Annals of Botany **91**: 85–89, 2003
doi:10.1093/aob/mcg012, available online at www.aob.oupjournals.org

Evidence that Carbon Dioxide Enrichment Alleviates Ureide-induced Decline of Nodule Nitrogenase Activity

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Received: 15 July 2002 Returned for revision: 11 September 2002 Accepted: 17 October 2002 Published electronically: 21 November 2002

The hypothesis that elevated [CO_2] alleviates ureide inhibition of N_2-fixation was tested. Short-term responses of the acetylene reduction assay (ARA), ureide accumulation and total non-structural carbohydrate (TNC) levels were measured following addition of ureide to the nutrient solution of hydroponically grown soybean. The plants were exposed to ambient (360 μmol mol^{-1}) or elevated (700 μmol mol^{-1}) [CO_2]. Addition of 5 and 10 mM ureide to the nutrient solution inhibited N_2-fixation activity under both ambient and elevated [CO_2] conditions. However, the percentage inhibition following ureide treatment was significantly greater under ambient [CO_2] as compared with that under elevated [CO_2]. Under ambient [CO_2] conditions, ARA was less than that under elevated [CO_2] 1 d after ureide treatment. Under ambient [CO_2], the application of ureide resulted in a significant accumulation of ureide in all plant tissues, with the highest concentration increases in the leaves. However, application of exogenous ureide to plants subjected to elevated [CO_2] did not result in increased ureide concentration in any tissues. TNC concentrations were consistently higher under elevated [CO_2] compared with those under ambient [CO_2]. For both [CO_2] treatments, the application of ureide induced a significant decrease of TNC concentrations in the leaves and nodules. For both leaves and nodules, a negative correlation was observed between TNC and ureide levels. Results indicate that product(s) of ureide catabolism rather than tissue ureide concentration itself are critical in the regulation of N_2-fixation.

Key words: Carbon dioxide, *Glycine max*, nitrogen fixation, nodules, soybean, ureides.

INTRODUCTION

Feedback regulation of legume symbiotic N_2-fixation by N compounds has been hypothesized as an explanation for decreases in nitrogenase activity under several stress factors (Hartwig *et al*., 1994), including mineral N application (Oti-Boateng and Silsbury, 1993; Bacanamwo and Harper, 1997; Vadez *et al*., 2000*a*), drought stress (Serraj and Sinclair, 1996; Serraj *et al*., 1999*a*) and P deficiency (Almeida *et al*., 2000). The N feedback hypothesis proposes that nitrogenous compounds feed back to the nodule when there is excess nitrogen in the shoot, inducing an inhibition of nitrogenase activity (Heim *et al*., 1993; Parsons *et al*., 1993; Streeter, 1993; Hartwig *et al*., 1994; Serraj *et al*., 1999*a*).

In soybean [*Glycine max* (L.) Merr.], the main nitrogenous compounds exported from nodules to the shoot, where they are catabolized, are ureides, e.g. allantoin and allantoic acid (Alac). We have previously shown that application of external ureide to the nutrient solution of hydroponically grown soybean increased shoot ureide concentration and inhibited nitrogenase activity (Serraj *et al*., 1999*b*; Vadez *et al*., 2000*a*). More recent results have indicated that, besides ureides, asparagine (Asn) is also accumulated in nodules upon Alac or Asn treatment (Vadez *et al*., 2000*a*). Therefore, both ureide and Asn are candidate feedback compounds from shoots, although it was difficult to separate their respective effects as both Alac and Asn application inhibited the acetylene reduction assay (ARA) with a concomitant increase of their nodule concentrations.

Leaf ureide catabolism is involved in the regulation of nitrogenase activity under stress (Vadez *et al*., 2000*b*) as indicated by the fact that treatments that stimulate rates of ureide degradation result in diminished inhibition of N_2-fixation (Serraj *et al*., 1998; Vadez *et al*., 2000*b*). Since ureides accumulate in plant tissues under water deficit, it has been proposed that ureide accumulation may be involved in a feedback decline of nodule activity under these conditions (Sinclair and Serraj, 1995; Serraj and Sinclair, 1996; Serraj *et al*., 1999*a*).

An intriguing observation with regards to ureide feedback was that exposing shoots to elevated [CO_2] resulted in substantially less drought-induced N_2-fixation inhibition (Serraj *et al*., 1998). The elevated [CO_2] treatment also resulted in less ureide accumulation in leaf and nodule tissues compared with that in the ambient [CO_2] treatment. These results are in agreement with the well-documented effect of elevated atmospheric [CO_2] on the reduction in nitrogen content of plant tissues (Wong, 1979; Norby *et al*., 1986). The large effect of [CO_2] on ureide accumulation in leaves, taken together with the negative relationship observed between ureide accumulation and TNC levels under drought (Serraj *et al*., 1998), is consistent with the involvement of ureide breakdown in the response of N_2-

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TABLE 1. *Effect of CO_2 level and various ureide treatments on plant dry weight, acetylene reduction assay (ARA) and daily transpiration rate, measured at the end of 5 d of treatment*

	Ureide concentration (mM)	Shoot d. wt (g per plant)	Root d. wt (g per plant)	Nodule d. wt (g per plant)	ARA (μmol h^{-1} per plant)	Transpiration (g per plant)
Ambient [CO_2]	0	2·98 ± 0·14	1·18 ± 0·05	0·47 ± 0·03	28·6 ± 3·9	80 ± 10
	5	3·95 ± 0·45	1·30 ± 0·08	0·45 ± 0·08	15·9 ± 2·2	111 ± 12
	10	3·45 ± 0·57	1·34 ± 0·17	0·40 ± 0·07	8·4 ± 2·7	85 ± 12
Elevated [CO_2]	0	3·53 ± 0·46	1·08 ± 0·09	0·48 ± 0·09	38·8 ± 3·2	54 ± 10
	5	3·99 ± 0·39	1·47 ± 0·10	0·48 ± 0·04	23·0 ± 5·9	67 ± 12
	10	4·68 ± 0·12	1·57 ± 0·06	0·49 ± 0·01	17·0 ± 2·7	81 ± 12
Source	d.f.	*P*	*P*	*P*	*P*	*P*
[CO_2]	1	0·13	0·29	0·48	<0·0001	0·02
[Ureide]	2	0·17	0·02	0·88	<0·0001	0·12
[CO_2] × [Ureide]	5	0·48	0·30	0·87	0·04	0·25

Mean of four replicates (± s.e.).

fixation to drought and with the hypothesis of feedback inhibition by ureides. If the main effect of elevated [CO_2] was to stimulate leaf ureide catabolism, N_2-fixation would not be inhibited and nodule activity would be sustained.

The hypothesis that elevated [CO_2] results in increased ureide catabolism in leaves is still speculative since there is no direct evidence linking CO_2 levels, ureide metabolism and N_2-fixation activity. The purpose of this work was to test the effects on N_2-fixation of a direct application of ureide to the nutrient solution of hydroponically grown soybean under ambient and enriched [CO_2] environments. It was hypothesized that CO_2 enrichment would counteract leaf ureide accumulation induced by exposure to external ureides, and the subsequent inhibition of N_2-fixation.

MATERIALS AND METHODS

Plant growth in hydroponic conditions

Soybean plants were initially grown in a glasshouse under ambient CO_2 for 4 weeks, in conditions described previously (Serraj and Sinclair, 1999*b*). Seeds of soybean (cultivar Braxton) were inoculated with a commercial preparation of *Bradyrhizobium japonicum* (Nitragin, Milwaukee, WI, USA) and germinated in soil. The emerged seedlings were inserted in rubber stoppers placed in 1-l Erlenmeyer flasks. Plants were grown on a nutrient solution containing the following concentrations of macro- and micro elements: 3·3 mM $CaCl_2$; 2·05 mM $MgSO_4$; 1·25 mM K_2SO_4; 4 μM H_3BO_3; 6·6 μM $MnSO_4$; 1·55 μM $ZnSO_4$; 1·55 μM $CuSO_4$; 0·12 μM $NaMoO_4$; and 40 μM FeEDTA. The solution used for the first 2 weeks immediately following transplanting contained 1 mM urea; thereafter, the nutrient solution was changed twice weekly and contained no urea. The pH of the solution was maintained close to 7·0 by adding 0·2 g l^{-1} $CaCO_3$, and air was continuously bubbled through the solution at a flow rate of 2 l min^{-1} (Serraj and Sinclair, 1999*b*). The volume of nutrient solution was maintained at 40 % of the flask volume so that the nodules developed above the nutrient solution. At 4 weeks, plants were divided into two groups and transferred to two adjacent glasshouses for exposure to differing atmospheric [CO_2]. In one glasshouse the [CO_2] was maintained at an ambient level (360 μmol mol^{-1} during the daylight period) and in the other it was maintained continuously at a concentration of 700 μmol mol^{-1}, as described previously (Serraj *et al*., 1998).

Ureide treatment

Following transfer to the CO_2-exposure glasshouses, plants were allowed to acclimate for 5 d before beginning the ureide treatments. The intact nodulated roots were exposed to ureide by replacing the nutrient solution in the flask with 400 ml nutrient solution containing 0, 5 or 10 mM Alac. There were four replicate plants for each CO_2 and ureide treatment. The volume of the nutrient solution was measured daily by weighing the flasks, and adjusted to the original level by adding fresh nutrient solution. The daily transpiration rate was computed for each plant as the daily loss of nutrient solution. The ureide treatment was maintained for 5 d.

In situ *acetylene reduction assays*

ARA was monitored daily on undisturbed, intact plants, sealed in Erlenmeyer flasks (Serraj and Sinclair, 1999*b*). Acetylene was added to the air stream normally flowing into the nutrient solution to give a 9 : 1 mixture of air : acetylene. The gas flow was adjusted to 1 l min^{-1}, and time (15 min) was allowed for steady-state conditions to be achieved. No evidence of an acetylene-induced decline in nodule activity was observed in this system. After steady-state conditions had been achieved, a gas sample was collected from the gas exit port of each flask and the ethylene concentration was measured using a gas chromatograph equipped with a flame ionization detector. After ARA measurement, acetylene flow was stopped and the flasks were returned to continuous flushing with acetylene-free gas.

Ureide and TNC measurements

After 5 d of ARA measurements, plants were harvested and oven-dried for 2 d to determine dry matter accumulation in the leaves, stems, roots and nodules. These samples were

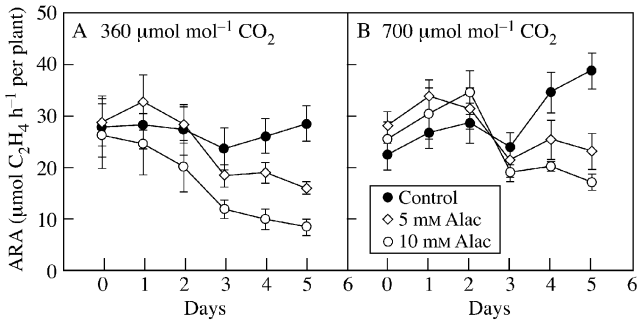

FIG. 1. Acetylene reduction assay (ARA) under 360 μmol mol^{-1} CO_2 (A) and 700 μmol mol^{-1} CO_2 (B), as a function of time, following exposure to 0, 5 or 10 mM allantoic acid (Alac). Bars represent s.e.m.

individually ground and analysed for ureide contents using a colorimetric method (Trijbels and Vogel, 1966). Ureides were extracted by boiling a 0·1-g sub-sample of ground tissue in 1 ml of 0·2 M NaOH for 30 min. Samples were centrifuged, refrigerated overnight, and 0·1–0·2 ml of supernatant used for ureide determination.

TNC contents were analysed in nodule, shoot and root tissues using the procedure described by Serraj *et al*. (1998). Approximately 0·1 g d. wt of ground tissue was refluxed twice, each time with 10 ml of an 80 % ethanol solution, at 95 °C for 1 h. After centrifugation (10 000 *g*, 15 min), the supernatants (containing soluble sugars) were combined and the pellet (starch) was oven-dried at 60 °C overnight. The ethanol extracts were evaporated to approx. 3 ml, and distilled water was added to a volume of 10 ml. The solution was mixed with approx. 20 mg of finely divided activated charcoal (Norit A; Sigma, St Louis, MO, USA), the tubes were then shaken gently and centrifuged (5000 *g*, 15 min) to produce a clear alcoholic extract. This charcoal purification step was essential to remove interfering ethanol-soluble materials in plant extracts (Hendrix, 1993). The purified extract was assayed for soluble sugars using an enzymatic kit (no. 716260; Boehringer Mannheim, Indianapolis, IN, USA). The pellet was suspended in 1·0 ml 0·2 N KOH and placed in boiling water for 30 min. After cooling, the pH was adjusted to 5·5 with 1·0 N acetic acid. One millilitre of dialysed amyloglucosidase solution (from *Aspergillus oryza*, Sigma Chemical Co., St Louis, MO, USA) (50 units of enzyme ml^{-1} Na acetate buffer, pH 4·5) was added to each sample, and the flasks were incubated at 55 °C for 1 h. Following incubation and centrifugation at 10 000 *g* for 30 min, glucose content in the supernatant was determined colorimetrically (procedure no. 635; Sigma Chemical Co.). TNC levels were computed from the sum of starch and soluble sugars. Data were analysed statistically using a two-way ANOVA with the SAS system (Cary, NC, USA).

RESULTS

Changes in shoot, root and nodule biomass in response to 5 d of [CO_2] and Alac treatments are given in Table 1. Probably as a result of the relatively short duration of the treatment, there was no significant effect of Alac or [CO_2] treatments on shoot and nodule dry weight, although a trend for increased shoot dry weight was observed in response to Alac and [CO_2] treatments. Root dry weight increased significantly following Alac treatment (Table 1). Exposure of soybean plants to elevated [CO_2] rather than ambient [CO_2] resulted in significantly lower transpiration rates for plants in the 0 and 5 mM Alac treatments.

Exposure to elevated [CO_2] stimulated ARA, irrespective of the concentration of Alac in the nutrient solution (Fig. 1). After 5 d, control plants (without Alac application) at elevated [CO_2] had 35 % higher ARA values ($P < 0·01$) than plants at ambient [CO_2] (Table 1). ARA data following 5 d of ureide treatment showed a significant interaction between [CO_2] and ureide treatments (Table 1). Application of 5 and 10 mM ureide to the nutrient solution inhibited nitrogenase activity under both ambient and elevated [CO_2] conditions (Fig. 1). However, the inhibition due to Alac seemed to have been delayed under elevated [CO_2] since ARA tended to increase under the elevated [CO_2] treatments for the first 2 d, whereas this was not the case under ambient [CO_2]. On day 3, there was a significant decrease in ARA after exposure to 5 and 10 mM ureide in both [CO_2] treatments (Fig. 1).

The application of 5 and 10 mM Alac under ambient [CO_2] resulted in a significant accumulation of ureide in nodule, root and leaf tissues (Fig. 2). However, increases in ureide concentration were much greater in the leaves than in the other tissues. Importantly, plant exposure to elevated [CO_2] resulted in no accumulation of ureides in response to Alac application in any tissue. Ureide levels remained unchanged under elevated [CO_2], although the plants were exposed to Alac application in the nutrient solution (Fig. 2).

TNC data showed significant effects of [CO_2] treatment in all plant tissues, with consistently higher TNC levels under elevated [CO_2] compared with those under ambient [CO_2] (Fig. 3). Application of 5 and 10 mM Alac induced a significant decrease of TNC concentrations in plants in both [CO_2] treatments, and in all plant tissues except roots under elevated [CO_2]. However, this effect was more marked in the leaves and nodules than in the roots. For both leaves and nodules, negative linear correlations were recorded between TNC and ureide levels, with a higher negative slope for nodules than for leaves (Fig. 4).

DISCUSSION

This work has demonstrated that ureide accumulation and inhibition of nitrogenase activity following exposure to external application of Alac were partially alleviated by CO_2 enrichment. The ureide-induced decline of ARA was significantly less under elevated [CO_2] as compared with ambient [CO_2] for both 5 and 10 mM Alac treatments (Table 1; Fig. 1). These findings bring new evidence to support the hypothesis of N feedback regulation of N_2-fixation.

Recently, Vadez *et al*. (2000*a*) suggested that there may be two possible origins for feedback inhibition of nodule activity: (1) a direct feedback within the nodule from accumulation of nitrogenous compounds, presumably ureides, that fail to be exported via the xylem; and (2) an indirect feedback originating from the leaves, with several

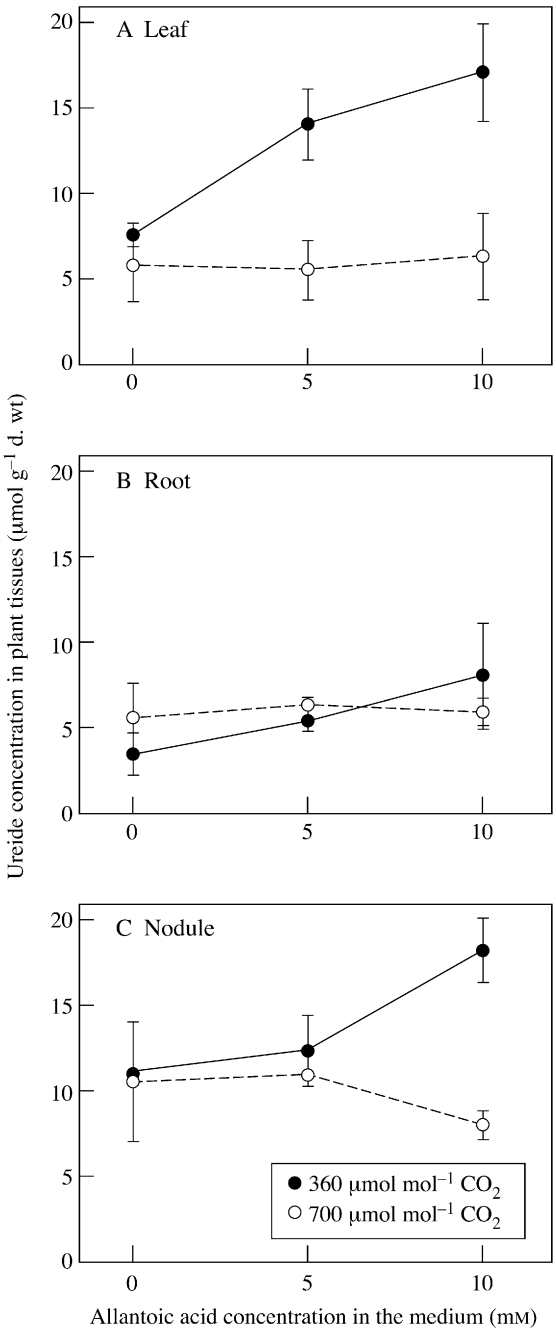

FIG. 2. Ureide concentration in plant tissues on day 5 of treatment, as a function of the concentration of allantoic acid in the nutrient solution, for soybeans exposed to two atmospheric CO_2 concentrations. Bars represent s.e.m.

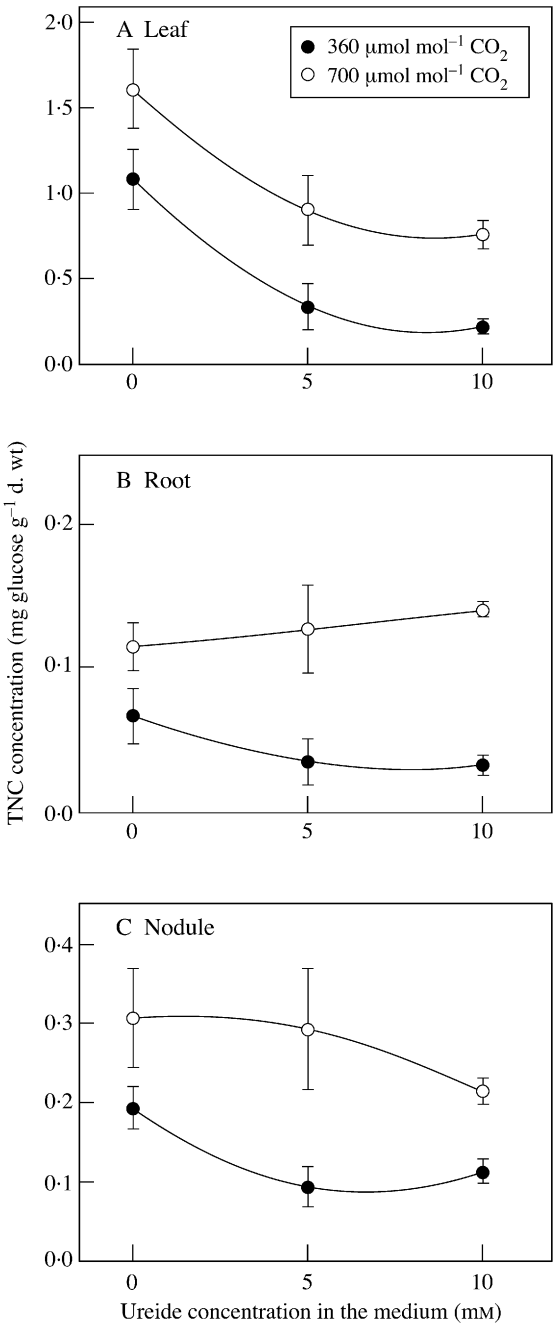

FIG. 3. Total non-structural carbohydrate (TNC) concentration in different plant tissues on day 5 of treatment, as a function of the concentration of allantoic acid in the nutrient solution, for soybeans exposed to two atmospheric CO_2 concentrations. Bars represent s.e.m.

likely candidates compounds including Asn and ureides, and depending on the interaction with ureide degradation metabolism. The ureide data in the present work do not allow a clear distinction between the two possibilities. However, ureide concentrations in the leaves appear to be more responsive to Alac application than those in nodules and roots (Fig. 2), which could indicate a role of leaf N in the feedback control of nodule activity. This agrees with the results of Neo and Layzell (1997) and Bacanamwo and Harper (1997) showing that feedback seems to result from compounds originating from the shoot. In fact, as concluded by Vadez *et al*. (2000*b*), it is possible that both currently fixed nitrogen and nitrogen recycled from the shoot may contribute to the feedback regulation of nitrogenase activity.

Under ambient [CO_2], the application of 5 and 10 mM Alac resulted in a significant accumulation of ureide in nodule, root and leaf tissues (Fig. 2). Importantly, plant exposure to elevated [CO_2] did not result in any accumulation of ureides in response to Alac application in any plant tissue. The lowered ureide levels under elevated [CO_2] are

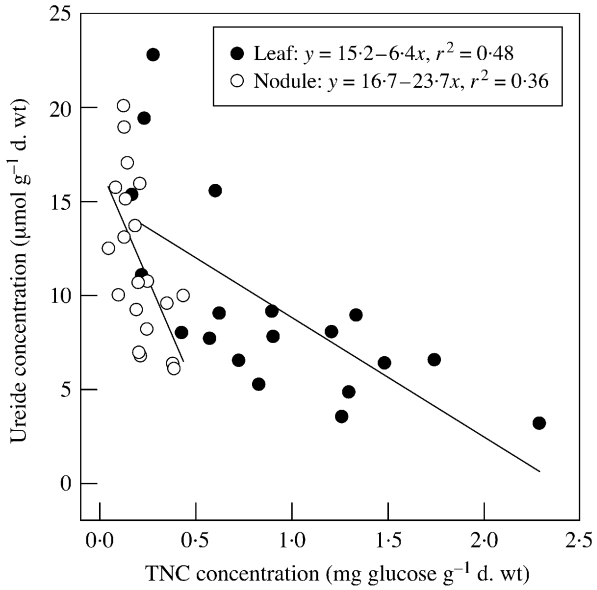

FIG. 4. Leaf and nodule ureide concentrations on day 5 of treatment, as a function of total non-structural carbohydrate (TNC) concentration, for soybeans exposed to two atmospheric CO_2 concentrations and three ureide treatments.

in agreement with the well-documented effect of elevated atmospheric [CO_2] on the reduction in N content of plant tissues (Wong, 1979; Norby *et al*., 1986; Soussana and Hartwig, 1996). It has also been reported recently that elevated [CO_2] triggered more than a 50 % reduction in leaf ureide content in soybean under both well-watered and drought conditions (Serraj *et al*., 1998). The decreased ureide concentration in leaves in response to elevated [CO_2] is consistent with the hypothesis of ureide breakdown as a critical feature of N_2-fixation regulation and feedback inhibition by ureides (Vadez *et al*., 2000*a*).

Ureide treatment resulted in decreased ARA rates under elevated [CO_2] even though there was no increase in ureide concentration in any tissue (Fig. 2). These results indicate that tissue ureide concentration itself was not directly responsible for the reduction in N_2-fixation activity. Seemingly, either ureide accumulation critical to the control of N_2-fixation is in a small pool, or a product of ureide metabolism may be responsible for the feedback on N_2-fixation rates. The second possibility is consistent with the hypothesis that Asn is crucial in the regulation of N_2-fixation activity. Under elevated [CO_2], the rate of ureide breakdown appears to have been high, resulting in no ureide accumulation in the leaves, but products of ureide catabolism may have accumulated to cause a feedback inhibition of N_2-fixation. Under ambient [CO_2], products of ureide catabolism may have accumulated more rapidly and to a greater concentration than under the elevated [CO_2] treatment. This could explain the difference between [CO_2] treatments in the dynamic response to ureide treatments, particularly in the 10 mM Alac treatment, in the first 2 d following ureide treatment.

ACKNOWLEDGEMENTS

Partial support for this research was provided by United Soybean Board project 8206P.

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