Nitrogen Fixation Control under Drought Stress. Localized or Systemic?[1][OA]

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Legume-Rhizobium nitrogen fixation is dramatically affected under drought and other environmental constraints. However, it has yet to be established as to whether such regulation of nitrogen fixation is only exerted at the whole-plant level (e.g. by a systemic nitrogen feedback mechanism) or can also occur at a local nodule level. To address this question, nodulated pea (*Pisum sativum*) plants were grown in a split-root system, which allowed for half of the root system to be irrigated at field capacity, while the other half was water deprived, thus provoking changes in the nodule water potential. Nitrogen fixation only declined in the water-deprived, half-root system and this result was correlated with modifications in the activities of key nodule's enzymes such as sucrose synthase and isocitrate dehydrogenase and in nodular malate content. Furthermore, the decline in nodule water potential resulted in a cell redox imbalance. The results also indicate that systemic nitrogen feedback signaling was not operating in these water-stressed plants, since nitrogen fixation activity was maintained at control values in the watered half of the split-root plants. Thus, the use of a partially droughted split-root system provides evidence that nitrogen fixation activity under drought stress is mainly controlled at the local level rather than by a systemic nitrogen signal.

The effect of drought (D) on biological nitrogen fixation (BNF) has been widely reported (for review, see Zahran, 1999) and is considered to be by far the most important environmental factor resulting in crop yield loss (Boyer, 1982). As such, a more precise understanding of the factors limiting and regulating the response of nitrogen fixation to D is of special interest.

Several mechanisms have been proposed to explain nitrogen fixation inhibition under abiotic stresses. Oxygen permeability appears to be a limiting factor for nodule functioning, and it has also been put forward as a controlling factor for BNF under a wide range of environmental stresses (Minchin, 1997; Denison, 1998). A reduction in nodule carbon flux has also been related to the inhibition of nitrogen fixation under D (Arrese-Igor et al., 1999). In these conditions, nodule Suc synthase (SS) activity sharply declines (González et al., 1995; Gordon et al., 1997), thus limiting the carbon flux required for bacteroid respiration. Indeed Suc accumulation and malate depletion take place in nodules as a result of SS down-regulation (González et al., 1995, 1998; Gálvez et al., 2005). Such carbon limitation can be mimicked by methyl viologen application, indicating a close relationship between the widely reported cell redox alteration caused by water stress and nitrogen fixation inhibition (Marino et al., 2006). However, the inhibition of BNF under D is not related to photosynthate depletion as it has been shown that BNF is more sensitive than CO_2 assimilation to moderate water deprivation (Durand et al., 1987). There is now evidence that signaling for BNF regulation can be provided by a nitrogen feedback mechanism involving shoot nitrogen status. Several molecules have been suggested to be involved in such a mechanism, including Gln (Neo and Layzell, 1997), ureides (Serraj et al., 2001), and Asn (Bacanamwo and Harper, 1997). More recently, King and Purcell (2005) suggested that a combination of ureide and Asp levels in nodules, plus the transport of several amino acids from leaves, might be involved in such a feedback inhibition mechanism in soybean (*Glycine max*).

The split-root system (SRS) approach has been widely used for mineral nutrition research and there are several reports on the use of SRS for BNF studies, mainly focused on the nodule formation process (George et al., 1992; Van Brussel et al., 2002). Silva and Sodek (1997) observed that aluminum provoked a decrease in nodule number and size only in the half root in contact with the metal. It has also been reported that nitrate produced a local reduction in nodulation and BNF activity (Tanaka et al., 1985; Daimon and Yoshioka, 2001) and moreover,

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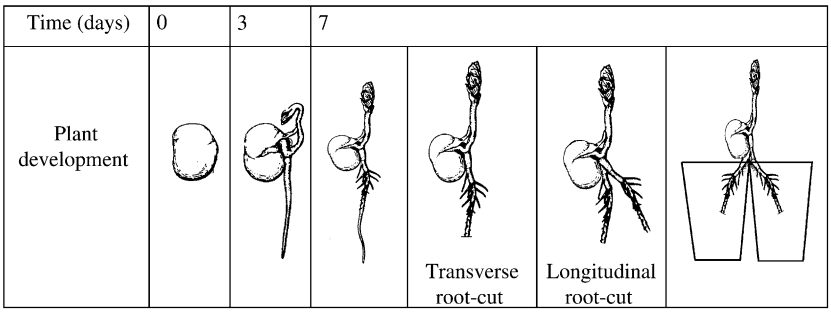

Figure 1. A scheme for the development of plants with a SRS.

nitrogen assimilation, whether from N_2 fixation or inorganic sources, had a localized effect on root development (Singleton and van Kessel, 1987). The SRS approach has also been used to investigate water stress effects on plant performance and the involvement of abscisic acid in the initial stages of stomatal control under D (Holbrook et al., 2002; Sobeih et al., 2004; Wakrim et al., 2005).

The goal of this work was to determine whether the root nodule response to D stress is influenced by systemic or local signal(s). Nodulated pea (*Pisum sativum*) plants were grown in a SRS, where half of the root system was irrigated at field capacity, while the other half was water deprived (see Fig. 1). If a systemic signal was involved than BNF activity should be reduced in both halves, while a local signal should only affect activity in the droughted half.

RESULTS

Effect of Partial and Total D on Evapotranspiration and Water Potential in Leaves and Nodules

Evapotranspiration (ET) was significantly reduced, both in partial D (PD) and D plants, 4 d after starting the D treatment (Fig. 2A). ET was progressively reduced in D plants, reaching a value close to zero at the end of the study. However, PD plants maintained an ET rate of around 60% of control plant values until the end of the study (Fig. 2A). The water potential of the first fully expanded leaf was already significantly reduced 7 d after the onset of treatment in D plants, whereas it was not significantly affected in PD plants at the end of the study (Fig. 2B). Water potential was also monitored for each individual plant leaf to assess whether lateral distribution of water occurred within the shoot of PD plants. This did occur, as evidenced by the lack of differences in water potential between leaves orientated to the drying half and those orientated to the watered half. The nodule water potential of D and PD-D plants showed the same pattern, with both being significantly affected after 7 d of treatment. In contrast, PD-control (C) nodules maintained a water potential very close to that of C nodules throughout the period of study (Fig. 2C).

Effect of Partial and Total D on Nitrogen Fixation

To test whether the PD treatment affected nodule functioning, nodule protein content and apparent nitrogenase activity (ANA) were measured. A significant

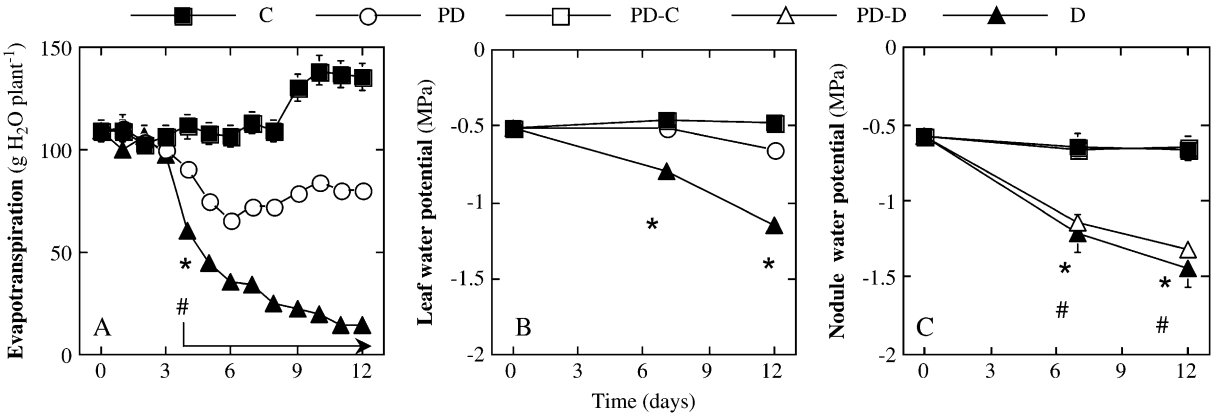

Figure 2. Effect of PD on ET (A), leaf water potential (B), and nodule water potential (C). For A and B, C denotes control, PD partial drought, and D drought. In C both root parts of the PD treatment are represented independently: PD-C denotes the irrigated part and PD-D the nonirrigated part. Values represent mean ± SE (n = 12). For A and B, an asterisk (*) represents significant differences ($P \leq 0.05$) between D and C plants and a hash (#) represents significant differences between PD and C plants. For C, an asterisk (*) represents significant differences ($P \leq 0.05$) between D and C nodules and a hash (#) represents significant differences between PD-D and C nodules. For ET (A), significant differences were observed from day 4 to 12. For nodule water potential (C), symbols and lines for C and PD-C treatments are overlapping.

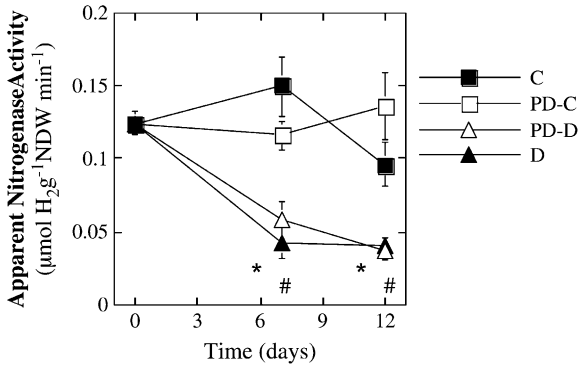

Figure 3. Effect of PD on nitrogen fixation. Both root parts of the PD treatment are represented independently: PD-C denotes the irrigated part and PD-D the nonirrigated part. NDW denotes nodule dry weight. Values represent mean ± SE ($n = 6$). An asterisk (*) represents significant differences ($P \leq 0.05$) between D and C nodules and a hash (#) represents significant differences between PD-D and C nodules.

diminution of protein content (expressed on a nodule dry weight basis) was observed at day 12 in D and PD-D nodules (39.21 ± 4.04 and 44.62 ± 3.77 mg g^{-1} nodule dry weight, respectively) compared to C and PD-C nodules (59.25 ± 2.85 and 58.72 ± 1.56 mg g^{-1} nodule dry weight, respectively). Moreover, a 70% reduction of ANA was observed after 7 d of D treatment of D and in PD-D nodules (Fig. 3). In contrast, C and PD-C nodules exhibited a similar ANA, which did not change significantly during the period of study.

Effect of Partial and Total D on SS and Isocitrate Dehydrogenase Activity and Gene Expression

SS and isocitrate dehydrogenase (ICDH) have been showed to be good biological markers of D stress. Indeed, both SS RNA levels and enzyme activity decreased in nodules of D-stressed plants (González et al., 1998; Ramos et al., 1999). In contrast, ICDH has been showed to be up-regulated both at the level of gene expression and enzyme activity (Gálvez et al., 2005; Marino et al., 2006). Thus, these two markers were used to study the effect of PD on nodule metabolism. Specific SS activity decreased significantly by day 7 in D and PD-D nodules with a further decline at day 12 (Fig. 4A). However, there was no significant difference in the SS activity of C and PD-C nodules. In agreement with the activity decline, a diminution of the quantity of SS transcripts was observed at day 12 in D and PD-D with respect to C and PD-C nodules (Fig. 4B). The discrepancy between the observed gene expression and the level of activity at day 7 probably reflects the posttranscriptional regulation of this enzyme (Geigenberger, 2003). Specific ICDH activity increased by 50% by day 12 in both D and PD-D as compared to C and PD-C nodules (Fig. 4A). ICDH gene induction was detectable after 7 d in D and PD-D nodules (Fig. 4B). The decline of

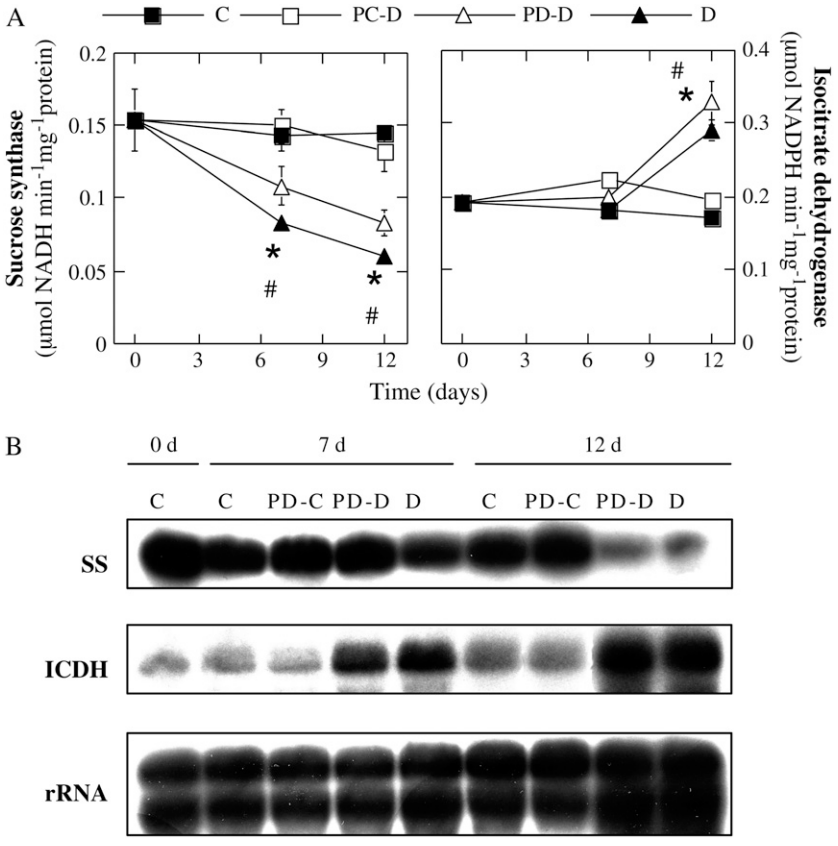

Figure 4. Effect of PD on nodule SS and ICDH enzyme activities (A) and gene expression (B). Both root parts of the PD treatment are represented independently: PD-C denotes the irrigated part and PD-D the nonirrigated part. NDW denotes nodule dry weight. RNA gel blots were probed with *SS*, *Icdh*, and ribosomal RNA. Values represent mean ± SE ($n = 6$). Symbols as in Figure 3.

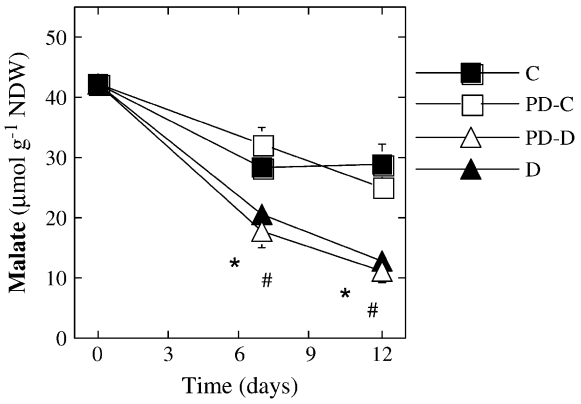

Figure 5. Water stress effect on nodule malate concentration. Both root parts of the PD treatment are represented independently: PD-C denotes the irrigated part and PD-D the nonirrigated part. NDW denotes nodule dry weight. Values represent mean ± SE ($n = 6$). Symbols as in Figure 3.

SS activity was correlated with a diminution of malate levels in D and PD-D nodules (Fig. 5).

PD Induces a Local Redox Imbalance in Nodules

Several works have shown that D stress modifies the redox state within nodules (e.g. Gogorcena et al., 1995). Moreover, it has been recently shown that D effects on nodule metabolism can be mimicked by methyl viologen, a compound that exacerbates the generation of reactive oxygen species (ROS; Marino et al., 2006). To test whether the PD stress modifies the cell redox metabolism in the nodules, ascorbate (ASC) content and catalase gene expression were used as markers.

Both ASC and dehydroascorbate (DHA) content sharply declined in D and PD-D nodules after 7 d of treatment as compared to the controls (Fig. 6, A and B). The ASC/(ASC + DHA) ratio also decreased significantly at day 12 in D and PD-D nodules (Fig. 6C). In parallel, catalase gene expression was up-regulated in D and PD-D nodules as compared with C and PD-C nodules (Fig. 6D).

DISCUSSION

BNF is an extremely complex biological process, which is known to be very sensitive to D stress (Sprent et al., 1988; Zahran, 1999). However, it still remains unclear whether nodule metabolism and thus the BNF process under D is controlled at the nodule level (local control) or by other parts of the plant (systemic control). To answer this question, a SRS was used to compare droughted and watered nodules on the same plant.

The diminution of ET observed in the shoot of partially droughted plants showed that they were affected by these conditions (Fig. 2A). Stomatal conductance measured in preliminary studies at the first, second, and the fourth leaf level was unaffected at day 5, but was significantly reduced at the end of the study period in both PD and D plants (data not shown). Taken together with the water potential measurements, this indicated that reduced transpiration in PD plants was caused by a homogenous stomatal closure in all the leaves, possibly related to some form of long-distance signaling as shown by Sobeih et al. (2004) in tomato (*Lycopersicon esculentum*) plants subjected to partial root-zone drying. Holbrook et al. (2002) observed that

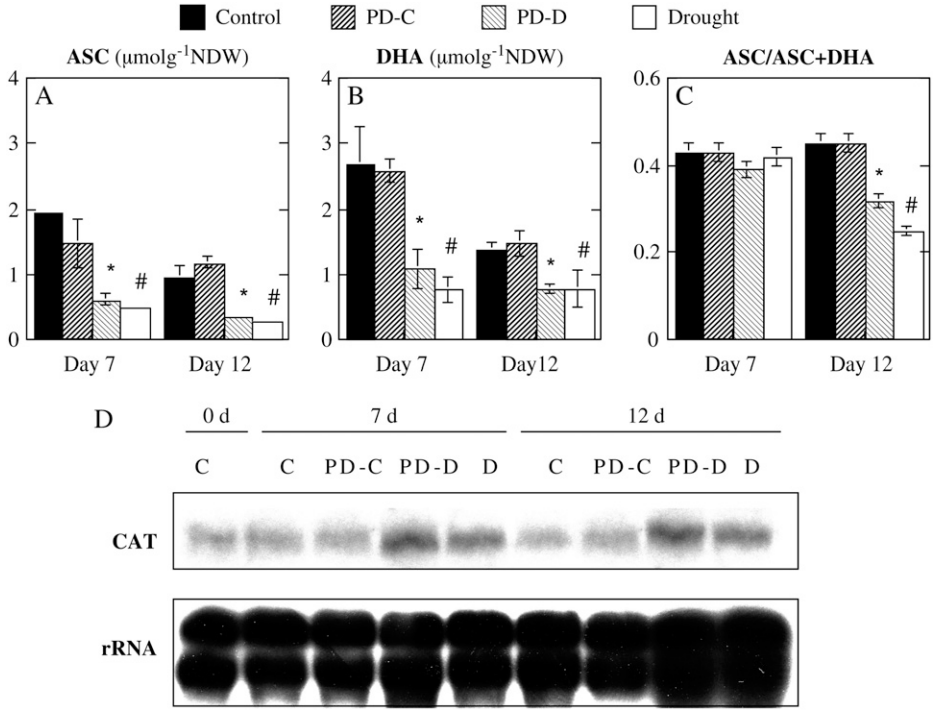

Figure 6. Effect of PD on components of the nodule antioxidant defense system. A, ASC content. B, DHA content. C, ASC/(ASC + DHA) ratio. D, Expression of catalase (CAT); RNA gel blots were probed with *Cat* and ribosomal RNA. Both root parts of the PD treatment are represented independently: PD-C denotes the irrigated part and PD-D the nonirrigated part. NDW denotes nodule dry weight. Values for A, B, and C represent mean ± SE ($n = 6$). Symbols as in Figure 3.

long-distance signaling is able to provoke stomatal closure in response to soil drying in the absence of leaf water deficit. Indeed, only a small diminution in leaf water potential was observed in PD plants, indicating that shoot water stress was not severe under partial droughting (Fig. 2B). In contrast, the use of partially droughted split-root plants produced a marked difference in the water status of nodules in the PD-D and PD-C treatments (Fig. 2C). It is known that phloem input is a major source of nodule water supply (Raven et al., 1989; Walsh, 1989; Walsh et al., 1989a, 1989b). Although this is unlikely to have been reduced in the PD plants, as evidenced by the maintained ANA of the PD-C nodules, it is evident that water input through the phloem was insufficient to maintain the water status of the PD-D nodules.

The correlation of nitrogen fixation activity of the PD plants with the water potential of the PD-D and PD-C nodules, rather than with the leaf water potential, strongly suggests that D stress exerts a local rather than a systemic control. Such local control could be expressed through decreased carbon metabolism, as evidenced by the significant down-regulation of SS gene expression and activity in PD-D nodules (Fig. 4). These parameters were slightly less affected in PD-D than in D nodules, but statistical analysis showed no significant difference between these treatments. As a result of the reduction in SS activity, the content of malate, the main carbon substrate for bacteroid's respiration and nitrogen fixation activity, declined significantly in both PD-D and D nodules (Fig. 5). The correlation between all these parameters in the D nodules of the partially droughted plants (Figs. 3–5) strengthen the hypothesis that nitrogen fixation may be regulated under D by carbon supply to the bacteroid (Arrese-Igor et al., 1999; Gálvez et al., 2005). An additional mechanism for local control of nitrogen fixation activity under D stress could be the overproduction of ROS (Gogorcena et al., 1995; Iturbe-Ormaetxe et al., 1998). Indeed, it has been shown that D effects on nodule metabolism can be mimicked by methyl viologen, a compound that exacerbates ROS production and induces alterations in redox status (Marino et al., 2006). In this study a redox imbalance occurred in PD-D nodules, but not in PD-C ones, suggesting a localized control (Fig. 6). This hypothesis is strengthened by the up-regulation of both catalase and ICDH in PD-D nodules. It has recently been suggested that the main role of ICDH under D stress is to regenerate the NADPH pool (Marino et al., 2007), an essential component of antioxidant defense systems.

Systemic regulation of nitrogen fixation under environmental stress is believed to involve nitrogen feedback (Parsons et al., 1993; Parsons and Sunley, 2001; Serraj et al., 2001; King and Purcell, 2005), possibly operating through reduced oxygen permeability (Neo and Layzell, 1997). The potential for such regulation existed in this study as nitrogen circulation between split roots is widely accepted (Fisher, 2000) and has been shown for nodulated soybeans (Tanaka et al., 1985). However, the maintenance of control level nitrogen fixation activity in PD-C nodules suggests that systemic nitrogen circulation from PD-D to PD-C roots was not relevant to control nitrogen fixation. Nevertheless, several studies suggest that a nitrogen feedback inhibition of BNF does operate in water-stressed plants (Serraj et al., 2001; King and Purcell, 2005). Serraj et al. (2001) proposed two possible origins for nitrogen feedback inhibition: (1) indirect feedback coming from the shoot, which would be in agreement with previous studies dealing with the effect of other nitrogen sources on BNF (Bacanamwo and Harper, 1997; Neo and Layzell, 1997), and (2) a direct feedback within the nodule coming from the accumulation of nitrogenous compounds. The latter mechanism could be involved in a local regulation of BNF as proposed in this study. Moreover, King and Purcell (2005) observed that leaf ureides or nodule Asn did not inhibit BNF, while elevated levels of ureides or Asp in nodules were consistently associated with a decline in nitrogenase activity. Clearly, a detailed analysis of plant component nitrogen status is required to clarify this situation. Such an analysis would also require the repetition of the split-root PD experiment using a legume species from which it is possible to extract phloem sap for nitrogen content analysis (e.g. soybean or lupin [*Lupinus albus*]; Neo and Layzell, 1997). Therefore, this study provides evidence for a local signal for reduced N_2 fixation under D but further studies are required to determine if nitrogen compounds are involved in this signaling.

MATERIALS AND METHODS

Experimental Procedures and Growth Conditions

Pea seeds (*Pisum sativum* L. cv Sugar-lace, provided by Bonduelle SA) were surface sterilized (Labhilili et al., 1995) and inoculated with *Rhizobium leguminosarum* biovar. *viciae* strain NLV8, which is hup$^-$. For production of split-root plants the tap roots of 7-d-old plants were transversely cut to produce a 2-cm length of root that was then divided longitudinally. The split-root plants were then placed in a double pot of 2 × 600 mL with a 1:1 (v:v) perlite:vermiculite mixture (Fig. 1) in a controlled environment chamber (22°C/18°C day/night temperature, 70% relative humidity, 500 μmol m^{-2} s^{-1} [photosynthetic photon flux density], and 15 h photoperiod), and watered with a nitrogen-free nutrient solution (Rigaud and Puppo, 1975). Four-week-old plants were separated randomly into three sets. Controls were supplied daily with nutrient solution to achieve field capacity to both sides of the SRS (C) whereas D treatment was achieved by withholding water/nutrients from both sides (D). PD plants were irrigating to field capacity to one side of the SRS (PD-C) while water/nutrients were withheld from the other side (PD-D). Plants were harvested at days 0, 7, and 12 after the onset of the D treatment. Nodules were harvested, immediately frozen in liquid nitrogen, and stored at −80°C for analytical determinations.

Water Relations

ET was determined gravimetrically on a daily basis throughout the study period. Leaf water potential was measured in the first fully expanded leaf 2 h after the beginning of the photoperiod using a pressure chamber (Soil Moisture Equipment) as described by Scholander et al. (1965). Nodule Ψ_w was measured in the same plants as leaf Ψ_w using C52 sample chambers coupled to a HR33T microvoltmeter (Wescor).

Nitrogen Fixation Determination

Nitrogen fixation was measured as ANA. H_2 evolution from sealed roots systems was measured in an open flow-through system under N_2:O_2 (79%:21%) according to Witty and Minchin (1998) using an electrochemical H_2 sensor (Qubit

System Inc.). The H_2 sensor was calibrated with high purity gases (Praxair) using a gas mixer (Air Liquid) outputing at the same flow rate as the sampling system (500 mL min^{-1}).

Extraction and Assay of Enzymes

Nodules were homogenized in a mortar and pestle with 50 mM MOPS, pH 7, 20% polyvinylpolypyrrolidone, 10 mM dithiothreitol, 10 mM 2-mercaptoethanol, 1 mM EDTA, 20 mM KCl, and 5 mM $MgCl_2$ at 0°C to 2°C (5 mL per g fresh weight). The homogenate was centrifuged for 30 min at 20,000*g*, 4°C.

An aliquot of the supernatant was retained for plant fraction protein determinations (Bradford, 1976). The rest of the supernatant was desalted by low speed centrifugation (180*g*, 1 min) through Bio Gel P6DG columns (Bio-Rad) equilibrated with 50 mM MOPS pH 7, 20 mM KCl, and 5 mM $MgCl_2$. The desalted extract was used to measure SS (EC 2.4.1.13) enzyme activity according to González et al. (1998) and $NADP^+$-dependent ICDH (EC 1.1.1.42) activity according to Marino et al. (2007). The nitrogenase components were not detectable by immunodetection in the host plant protein extracts, confirming that the enzyme activities did not include contamination from the bacteroid.

Malate Content

Malate was determined by ion chromatography in a DX-500 system (Dionex Sunnyvale) as described by Gálvez et al. (2005).

Extraction and Determination of ASC and DHA

Frozen nodules (200 mg) were crushed with liquid nitrogen to a fine power and subsequently homogenized with 1.5 mL of ice-cold 2% metaphosphoric acid and 1 mM EDTA (Schutzendubel et al., 2002). The homogenate was centrifuged (4,400*g*, 4°C, 2 min) and filtered (Millex GV, 0.22 μm). Antioxidants were analyzed by high-performance capillary electrophoresis as described by Zabalza et al. (2007). Recovery of externally added ASC was always greater than 92%, while that of DHA was greater than 98%.

Cloning of the Probes Used for the Gene Expression Analysis

Probes corresponding to the different genes were amplified by reverse transcription (RT)-PCR. Two micrograms of pea nodule total RNAs were used as template in the RT experiment. One tenth of the RT reaction and 50 pmol of each primer was used to amplify DNA during 30 cycles of sequential incubations at 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s, in a final 50 μL reaction mixture containing 5 units of *Taq* DNA polymerase (Stratagene-Oncor). The PCR reaction products were purified from agarose gel using the QIAEX II kit (Qiagen) and inserted in the pGEM-T vector (Promega). The PCR products were verified by sequencing and used as probes for the RNA analysis.

RNA Analysis

RNA was extracted from pea nodules using Trizol (Invitrogen) according to the manufacturer's recommendations. For RNA gel-blot analysis, RNA samples (10 μg) were fractionated on 1.4% formaldehyde-agarose gels, transferred onto Hybond N membranes (Amersham), and hybridized with *Ss*, *Icdh*, and *Cat* probes. Ribosomal RNA hybridization served as the RNA loading control.

Statistical Analysis

All the presented results were examined by two-way analysis of variance, using Fisher's protected LSD tests between means, and all significant differences for a given time point were at $P \leq 0.05$.

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