mol m⁻³ 6-phosphogluconate, 0.2 mol m⁻³ NADP⁺, 15 mol m⁻³ MgCl₂, 0.13 mol m⁻³ PMS, and 0.5 mol m⁻³ MTI.

**Sucrose and sugar-phosphates determination**

For sucrose determination, frozen nodules were extracted in boiling 80% (v/v) ethanol. Ethanol soluble extracts were dried in a Turbobov LV evaporator (Zymark Corp. Hopkinton, MA, USA) and soluble compounds were redissolved with 4 cm³ of distilled water, mixed and centrifuged at 20 000 g for 10 min. Sucrose content was measured in the supernatant according to González et al. (1995).

**Effects of water stress on nodule carbon metabolism**

For sugar-phosphates determination, nodule samples were stored at −80 °C in 16% (w/v) trichloroacetic acid (TCA) dissolved in diethyl ether. Samples were processed according to Curioni et al. (1999) with minor modifications. Frozen nodules were homogenized to a fine powder in liquid N₂ with a mortar and pestle. A 1.5 cm³ aliquot of 16% (w/v) TCA in distilled water containing 5 mol m⁻³ EGTA was added. The homogenate was centrifuged for 5 min at 15 000 g at 4 °C. The supernatant was washed three times with diethyl ether and neutralized with 5 mol dm⁻³ KOH with 1 mol dm⁻³ triethylamine until pH 7.3 was reached. Samples were purified through a resin (Dowex® 50 mx8 AG, SERVA Electrophoresis GmbH, Heidelberg, Germany) previously equilibrated with water and then filtered through 0.2 μm PVDF filters. Glucose-1-phosphate (G1P), glucose-6-phosphate (G6P), fructose-6-phosphate (F6P), and UDP-glucose (UDPG) were determined by ion chromatography in a DX-500 system (Dionex, Salt Lake City, UT, USA) by gradient separation with a CarboPac 10 column according to the application method suggested by the supplier (100 mol m⁻³ NaOH/100 mol m⁻³ sodium acetate to 100 mol m⁻³ NaOH/500 mol m⁻³ sodium acetate in 40 min).

**Organic acids and nucleotides determination**

Nodule samples were stored at −80 °C in 9.8% (w/v) TCA dissolved in diethyl ether. Samples were processed according to Wilson and Harris (1966) with minor modifications. Frozen nodules were homogenized to a fine powder in liquid N₂ with a mortar and pestle. A 1.5 cm³ aliquot of 5% (w/v) TCA in water was added. The homogenate was centrifuged for 10 min at 1750 g, at 4 °C. The aqueous phase was washed three times with diethyl ether saturated with water. The ether was discarded and the aqueous phase was kept. This phase was purged with N₂ for 2 min and then filtered through a 0.45 μm syringe filter.

Succinate, malate, α-ketoglutarate and citrate levels were determined by ion chromatography in a DX-500 system (Dionex) by gradient separation with a Dionex IonPac AS11 column according to the application method suggested by the supplier (2.5 mol m⁻³ NaOH/18% methanol to 45 mol m⁻³ NaOH/18% methanol in 13 min).

AMP, ADP, ATP, and UDP were determined by High-Performance Capillary Electrophoresis in a PACE System 5500 (Beckman, Fullerton, CA, USA). The electrophoreses were 20 mol m⁻³ TRIS and 20 mol m⁻³ Na₂HPO₄·7H₂O pH 7.0, containing 100 mol m⁻³ DTAB and 1 mol m⁻³ EDTA. The applied potential was 30 KV and the capillary was 50 μm inside diameter and 62/69 cm long. Detection was performed at 260 nm with an UV detector. Adenylate energy charge (AEC) was calculated as [ATP]+0.5 [ADP]+[AMP]+[ADP]⁺[ATP] according to Pradet and Raymond (1983).

**Statistical analysis**

The whole experiment was repeated four times with five replicates per treatment and day. Physiological determinations were measured in all runs and nodule material was divided for the different extractions. Results were examined by two-way analysis of variance. Field effects discussed in this study were significant at P < 0.05 in Fisher’s (protected) least significant difference (LSD) among means. Principal component analysis (PCA) rotated with the orthogonal transformation varimax was performed with those variables significantly affected by mild water stress (day 7). The SPSS software package 12.0 for Windows was used for PCA and visualization.

**Results**

Water stress provoked a gradual and progressive decline of both leaf and nodule water potential (Fig. 1A, B) that...
showed significant differences after 4 d of water stress imposition compared with well-watered plants which maintained a water potential about $-0.50/-0.60$ MPa throughout the study (Fig. 1A, B). A significant decline in net photosynthesis was also detected after 4 d of withholding water (Fig. 1C) caused by stomatal closure (data not shown). In addition, a slight decline of the shoot growth together with an increase of the root growth (data not shown), which is considered an adaptive response to drought, was also observed.

Nodule plant fraction protein content, along with nodule enzyme activities related to carbon and nitrogen metabolism and nitrogen fixation showed a decreasing trend as water stress progressed. The most immediate and drastic response to drought was that observed in ANA (Fig. 2A) and SS (Fig. 2C) and G6PDH (Fig. 3B) activities, which experienced a decrease of 27%, 40%, and 100%, respectively, by day 4, compared with their corresponding controls, before any change in other enzyme activities related to carbon or nitrogen metabolism could be detected. The decline in ANA was not statistically significant at that stage, mostly because of the variation of controls, but its further decrease resulted in significant differences thereafter (Fig. 2A). SS activity showed a significant decline in water-stressed nodules throughout the studied period (Fig. 2C). Plant fraction protein content, as well as Al, UDPGPP, GS, and AAT activities were affected after 9 d of water deprivation (Fig. 2B, D, E, G, H) while PEPC activity only significantly declined in drought-stressed nodules by day 12 (Fig. 2F). No differences were found between unstressed and stressed nodules in MDH and GOGAT activities, whose values were $13.4\pm0.6$ and $0.045\pm0.001$ \(\mu\)mol NADH mg\(^{-1}\) protein min\(^{-1}\), respectively. Furthermore, PDC and ADH activities did not differ in nodules under drought conditions from the values of control nodules ($5\pm0.5$ nmol NADH mg\(^{-1}\) protein min\(^{-1}\) and $0.15\pm0.01$ \(\mu\)mol NADH mg\(^{-1}\) protein min\(^{-1}\), respectively) indicating that fermentative metabolism was not induced by water stress.

From the NADPH-generating enzymes, ICDH activity showed opposite behaviour to that shown by the rest of activities, since a gradual increase as nodule water potential declined was observed, being significant at days 9 and 12 (Fig. 3A). This increment in ICDH activity was further confirmed by activity staining following native-PAGE. Four ICDH isoenzymes were detected in pea nodules, namely I, II, III, and IV which were all activated under water deficit (Fig. 3A). Isoenzyme II, and to a lesser extent isoenzyme IV, showed an enhanced signal with respect to isoenzymes I and III, under both control conditions and also under the mildest stages of water stress (days 4 and 7). As drought stress progressed (day 9), the most activated isoenzymes were I and III. Nevertheless, all the isoenzymes showed a strong activation when water stress became more severe (day 12). However, other NADPH-generating enzyme activities showed different responses to water stress: a sharp decline in G6PDH activity was observed 4 d after the onset of water deficit in nodules, reaching no detectable values (Fig. 3B). Activity staining of native-PAGE confirmed the above results of G6PDH: five isoenzymes were found, with a prominent isoenzyme (III) in control nodules that was undetectable at any level of water stress (Fig. 3B). In turn, no significant differences were observed in 6PGDH activity between control and water-stressed nodules (Fig. 3C). Two prominent bands (I and II) and a much fainter third band (III) of 6PGDH activity were observed. Only isoenzyme I appeared to be gradually affected by water stress, with this response being attenuated at day 4 (Fig. 3C).

Water stress caused a decline in nodule sugar-phosphates concentration (Fig. 4). Nodule UDPG, G1P, and G6P concentrations significantly declined 9 d after the onset of water stress (Fig. 4A, B, C), experiencing further slight reductions afterwards. Thus, the levels of these metabolites at the end of the study represented 37% (Fig. 4A), 52% (Fig. 4B), and 21% (Fig. 4C) of their corresponding controls. Nodule F6P concentration was only significantly affected at the end of the drought period, showing a decline of 40% (Fig. 4D).
Both nodule succinate and malate content decreased as nodule water potential became more negative (Fig. 5A, B). Malate concentration was remarkably higher than any of the other organic acids determined (Fig. 5B). While a significant reduction in succinate content was only observed at the end of the study period (Fig. 5A), malate content decreased by day 7, further decreasing thereafter (Fig. 5B). A significant decrease in the level of $\alpha$-ketoglutarate occurred 7 d after the onset of the treatment, although by day 9, $\alpha$-ketoglutarate content regained control values (Fig. 5C). Citrate content in stressed and unstressed nodules did not show any significant variation throughout the studied period keeping an average value of $1.01 \pm 0.04$ mM. On the other hand, sucrose content of drought-stressed nodules experienced a gradual increase that became significant at day 7, and was more pronounced as water potential declined (Fig. 5D).

Nodule AMP, ADP, and ATP contents were close to control values at the first stages of drought, experiencing a gradual decline afterwards. AMP content showed a significant decline at the end of the study (Fig. 6A). An earlier effect of drought was observed in nodule ADP and ATP contents, which declined significantly by day 7, further decreasing after 9 d and 12 d of water deficit imposition (Fig. 6B, C). However, AEC maintained a fairly constant value throughout the study, showing no differences between control and water-stressed nodules ($0.72 \pm 0.02$), which indicates that adenylate synthesis might be affected by water stress, but not energy availability. The response of UDP content in nodules subjected to drought was rather particular: it experienced a transient significant accumulation at day 4, sharply decreasing at day 7 with respect to its corresponding control. This decline became significant after 9 d of withholding water (Fig. 6D).

Fig. 2. Effect of water stress on apparent nitrogenase activity, nodule protein content and nodule enzyme activities of carbon and nitrogen metabolism. Apparent nitrogenase activity (A), nodule plant fraction protein content (B), and sucrose synthase (C), alkaline invertase (D), UDP-glucose pyrophosphorylase (E), phosphoenolpyruvate carboxylase (F), glutamine synthetase (G), and aspartate aminotransferase (H) activities of control and water-stressed pea nodules. Prot, NDW, and Gh denote protein, nodule dry weight, and glutamylhydroxamate, respectively. For each parameter, an asterisk represents significant differences with the corresponding control at $P < 0.05$. Values represent mean ± standard error ($n=12$).

Both nodule succinate and malate content decreased as nodule water potential became more negative (Fig. 5A, B). Malate concentration was remarkably higher than any of the other organic acids determined (Fig. 5B). While a significant reduction in succinate content was only observed at the end of the study period (Fig. 5A), malate content decreased by day 7, further decreasing thereafter (Fig. 5B). A significant decrease in the level of $\alpha$-ketoglutarate occurred 7 d after the onset of the treatment, although by day 9, $\alpha$-ketoglutarate content regained control values (Fig. 5C). Citrate content in stressed and unstressed nodules did not show any significant variation throughout the studied period keeping an average value of $1.01 \pm 0.04$ mM. On the other hand, sucrose content of drought-stressed nodules experienced a gradual increase that became significant at day 7, and was more pronounced as water potential declined (Fig. 5D).

Nodule AMP, ADP, and ATP contents were close to control values at the first stages of drought, experiencing a gradual decline afterwards. AMP content showed a significant decline at the end of the study (Fig. 6A). An earlier effect of drought was observed in nodule ADP and ATP contents, which declined significantly by day 7, further decreasing after 9 d and 12 d of water deficit imposition (Fig. 6B, C). However, AEC maintained a fairly constant value throughout the study, showing no differences between control and water-stressed nodules ($0.72 \pm 0.02$), which indicates that adenylate synthesis might be affected by water stress, but not energy availability. The response of UDP content in nodules subjected to drought was rather particular: it experienced a transient significant accumulation at day 4, sharply decreasing at day 7 with respect to its corresponding control. This decline became significant after 9 d of withholding water (Fig. 6D).

Discussion

Water potential and photosynthesis

This study has been focused on the kinetics of water stress installation in order to detect primary responses of nodule physiology to water shortage. In that sense, four water-stress stages have been considered, namely very mild, mild, moderate, and severe, which correspond to 4, 7, 9, and 12 d, respectively, after the onset of drought. This study was carried out with the pea cultivar Sugar snap which, according to the response shown by the photosynthetic rate, is more sensitive to water deprivation than other pea cultivars whose results have previously been reported (González et al., 1998; Iturbe-Ormaetxe et al., 1998). Differences in the responses of different pea cultivars to water and oxidative stress have been highlighted elsewhere (Iturbe-Ormaetxe et al., 1998).
conversion of sucrose to malate occurs via PEPC and MDH. It has been suggested that PEPC activity rates are closely related to N\textsubscript{2} fixation rates and decreasing PEPC expression resulted in impaired N\textsubscript{2} fixation (Schulze et al., 1998).

However, the rapid response of SS activity provides further evidence of the key regulatory role that SS activity, and not PEPC, plays in the supply of glycolytic carbon flux. Indeed, malate concentration declined prior to the observed decrease in PEPC activity.

**Effect of SS down-regulation on carbon metabolites**

As a result of SS down-regulation, a significant increase in sucrose content at mild drought was detected and sugarphosphates concentration experienced a general decline under moderate to severe water deficit. Moreover, under mild water stress, malate content was significantly reduced, which gives clear proof of a limitation in carbon availability to bacteroids that is likely to cause a decline in N\textsubscript{2} fixation. In this sense, the pivotal role that malate plays in legume nodules is proved by the fact that ineffective nodules show reduced levels of malate when compared with effective nodules (Schulze, 2004) of the same magnitude of the values shown in the present study.

\(\alpha\)-ketoglutarate plays an important role providing carbon skeletons for ammonia assimilation through the GS/GOGAT pathway (Scheible et al., 2000). Chen and Gadal (1990) showed that the cytosol is the main site of \(\alpha\)-ketoglutarate synthesis by the action of the cytosolic ICDH activity. In the present study, \(\alpha\)-ketoglutarate concentration declined significantly at moderate drought, but totally recovered thereafter. This fact was coincident with the increase of ICDH activity. It has been suggested that this metabolite could be involved in the monitoring and
signalling of C/N balance to the plant regulatory machinery (Hodges, 2002).

**Effect of water stress on the OPPP and ICDH activity**

The relevance of OPPP relies on its role in the biosynthesis of intermediates, such as ribose-5-phosphate for the biosynthesis of nucleotides, and the generation of NADPH in heterotrophic tissues. A dramatic inhibition of G6PDH activity was already observed at the mildest level of water stress and throughout drought levels. Although two isoenzymes were described for soybean nodules (Hong and Copeland, 1991), five isoenzymes were detected in pea control nodules that became totally undetectable under water stress. These findings agreed with previous observations in drought-stressed soybean nodules where an inhibition of G6PDH activity was detected (Chen and Sung, 1983).

Conversely, the isoenzyme pattern of 6PGDH was similar to that reported for soybean nodules (Hong and Copeland, 1992), and only isoenzyme I seemed to be slightly affected by water stress. It has been described that both G6PDH and 6PGDH activities tend to increase in response to different environmental stresses such as low pH, salicylic acid treatments, disease and high soil temperature (Chen et al., 2003, and references therein).

The OPPP together with ICDH and the malic enzyme are considered to provide reductant and substrates for nodule metabolism as well as carbon skeletons for ammonia assimilation (Chopra et al., 2002). Moreover, oxidative stress has been shown to occur within nodules under water stress conditions (Gogorcena et al., 1995). G6PDH activity has been demonstrated to be essential for defence against oxidative stress (Pandolfi et al., 1995), supplying the required NADPH to the antioxidant ascorbate/glutathione cycle to cope with the stress. Since G6PDH activity completely declined even under the mildest level of water deficit, ICDH could play a relevant role under oxidative stress conditions. Indeed, the function of ICDH as the NADPH source becomes particularly relevant in circumstances of metabolic limitation of the OPPP (Fieuw et al., 1995). Conversely to the other enzyme activities assayed, ICDH activity showed a gradual increase in nodules from moderate drought. The isoenzyme pattern further supported the observation of a higher ICDH activity in drought-stressed nodules. In addition to this role, it has been reported that, in leaves, this activity balances C/N metabolic fluxes (Gallardo et al., 1995; Gálvez and Gadal, 1995). Thus, this alteration in ICDH activity as water potential declined suggests that C/N balance can be impaired in these nodules and ICDH enhancement might compensate for the carbon limitation that occurs in nodules under water stress (González et al., 2001). All this evidence points to a crucial role of ICDH under water stress, and suggests that ICDH activation under those conditions could carry out a possible double role; balancing carbon and nitrogen metabolisms and supplying NADPH.

**Fig. 5.** Effect of water stress on nodule organic acids and sucrose concentration. Nodule succinate (A), malate (B), α-ketoglutarate (C), and sucrose (D) concentrations of control and water-stressed pea nodules. Note the different scales of the y-axis for the different organic acids. For each parameter, an asterisk represents significant differences with the corresponding control at $P < 0.05$. Values represent mean ± standard error ($n=12$).

**Fig. 6.** Effect of water stress on nodule nucleotides concentration. AMP (A), ADP (B), ATP (C), and UDP (D) concentrations of control and water-stressed pea nodules. For each parameter, an asterisk represents significant differences with the corresponding control at $P < 0.05$. Values represent mean ± standard error ($n=16$).
Fig. 7. (A) Principal component analysis rotated with the orthogonal transformation varimax, carried out with the parameters affected by mild water stress (day 7), where components 1, 2, and 3 account for 48%, 11%, and 10% of the total variance respectively. (B) Comparison of the effects of water stress on pea metabolism, expressed as percentage of variation with respect to control plants. Original data are shown in Figs 1, 2, 3, 5, and 6. For this comparison all enzyme activities are expressed on a nodule dry weight basis. (C) Scheme of the main metabolic pathways occurring in the cytosol, symbiosomes, and plastids of a nodule infected cell of amide exporting legumes. Monitored substrates and enzymes are represented underlined and in bold and italics, respectively. Abbreviations: AAT, aspartate aminotransferase; AI, alkaline invertase; ANA, apparent nitrogenase activity; DHAP, dihydroxyacetone phosphate; GA3P, glyceraldehyde-3-phosphate; GOGAT, glutamate synthase; G6PDH, glucose-6-phosphate dehydrogenase; GS, glutamine synthetase; ICDH, isocitrate dehydrogenase; KG, α-ketoglutarate; MDH, malate dehydrogenase; Nase, nitrogenase; PEP, phosphoenolpyruvate carboxylase; Ph, photosynthesis; 6PGDH, 6-phosphogluconate dehydrogenase; SS, sucrose synthase; UDPGPP, UDP-glucose pyrophosphorylase.
Effects on adenylates and UDP

A significant decline in both ADP and ATP concentration was observed at mild drought, whilst the decline of AMP content was only significant at severe drought. Although this general reduction of adenylates concentration might suggest an impaired respiratory process, this seems to be unlikely as AEC was fairly constant throughout drought levels. Likewise, soybean nodules exposed to Ar:O2 showed a marked inhibition of nitrogenase activity whilst AEC was not affected (de Lima et al., 1994).

UDP content experienced a significant increase of 33% at very mild drought as a direct consequence of SS activity down-regulation. However, the increase in nodule UDP content was transitory, since a progressive decline was observed thereafter. Both the decline in nodule adenylate content and this further decrease in UDP levels as water deficit progressed are likely to be provoked by OPPP inhibition, due to the drastic decline of G6PDH activity.

Concluding remarks

Figure 7A represents the result of PCA performed with those parameters that experienced significant variations by mild water stress, in order to establish likely relationships among them, whilst Fig. 7B shows the responses of those same parameters to water stress in order to establish the temporal sequence of events that takes place after water stress imposition. The first three components obtained from PCA accounted for 69% of the total variance observed within the whole data set. According to PCA, the responses of the parameters to water stress can be differentiated into three main groups. In a first group, net photosynthesis rate and N2 fixation, together with SS and G6PDH enzyme activities, and the metabolites malate, ADP, ATP, and UDP are included. Within this group, two slightly different responses can be observed: on one hand, photosynthesis, N2 fixation, and the enzyme activities which reflect the earliest responses, and, on the other hand, metabolite levels. A second group would consist of ICDH activity and nodule sucrose content. These are the only parameters that experienced an increase under water stress conditions. Finally, nodule α-ketoglutarate content is shown to be clearly individualized from the other variables, because of its rather specific response to water stress.

In conclusion, this study presents evidence supporting that a carbon flux shortage is taking place in nodules experiencing a range of mild drought conditions as a result of SS activity down-regulation, as shown not only by direct measurements of in vitro SS activity, but as revealed by the accumulation of sucrose and UDP and the decrease of sugar-phosphates and malate. However, it is still debatable whether a carbon shortage would actually be limiting N2 fixation. Thus, whilst SS declines more markedly than ANA at very mild water stress, the decline of organic acids and sugar-phosphates occurs after ANA decline. It should be noted that methods used in the present study are not capable of providing information about the subcellular concentrations of these compounds. Therefore, the issue of whether the decline of SS activity is a causative effect of N2 fixation inhibition or whether both processes may share a common signal transduction pathway responsive to drought perception, as may be derived from the work of Gordon et al. (2002), is still elusive. The observation that drought provokes a dramatic impact on OPPP functionality which, in turn, seems to affect nucleotides concentration and an increase in ICDH activity suggests that strong interactions among different pathways involved in C and N metabolism take place at early stages of drought in legume nodules affecting N2 fixation.

Acknowledgements

The authors thank Gustavo Garrijo and Elena Denia for technical assistance and Dr Mercedes Royuela for her contribution to set up methods related to adenylate determinations. Loli Gámez was the holder of a grant from the Spanish Ministry of Education and Science. This work was supported by DGI-MEC (Spain) grant AGL2002-02730, and its associated FEDER funding, and European Commission FOOD-CT-2004-506223 (Grain Legumes).

References


Chen KM, Gong HJ, Chen GC, Wang SM, Zhang CL. 2003. Up-regulation of glutathione metabolism and changes in redox status...
involved in adaptation of reed (Phragmites communis) ecotypes to drought-prone and saline habitats. Journal of Plant Physiology 160, 293–301.

Chen R, Gadal P. 1990. Do mitochondria provide the 2-oxoglutarate needed for glutamate synthesis in higher plants chloroplasts? Plant Physiology and Biochemistry 28, 141–145.


