Comparison of inhibition of N2 fixation and ureide accumulation under water deficit in four common bean genotypes of contrasting drought tolerance

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INTRODUCTION

Common bean (Phaseolus vulgaris) is the main food protein crop for direct consumption worldwide. It is a staple food in developing countries, where beans are the main dietary supply of plant proteins (Broughton et al., 2003; Graham and Ranalli, 1997; Graham and Vance, 2003). Beans, like other legumes, are able to use atmospheric nitrogen through symbiotic associations with soil N2-fixing rhizobia. This capacity is one of the main advantages of this crop because it reduces the nitrogen fertilizer demand and thus has a positive impact on the economy, especially in developing countries, and on the environment, contributing to reduction in the emission of greenhouse gases (NOx). The existence of genetic variation in the response of N2 fixation to abiotic stresses among legume genotypes opens the possibility of enhancing N2 fixation tolerance through selection and breeding. Among the constraints that affect legume crops (Zahran, 1999), drought is the principal factor limiting the yield of the common bean. In particular, biological nitrogen fixation (BNF) in the symbiotic system of bean rhizobia is strongly inhibited under water deficit, so that the amount of nitrogen compounds required for biomass and seed production is seriously compromised under water-stress conditions (Sinclair and Serraj, 1995; Serraj et al., 1999a; Serraj, 2003; Purcell et al., 2004; Arrese-Igor et al., 2011).

Ureides (allantoin and allantoate) are the main N-rich molecules used by ureidic legumes, such as soybean (Glycine max) and common bean, to transport the nitrogen fixed in the nodules to tissues throughout the plant (Kohl et al., 1990; Boldt and Zrenner, 2003; Zrenner et al., 2006). In ureidic legumes, ureides are formed through the oxidative catabolism of purines synthesized de novo in the nodules, incorporating recently fixed nitrogen. Among the enzymatic steps leading to the synthesis of ureides, urate oxidase (uricase; EC 1:7:3:3), catalysing the oxidation of uric acid, has a key role in nodule development and function (Bergmann et al., 1983; Le et al., 1993). The direct product of uricase, 5-hydroxyisourate, is transformed by the bifunctional enzyme allantoin synthase (Lamberto et al., 2010; Pessoa et al., 2010) to allantoin, which is then oxidized to allantoate by allantoin amidohydrolase (allantoinase, ALN; EC 3:5:2-5) (Webb and Lindell, 1993; Raso et al., 2007). Allantoinase is a key enzyme in the metabolism of ureides,
In a common bean breeding line belonging to the Great Northern market class (Santalla et al., 2001), we recently showed that prolonged periods of water deficit led to the progressive accumulation of the ureide allantoin in roots, stem and leaves but not in nodules of plants subjected to drought (Alamillo et al., 2010). Moreover, this accumulation was attributed to the induction of allantoinase activity, which catalyses the synthesis of allantoin, and not to a decrease in the degradation of allantoin. Moreover, it was found that allantoin increased even when nitrogen fixation was nearly or completely inhibited by drought, as well as in non-nodulated plants, therefore indicating that these ureides could not be the result of nitrogen fixation held in nodules, or the cause of its inhibition, but instead that the drought would have induced alternative routes of ureide synthesis in aerial tissues (Alamillo et al., 2010).

The aim of this work was to analyse ureide metabolism in two breeding lines of common bean that are reported to differ in their tolerance to soil water deficit (Frahm et al., 2003) and two landraces that showed differential yields under elevated or restricted rainfall conditions (Riveiro, 2012). For this purpose, we determined whether there are genotypic differences in the accumulation of ureides in common bean germplasms differing in sensitivity of nitrogen fixation to drought. Moreover, we measured the levels of gene expression and enzyme activities of the key ureide metabolism enzymes, ALN and AAH, to further determine the actual cause of ureide accumulation under these conditions.

MATERIALS AND METHODS

Plant materials, growth conditions and drought treatments

The Phaseolus vulgaris bean genotypes studied were the landraces PHA-0246 and PHA-0683 and the breeding lines PBM-0285 and PBM-0306 (Table 1; Supplementary Data Fig. S1), from the germplasm collection at the MBG-CSIC (Misión Biológica de Galicia-Consejo Superior de Investigaciones Científicas, Pontevedra, Spain) (De Ron et al., 1997). The seeds are maintained in cold storage rooms at 4 °C and 40 % relative humidity. Rhizobium leguminosarum ISP 14 (HUP−) strain was provided by CIFA-Las Torres (Centro de Investigación y Formación Agraria, Sevilla, Spain). Seeds were surface-sterilized by sequential dipping in ethanol (30 s) and 0.2 % (w/v) sodium hypochlorite (5 min) and then washed thoroughly with distilled water. Soaked seeds were allowed to germinate in Petri dishes (120 mm diameter) with wet paper under sterile conditions. After germination, three seedlings were sown on each pot (16 cm diameter, 18 cm height) filled with an artificial substrate composed of vermiculite/perlite mixture (2/1 w/w) and inoculated with a fresh suspension of Rhizobium, which had been cultured at low temperature (28 °C) for less than 30 h. Plants were cultured in a growth chamber under a long-day photoperiod (16 h light, 8 h dark), 300 µE m−2 s−1 lighting and 70 % relative humidity at 26 °C (day)/21 °C (night). Inoculated plants were watered three times a week with nitrogen-free nutrient solution (Rigaud and Puppo, 1975).

Drought stress treatments started when the plants came into flowering (25–28 days old). Plants were randomly separated into two sets; watering was withheld from one half of the plants and the second half was regularly irrigated. Nitrogen fixation measurement and plant collection was done at 0 (before

catalysing the first step in the degradation of the ureide allantoin and the synthesis of allantoin, the second most prominent ureide. Allantoate amidohydrolase (AAH; EC 3.5.3.9) then catalyses the breakdown of allantoin to the unstable ureidoglycine compound (Winkler et al., 1985, 1987; Serventi et al., 2010; Werner et al., 2008, 2010). At least two more enzymatic reactions are needed to yield glyoxylate, the last product of ureide degradation, releasing the four nitrogen atoms from the ureide molecule (Werner et al., 2013).

In soybean, several studies have shown that differences in the tolerance of nitrogen fixation to drought among genotypes are inversely correlated to plant ureide concentrations under water deficit conditions (Serraj and Sinclair, 1996; Purcell et al., 2000; Sinclair et al., 2000; Charlson et al., 2009; Gil-Quintana et al., 2013). The accumulation of ureides in shoots of susceptible soybean genotypes under water deficit has been shown in various reports, supporting the idea that high ureide levels are likely associated with nitrogen fixation inhibition (Purcell et al., 2000; Vadez and Sinclair, 2001; King and Purcell, 2005; Charlson et al., 2009). Moreover, Ladrera et al. (2007) also proposed that ureide accumulation is related to nitrogen fixation inhibition, but, in contrast to previous reports, accumulation of ureides was only observed in the nodules and not in aerial tissues of the drought-stressed soybeans.

In addition, it has been proposed that ureide-exporting legumes are more drought-sensitive than the amide-exporting ones (Sinclair and Serraj, 1995). Thus, an N-feedback regulation hypothesis, in which ureides would be among the signalling molecules triggering the inhibition, has been proposed to explain the reduction in nitrogen fixation under drought stress conditions in soybean (Serraj et al., 1999b; King and Purcell, 2005), although the actual mechanism involved in the inhibition remains unclear.

The differential ureide accumulation between tolerant and sensitive genotypes has been associated with inhibition of the Mn-dependent enzyme AAH in the most sensitive genotypes (Vadez and Sinclair, 2001; Sinclair et al., 2003). However, expression of AAH did not change under drought conditions (Charlson et al., 2009), or was slightly stimulated (Alamillo et al., 2010), and posttranscriptional inhibition of AAH protein has been proposed as the possible cause of allantoinate accumulation.

In the genetic pools (Andean and Mesoamerican) of common bean there is wide variability in phenotypic characters, including morpho-agronomic traits (Sing et al., 1991; Escrivano et al., 1994; Casquero et al., 2006; González et al., 2006), stress tolerance and the ability to establish an effective symbiotic association with rhizobia (Rodrigo et al., 2011). In contrast to the intensive search in soybean, there have been only a few studies on the genotypic diversity of the tolerance to drought of N2 fixation in common bean (Riveiro, 2012) and its possible relationship to the low accumulation of ureides. In an early work, Serraj and Sinclair (1998) showed variation in ureide concentrations despite the lack of significant differences in the N2 fixation response to drought in two genotypes of common bean. Genetic variability in the resistance of N2 fixation to drought has been further corroborated in a comparison of 12 common bean genotypes by Devi et al. (2013). However, the possible correlation of this resistance to ureide concentration was not examined in this study.
Tissue samples were weighed fresh and desiccated in an oven and treated plants were determined at the sampling times. of nitrogen fixation capacity. individual plant were weighed and photographed as a visual control after careful removal of nodules. Nodules collected from each in- apical stem after removal of leaves; and whole roots collected leaves; shoot tissue portions including basal, medium and routine, the following samples were collected: fourth trifoliate content was calculated using the following formula: RWC = weight of the plant samples was determined. Relative water content (RWC) was measured according to Barrs and Weatherly (1962). The leaves were weighed immediately after sampling and soaked overnight in distilled water at 4 °C. After incubation, the leaves were blotted dry and weighed prior to drying at 80 °C for 24 h. Subsequently, dry weight of the plant samples was determined. Relative water content was calculated using the following formula: RWC = [(FW – DW)/(TW – DW)] × 100, where FW is fresh weight, DW is dry weight and TW is turgid weight (weight after the leaf was kept overnight in distilled water). Plant material collected at the indicated times was frozen with liquid nitrogen and stored at −80 °C until further analysis. As a routine, the following samples were collected: fourth trifoliate leaves; shoot tissue portions including basal, medium and apical stem after removal of leaves; and whole roots collected after careful removal of nodules. Nodules collected from each individual plant were weighed and photographed as a visual control of nitrogen fixation capacity. For biomass estimation, fresh and dry weights of the control and treated plants were determined at the sampling times. Tissue samples were weighed fresh and desiccated in an oven at 72 °C for 48–72 h before dry weight determination.

Nitrogen fixation

Nitrogenase (EC 1.7.9-92) activity was measured as the representative H₂ evolution in an open-flow system (Witty and Minchin, 1998) using an electrochemical H₂ sensor (Qubit Systems Inc., Canada). For this purpose, nodulated roots were excised from their shoots and potting medium was removed just before the activity assay. Just after separation from the shoot, whole roots from each plant were sealed in 0.125 L cylinders and H₂ production was recorded according to the manufacturer’s instructions. Apparent nitrogenase activity (rate of H₂ generation in air) was determined under N₂:O₂ (80%:20%) with a total flow of 0.4 L min⁻¹. After reaching steady-state conditions (5–10 min), total nitrogenase activity was determined under Ar:O₂ (79%:21%). Standards of high-purity HB₂ were used to calibrate the detector. Roots attached to whole plants were also measured to ascertain that the separation from shoots did not cause a drastic reduction in nodule activity during the assay. Mean nitrogenase activity values were about 15–20% lower in excised roots than in roots of intact plants. However, this effect was similar for all tested cultivars and treatments. After the nitrogenase activity assay, nodules were collected and dried at 70 °C for 48 h. Data are given as nitrogen fixed per plant.

Gene expression analysis

Total RNA was isolated from the different tissues using TRI Reagent® RNA/DNA/Protein Isolation Reagent (Molecular Research Center, Cincinnati, OH, USA) following the manufacturer’s instructions. Prior to reverse transcription (RT)–PCR, total RNA from control and treated tissues was incubated with RNAase-free DNAase I (Promega, Madison WI, USA) at 37 °C for 30 min to eliminate any traces of genomic DNA. Lack of amplification of 18S rRNA was used to check the successful removal of DNA. First-strand cDNA synthesis was done from 2.5 μg of DNAase-treated RNA using SuperScript® reverse transcriptase (Invitrogen, Carlsbad, CA, USA), following the manufacturer’s instructions. Expression analysis was performed by quantitative RT–PCR in an iCycler iQ System (Bio-Rad, Hercules, CA, USA) using iQ SYBR-Green Supermix (Bio-Rad) and gene-specific primers (Supplementary Data Table S1). Expression levels of Actin-2 and 18S rRNA were used as internal controls, after checking that levels of expression were similar in all samples. The PCR programme consisted of initial denaturation and a Taq polymerase activation step of 5 min at 95 °C, followed by 50 cycles of 15 s at 95 °C and 1 min at 60 °C. The specificity of primers was verified using amplicon dissociation curves and gel electrophoretic analysis. The amplification efficiency of each primer pair, calculated by PCR using serial dilutions of root and leaf cDNAs, was >80%. The threshold cycle (C_T) values were in the range of 20–23 for Tactin-2, 21–30 for the genes of ureide metabolism and 14–17 for 18S rRNA. Analysis of relative gene expression was calculated from ΔC_B values (Pfaffl, 2001), using expression of Actin-2 for data normalization. All reactions were set up in triplicate (three technical replicates) using three to five RNA preparations (biological replicates) from different plants. Sequences used to synthesize the specific primers were available in databases (Supplementary Data Table S1). In all cases,
identity of the products amplified by the specific primers was verified by sequencing.

**Allantoinase and allantoate amidohydrolase enzyme assays**

Allantoinase degrading activity was measured as described in Alamillo et al. (2010), following the production of allantoin as indicated by Raso et al. (2007).

Allantoate amidohydrolase activity was determined according to a modification of the assay described in Werner et al. (2008) (Diaz-Leal et al., 2014). All procedures for crude extract preparation were carried out at 0–4 °C. Frozen plant material was ground to a fine powder under liquid nitrogen. Plant extracts were obtained by adding 2 ml of extraction buffer per gram of tissue. The extraction buffer for the AAH assay was 100 mM TEA–NaOH pH 8, 150 mM NaCl, 15 mM dithiothreitol (DTT), 1 mM MnCl₂, 0.5 % (w/v) Triton X-100 and 50 μM phenylphosphorodiamidate (PPD). The resulting homogenate was centrifuged at 15,000 g for 10 min and the supernatant was dialysed through SpinTrap G25 columns previously equilibrated with wash buffer, and the flow-through was used as crude extract. The wash buffer contained 100 mM TEA–NaOH, pH 8.0, 40 % (v/v) wash buffer and 10 % (v/v) of the previously dialysed crude extract. Aliquots were taken at several time points and diluted in chilled water to stop the reaction, and allantoate degrading activity was determined by following the production of ammonium as indicated by Witte and Medina-Escobar (2001).

One unit (U) of enzymatic activity is the amount of enzyme that catalyses the transformation of 1 micromole of substrate per minute. Results of enzymatic activity are given as mU mg⁻¹ soluble protein. The results are expressed as means of the values from at least three independent experiments. Enzymatic assays and analytical determinations from each biological experiment were done at least twice (two independent samples) and each assay was measured in duplicate (technical duplicate).

**Analytical determinations**

The concentration of ureides was determined by the colorimetric assay of glyoxylate derivatives (Vogels and Van der Drift, 1970). In this method, allantoin and allantoate are determined independently after their chemical transformation to glyoxylate. The values of total ureides in crude extracts correspond to the sum of allantoin plus allantoate. For each assay, 100 mg of plant tissue was homogenized in 0.4 ml of 50 mM Tris–HCl, pH 7.8, 100 mM MgSO₄ and 0.15 % (w/v) deoxycholic acid, centrifuged to remove cell debris and used immediately to estimate the ureide content. Soluble protein was measured in the same extracts in which ureides from control and drought-stressed tissues were determined (Bradford, 1976), and the concentration of ureides per milligram of protein is given in the figures. For each biological experiment, determinations were done in duplicate (technical repetition).

**Results**

Plants cultured under well irrigated conditions were randomly distributed into control and treated groups. The experiments were designed as a randomized block with two replications (independent experiments). Each replication consisted of 30 plants from each genotype that were randomly distributed, 18 plants for well irrigated controls (six plants at each of the 0, 7 and 14 days of analysis) and 12 plants for the water-stress treatment (7 and 14 days). Nitrogen fixation was measured in all individual plants, whereas for ureide content and molecular analysis samples from two or three plants were combined. Analysis of the data was done considering the measurements of the two replicates; therefore 12 individuals per sampling and genotype (60 plants per genotype) were considered for the ANOVA of nitrogen fixation data and five individuals per sample from each genotype were used for the analysis of ureide content.

Student’s t-test was performed using the GraphPad software package. ANOVA, Fisher’s LSD test and the Pearson correlation matrix were computed using XLSTAT software, version 2011.4.02 (Addinsoft, 2011).

**Differential nitrogen fixation inhibition**

Nitrogen fixation was measured in PHA-0246, PHA-0683, PMB-0285 and PMB-0306 plants 0, 7 and 14 days from the beginning of treatment in control (fully irrigated) and at 7 and 14 days of treatment in drought-stressed plants. Nitrogen fixation was measured as the apparent nitrogenase activity according to Witty and Minchin (1998). As shown in Fig. 1A–D, water deprivation caused variable inhibition of N₂ fixation among the different genotypes, with lower inhibition of N₂ fixation in the PHA-0246 and PHA-0683 landraces than in the breeding lines PMB-0285 and PMB-0306. After the first 7 days of water deficit treatment, PHA-0246 and PHA-0683 plants did not show a lower N₂ fixation rate than the corresponding controls, whereas PMB-0285 and PMB-0306 plants showed 54 and 23 % inhibition, respectively. After 14 days of drought (severe stress) PHA-0246 and PHA-0683 plants were still able to maintain around 50 % of their N₂ fixation compared with control plants (Fig. 1A–D). In contrast, N₂ fixation in PMB-0306 declined to about 20 % of that in well irrigated controls and was completely inhibited in PMB-0285 (Fig. 1A–D).

Statistical analysis of the data revealed that the four accessions did not differ significantly in their nitrogen fixation rates under well irrigated conditions. In contrast, the differences in nitrogen fixation among the accessions were significant after 14 days of drought, with PHA-0683 and PHA-0246 showing higher performance under drought stress than lines PMB-0306 and PMB-0285 (Supplementary Data Tables S2–S4).

Soil RWC was measured during the treatments. Results shown in Fig. 1E indicate that the decrease in soil water content was similar for all four genotypes undergoing the water deficit treatment. Estimation of leaf relative water content also showed that leaves from all the genotypes were similarly affected by water deficit (Fig. 1F).

Nodules from each genotype were differently affected by the stress. Nodules from PHA-0246 and PHA-0683 showed only a slight decrease in their dry mass in response to stress (18 and
38% reduction, respectively) and exhibited typical nodule senescence symptoms later than nodules from PMB-0285 and PMB-0306 plants. In contrast, water deficit caused a drastic reduction in PMB-0306 nodule dry weight (65%) and complete lack of measurable nodules in PMB-0285 roots (Table 2; Supplementary Data Fig. S1). Root and shoot biomass was also affected by the water deficit treatment, although the differences among genotypes were less pronounced than the effects observed in nodules. In general, root biomass increased in response to the stress in all genotypes, whereas shoot biomass showed a 10% reduction in PHA-0683, 20% in PHA-0246 and around 25% in PMB-0285 and PMB-0306 plants (Table 2).

**Ureide accumulation in aerial tissues under drought conditions**

To assess whether the N₂ fixation sensitivity of each genotype was related to the ureide content in plant tissues, the ureide concentration was determined in samples of nodules, roots, stems and leaves of plants from each genotype at 0, 7 and 14 days of drought treatment. Tissues from controls (well watered) and drought-stressed (drought) before (0) and during drought treatment (7 and 14 days). (E). Relative soil water capacity after 7 and 14 days of water deprivation. (F) Relative leaf water content after 7 and 14 days of water deprivation. Genotypes are ordered according to their degree of tolerance to N₂ fixation. Data are means of two independent experiments with 10–12 plants at each sampling time. Asterisks indicate statistically significant differences.

### Table 2. Plant biomass changes in response to drought

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Time (days)</th>
<th>Treatment</th>
<th>Nodules (g DW)</th>
<th>Ratio (drought/control)</th>
<th>Roots (g DW)</th>
<th>Ratio (drought/control)</th>
<th>Shoots (g DW)</th>
<th>Ratio (drought/control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PHA-0683</td>
<td>7</td>
<td>Control</td>
<td>0.027 ± 0.002</td>
<td>1.20</td>
<td>0.168 ± 0.024</td>
<td>1.70</td>
<td>1.700 ± 0.130</td>
<td>0.85</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>Drought</td>
<td>0.033 ± 0.001</td>
<td>0.82</td>
<td>0.302 ± 0.067</td>
<td>2.30</td>
<td>1.460 ± 0.132</td>
<td>0.91</td>
</tr>
<tr>
<td>PHA-0246</td>
<td>7</td>
<td>Control</td>
<td>0.030 ± 0.003</td>
<td>1.03</td>
<td>0.123 ± 0.025</td>
<td>1.84</td>
<td>1.700 ± 0.120</td>
<td>0.95</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>Drought</td>
<td>0.031 ± 0.005</td>
<td>0.82</td>
<td>0.227 ± 0.030</td>
<td>1.45</td>
<td>1.620 ± 0.066</td>
<td>0.81</td>
</tr>
<tr>
<td>PMB-0306</td>
<td>7</td>
<td>Control</td>
<td>0.035 ± 0.002</td>
<td>1.20</td>
<td>0.250 ± 0.077</td>
<td>1.25</td>
<td>1.840 ± 0.170</td>
<td>0.81</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>Drought</td>
<td>0.036 ± 0.005</td>
<td>0.35</td>
<td>0.363 ± 0.035</td>
<td>0.923 ± 0.127</td>
<td>0.850 ± 0.165</td>
<td>0.77</td>
</tr>
<tr>
<td>PMB-0285</td>
<td>7</td>
<td>Control</td>
<td>0.011 ± 0.004*</td>
<td>1.20</td>
<td>0.300 ± 0.030</td>
<td>1.84</td>
<td>1.850 ± 0.270</td>
<td>0.77</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>Drought</td>
<td>0.029 ± 0.006</td>
<td>0.87</td>
<td>0.218 ± 0.014</td>
<td>1.53</td>
<td>0.602 ± 0.053</td>
<td>1.00</td>
</tr>
</tbody>
</table>

*Significant difference between control and stressed samples.
irrigated plants) were collected and ureides measured at each sampling time.

In all four genotypes, levels of ureides in stems and leaves increased during the drought treatment, mainly in the form of allantoate (Fig. 2). However, the ureide levels in roots and nodules did not show any significant changes during the experiment in any plants (Fig. 2). There was no increase in ureides in the stressed nodules after 7 or 14 days of drought, although N₂ fixation was highly inhibited in the most sensitive lines, the breeding lines PMB-0285 and PMB-0306 (Figs 1 and 2).

In contrast to the lack of effect on ureide concentration in roots and nodules, there was a significant accumulation of ureides under drought conditions in the stems of all genotypes. Nevertheless, ureide levels in stems did not increase with increasing N₂ fixation sensitivity of the plants (Fig. 2). In contrast, leaves of the breeding lines (PMB-0306 and PMB-0285), in which N₂ fixation was more inhibited by the treatment, accumulated more ureides that the more tolerant PHA-0246 and PHA-0683 landraces. Moreover, differences in the changes in leaf ureide levels among genotypes increased when the stress became more severe (Fig. 2).

**Fig. 2.** Ureide (allantoin and allantoate) levels in nodules, roots, stems and trifoliate leaves from common bean genotypes after 0, 7 and 14 days of watered (W) or drought stress (D) treatment. Genotypes are ordered according to their degree of tolerance. Results are means of 10–12 measurements from at least two independent experiments. *P < 0.05.
Statistical analyses of the data confirmed that the ureide concentrations in the drought-stressed leaves of PMB-0285 and PMB-0306 were significantly higher than in leaves of control plants (Supplementary Data Table S2), and that there were significant differences in the accumulation of ureides among the genotypes (Supplementary Data Tables S3 and S4). The differences between 7 and 14 days of drought were higher in lines PMB-0306 and PMB-0285 than in PHA-0246 and PHA-0683 (Supplementary Data Tables S2–S4). Moreover, Pearson’s scores for N₂ fixation and ureides, as comparisons between control and stressed plants, were significantly correlated, both at 7 and 14 days of drought (0.88** and 0.89**, respectively).

Changes in ureide metabolism gene expression in response to drought

To determine whether the increasing values of tissue ureides (mainly allantoate) resulted from changes in the transcription of genes related to ureide metabolism in response to water stress, we used quantitative real-time PCR (qRT-PCR) to determine the mRNA levels of genes coding for the two key enzymes of allantoate metabolism, ALN and AAH. The activity of ALN is responsible for the synthesis of allantoate from allantoin, whereas AAH catalyses allantoate degradation. Expression of these two genes was measured in nodules, stems and leaves from control, well irrigated plants and from plants subjected to drought stress for 7 days. Expression levels of ureide metabolism genes in each sample were normalized with respect to the expression of Actin-2, used as internal control. The relative expression levels of the allantoate metabolism genes in tissues from drought-stressed plants, compared with their expression in well irrigated controls, are shown in Fig. 3. Although there are two genes coding for ALN in common bean (Díaz-Leal et al., 2012), expression levels of one of them were negligible and are not shown in Fig. 3. There were no major changes in the transcript levels of either ALN or AAH in the nodules of any of the genotypes after 7 days of water stress. In contrast, ALN mRNA expression was induced in stems and leaves of the breeding lines PMB-0285 and PMB-0306 and in the PHA-0246 landrace, but not in PHA-0683, which showed similar expression levels in control and drought-treated tissues. Moreover, the expression of ALN mRNA was higher in the drought-sensitive than in the tolerant genotypes (Fig. 3A). In contrast to ALN, the expression of AAH mRNA did not show any relevant changes in tissues from drought-stressed plants compared with well irrigated controls, for any of the four genotypes (Fig. 3B).

Accumulation of ureides is mainly due to induction of ALN activity

Besides gene expression levels, enzymatic activities of ALN and AAH, which catalyse the synthesis and degradation of allantoate, respectively, were measured for all four accessions, using tissue samples from control plants and plants subjected to 7 and 14 days of drought stress. Activity of ALN was determined in the same tissues in which ureides were determined, whereas AAH activity was determined only in leaves, because its activity level was too low for reliable measurement in other samples.

ALN activity increased together with the progressive water deficit in aerial tissues (stems and leaves), whereas it remained at levels comparable to those in control samples in roots and nodules from stressed plants (Fig. 4). Moreover, ALN activity levels in shoots from the four genotypes followed a trend similar to that of increments in ureide levels in the corresponding samples (Figs 2 and 4). In the stems of the stressed plants the ALN induction level was similar among all four genotypes, and increased with increasing stress (Fig. 4). In contrast to the similar induction in stems in the four genotypes, leaves showed a differential pattern of ALN induction. After 7 days of drought treatment, ALN activity in stressed leaves of PHA-0246 and PHA-0683 was similar to that in controls, whereas leaves from PMB-0285 and PMB-0306 showed a slight ALN increment. Allantoainase activity was induced in all plant genotypes after 14 days of drought treatment (Fig. 4). Nevertheless, ALN activity levels at 14 days of stress showed higher induction in PMB-0285 and PMB-0306 than in PHA-0246 and PHA-0683. Remarkably, PMB-0285 and PMB-0306 were the lines responding to stress with the largest increments in leaf ureides and whose N₂ fixation was most sensitive to drought (Figs 1 and 2).

In contrast to ALN induction in the stressed leaves, AAH activity was only slightly reduced under severe stress (14 days of water deprivation) in the leaves of the most drought-sensitive line, PMB-0285 (Fig. 5).
DISCUSSION

Nitrogen fixation is among the more severely affected processes in situations of water scarcity in legumes (Sinclair and Serraj, 1995; Zahran, 1999). Genotypic differences in the drought tolerance of N\textsubscript{2} fixation have been reported in soybean (Sall and Sinclair, 1991; Serraj and Sinclair, 1996, 1997; Purcell et al., 1997) and also in common bean (Ramos et al., 1999; Teran and Singh, 2002; Riveiro, 2012; Devi et al., 2013). The objective of this work has been to analyse whether the genotypic differences concerned not only the tolerance or sensitivity of the N\textsubscript{2} fixation process, but also the accumulation of ureides under these stress conditions.

Genotypic variations in the N\textsubscript{2} fixation response to water deficit have been found to be related to the accumulation of ureides in soybean genotypes (Sall and Sinclair, 1991; Serraj and Sinclair, 1996; Purcell et al., 2000, 2004). However, in one of the few studies in which the N\textsubscript{2} fixation response and changes in ureide levels were compared among common bean cultivars, there was no consistent variability in the N\textsubscript{2} fixation response to drought, although the cultivars showed differences in the levels of ureides.
Comparison of the sensitivity of N₂ fixation to drought also takes place among common bean genotypes. Serraj and Sinclair (1998) showed that landraces PHA-0246 and PHA-0683 were able to maintain higher N₂ fixation rates with moderate water stress than the breeding lines PMB-0285 and PMB-0306 (Fig. 1A–D). PMB-0306, considered an improved tolerant breeding line, showed moderate sensitivity, whereas, as expected, N₂ fixation was highly inhibited in the line PMB-0285, reported to be sensitive (Frahm et al., 2003) (Table 1).

In contrast to the differential effect of water stress on N₂ fixation, there were no substantial differences in the relative water deficit among the four genotypes (Fig. 1F). Moreover, comparison of nodule and plant biomass among control and stressed tissues showed that the drought treatment affected nodules more severely than plant tissues (Table 2). Drought had little effect on plant growth in any of the genotypes, with a shoot biomass reduction after 14 days of water deprivation that ranged from 10 to 24 % among sensitive and tolerant genotypes. However, despite its relatively mild effect on shoots, the water deficit treatment caused a severe dry weight reduction in the nodules of the sensitive genotypes while the effect was moderate in the more tolerant genotypes (Table 2). This effect was fully coincident with the degree of N₂ fixation inhibition in the different accessions (Fig. 1). In accordance with these results, a similar study using a common bean genotype considered to be drought tolerant, Ramos et al. (1999) reported a relatively small effect on both leaf water potential and plant dry mass after 10 days of exposure to two regimes of water deficit. These authors also found that nitrogenase activity and nodule dry mass were more affected by the treatment than the plant growth parameters.

Reports in soybean have shown that under drought stress a high increment of ureide concentrations occurs in the shoots of the most sensitive genotypes (Purcell et al., 2000; Sinclair et al., 2000), and it was proposed that ureides could be involved in the inhibition of N₂ fixation, by means of feedback signalling (Serraj et al., 1999b; King and Purcell, 2005). In this work we observed that, in contrast to soybean, in the common bean genotypes analysed the relationship between the accumulation of shoot ureides and drought sensitivity was not straightforward, since all four genotypes showed accumulation of ureides in their stems under drought conditions. Moreover, ureide concentrations increased in stems under water deficit independently of the degree of nitrogen fixation tolerance or sensitivity to drought in each genotype (Figs 1 and 2). Furthermore, nitrogen fixation in the more tolerant genotypes was not inhibited by the ureides already accumulated in their stems at 7 days of treatment (Figs 1 and 2). These data suggest that, contrary to reports in soybean, ureide accumulation in common bean shoots is not the primary signal of nitrogen fixation inhibition.

In contrast to the results in stems, we found that ureide accumulation in stressed leaves was fully consistent with previous reports in soybean. There was a higher accumulation of ureides in the leaves from the genotypes in which N₂ fixation was more heavily inhibited than in the leaves of those in which N₂ fixation was more tolerant (Figs 1 and 2).

There is still some controversy on whether the accumulation of ureides, and hence their supposedly inhibitory effect on N₂ fixation, occurs at a local level, in the nodules, or whether the increase in ureides in shoots may be able to inhibit nodule activity through systemic signalling. Several studies in soybean have suggested that the ureides accumulated in shoots would be transported by the phloem to nodules, where they inhibited nitrogenase activity (Charlson et al., 2009; Vadez and Sinclair, 2003).
Moreover, Ladrera et al. (2007) and King and Purcell (2005) attributed the inhibition of nitrogen fixation in sensitive soybean genotypes to ureide accumulation at the local level, within nodules. Instead, in recent work using a split-root system, the role of ureides as compounds causing inhibition of nitrogen fixation in soybean nodules was rejected, since, although ureide levels increased in the stressed nodules, the increment occurred later than the inhibition of N2 fixation (Gil-Quintana et al., 2013). In contrast to these studies, our results show that, in common bean, ureide levels increase in aerial tissues (stem and leaves) but not in nodules during the experimental drought period (Fig. 2). These results are consistent with recent work from our group performed in the common bean breeding line ‘Matterhorn’ (Great Northern market class), in which accumulation of ureides in leaves and stems occurred even after nitrogen fixation was inhibited by the drought treatment, or even in the total absence of nodules, in nitrate-fed plants (Alamillo et al., 2010). In the present study, the near or total inhibition of nitrogen fixation, with already senescent nodules, in the most sensitive genotypes (Fig. 1A–D; Table 2) was, however, accompanied by progressive increases in ureide concentration in the stems and leaves (Fig. 2). Since the accumulation of ureides in the leaves occurred later than the inhibition of nitrogen fixation in the sensitive genotypes, this indicates that there is no increment in the synthesis of ureides in nodules under such conditions (Figs 1 and 2). Moreover ureides did not accumulate in nodules from either tolerant or sensitive genotypes. Lack of ureide accumulation in nodules subjected to drought stress was also observed in a recent study using a broad-range analysis of drought-induced metabolic adjustment in two common bean genotypes (Silvente et al., 2012).

It remains an intriguing question why there is ureide accumulation in response to drought in the nodules of susceptible soybean genotypes but not in common bean ones, since both plants are ureidic legumes, and it would be interesting to check whether the symbiotic counterpart may contribute to these differences. The different kinetics of N2 fixation inhibition and ureide accumulation shown in this and in our previous report (Alamillo et al., 2010) further support that, at least in common bean, ureide accumulation in shoots does not seem to be the primary source of nitrogenase inhibition in drought-stressed nodules. Moreover, the increase in ureide levels after nitrogenase inhibition suggests that drought induces signalling pathways that lead to ureide accumulation, probably through the remobilization of other compounds from senescent tissues.

King and Purcell (2005) proposed that inhibition of AAH activity mediated by Mn deficiency could be the actual cause of allantoate accumulation under drought. However, Charlson et al. (2009) did not find any relevant changes in AAH expression between controls and drought-stressed plants. Moreover, in common bean, changes in neither AAH expression nor AAH activity were observed in response to drought, but in contrast there was a high induction of ALN, the enzyme that catalyses the synthesis of allantoate (Alamillo et al., 2010). In the present work we observed that the rise in ureide concentration in the water-stressed leaves of the more sensitive genotypes was correlated with higher induction of the expression and activity of allantoinase in these plants (Fig. 4). Nevertheless, in this work we also found slight inhibition of AAH activity in the most sensitive line (Fig. 5), although the expression of its gene was largely unaffected (Fig. 3B), thus suggesting that inhibition of allantoinase degradation may also contribute to the high allantoate accumulation in the leaves of highly sensitive genotypes.

Treatment with abscisic acid (ABA), the main hormone that regulates the plant response to drought, induced the expression of key genes for the synthesis of ureides, such as XDH (Yesbergenova et al., 2005), UO and ALN (Alamillo et al., 2010). Ureide synthesis induced in drought-stressed leaves seems to be part of a general response mediated, at least in part, by ABA. This response probably follows the same pattern as the inhibition of N2 fixation and therefore could be related to the drought sensitivity of each genotype. Results presented in this work suggest that the accumulation of ureides in drought-stressed tissues cannot be responsible for the feedback inhibition of N2 fixation. Moreover, lack of ureide accumulation in the nodules and the increment in ureide levels after the inhibition of N2 fixation in the more sensitive genotypes strongly suggests that ureides accumulating in leaves do not originate solely in the nodules of stressed plants. In contrast, our results indicate that, under the condition of water deficit, alternative pathways of ureide production are activated in aerial tissues of common bean. It is noteworthy that higher levels of allantoin have been observed in the desiccation-tolerant plant Sporobolus stapfianus compared with the desiccation-sensitive Sporobolus pyramidalis (Oliver et al., 2011) and also in the dry stages of the extremely drought-tolerant moss Selaginella lepidophylla (Yobi et al., 2013). Similarly, high levels of grain allantoin in some rice genotypes have been shown to be beneficial in drought and other abiotic stresses (Wang et al., 2012), pointing to nitrogen-rich ureides as part of an ancient and broad-range plant strategy to survive drought stress. Ureides have been suggested as the best compounds to channel remobilized nitrogen to seed filling (Diaz-Leal et al., 2012). Therefore, it is tempting to speculate that they may act as a nitrogen and perhaps a carbon source to accelerate seed production under drought conditions. Nevertheless, further studies will be needed to ascertain the actual function of the accumulation of ureides under stress conditions.

In summary, the results shown here indicate that, as in soybean, there are large genotypic differences in the drought sensitivity of nitrogen fixation among common bean germplasms, and that the genetic variation is linked to ureide accumulation in stressed leaves. In addition, we have found that the two landraces studied (PHA-0246 and PHA-0683) had better performance under drought stress that the tolerant breeding line PMB-0306, and could therefore be used in breeding programmes designed to improve drought tolerance and the efficiency of biological fixation of nitrogen in common bean.

**SUPPLEMENTARY DATA**

Supplementary data are available online at www.aob.oxfordjournals.org and consist of the following. Table S1: primers used for real time qRT-PCR. Table S2: Student’s t-test analysis of nitrogen fixation and leaf ureide levels between control and drought-stressed plants. Table S3: analysis of variance of nitrogen fixation and leaf ureide levels after 7 and 14 days of water deprivation. Table S4: LSD analysis of apparent nitorgenase activity and leaf ureide concentrations after 7 and 14 days of water deprivation. Figure S1: seeds of the four genotypes used in this
study and effect of water stress on the appearance of the nodules during the course of the treatment.

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LITERATURE CITED


