Soybean N_2 Fixation Estimates, Ureide Concentration, and Yield Responses to Drought

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ABSTRACT

Increasing N_2 fixation tolerance to drought has been hindered by the labor and costs of quantifying N_2 fixation using ^{15}N methodologies. The relative abundance of ureides (RAU) in plant tissues has been used for estimating N_2 fixation in soybean [*Glycine max* (L.) Merr.] grown under well-watered conditions, but it has not been evaluated for drought conditions. The present research evaluated the response of N accumulation to N fertilization, the ability of the RAU technique to predict N_2 fixation under drought conditions, and the response of yield to N fertilization under well-watered and drought conditions. Under drought, shoot N accumulation rate during vegetative growth approximately doubled as the amount of N fertilizer was increased from 10 to 200 kg N ha^{-1}, indicating a greater sensitivity of N_2 fixation to drought than uptake and assimilation of inorganic N. Under well-watered conditions, the relationship between estimates of N_2 fixation made by ^{15}N-dilution and RAU agreed within 15% of published reports. Under drought conditions, however, this relationship was greatly different (13 to 43%) from published reports. Fertilization with inorganic N in 1 yr increased grain yield 15 to 25% for the drought treatment and 12 to 15% for the well-watered treatment. In a second year, N fertilization increased yield of both drought and well-watered treatments approximately 9%. This research indicates that the RAU technique for estimating N_2 fixation under drought conditions may be invalid without further refinement, that N_2 fixation is more sensitive to drought than the uptake and assimilation of inorganic soil N, and that increasing the tolerance of N_2 fixation to drought would likely result in yield increases.

Nitrogen nutrition in soybean is met by a combination of the uptake and assimilation of inorganic-soil N and symbiotic N_2 fixation. The relative importance of these two sources of N in meeting the crop's N needs changes depending upon the availability of inorganic-soil N (Harper, 1987). Thus, when inorganic-soil N is abundant, N_2 fixation is inhibited or delayed and the proportion of N in the crop derived from N_2 fixation is decreased. Conversely, when there is little inorganic-soil N available, N_2 fixation provides the majority of the crop's N needs.

The proportion of a soybean crop's N derived from inorganic-soil N or from N_2 fixation may also change depending upon whether or not the crop is exposed to drought or other environmental constraints. Field (Sinclair et al., 1987; Sall and Sinclair, 1991; Serraj et al., 1997) and greenhouse (Sall and Sinclair, 1991; Purcell et al., 1998) experiments have shown that N_2 fixation in soybean is especially sensitive to water deficits and decreases before transpiration or photosynthesis. Furthermore, soybean grain yield under drought conditions was increased by 18% by applying 336 kg N ha^{-1} of NH_4NO_3 compared with treatments receiving no N fertilizer (Purcell and King, 1996). We are unaware of any soybean data that address how the proportion of N derived from N_2 fixation or from the soil may change in response to drought.

Obstacles for measuring N_2 fixation in field experiments include the labor required for collecting plant samples and the costs of ^{15}N-enriched fertilizer and isotope analysis of plant samples. Typically, soybean and a reference crop that does not fix N_2 are grown on soil enriched with ^{15}N fertilizer, plant samples are harvested from an area of the plots, and the samples are dried, weighed, ground, and analyzed by mass spectroscopy. As the soybean crop fixes N_2 from the atmosphere, ^{15}N derived from the soil is diluted relative to that of the reference crop, and this relationship can be used to estimate the N derived from the atmosphere (NDA) (Fried and Middleboe, 1977; Herridge and Peoples, 1990):

$$\text{NDA} = 1 - [(\text{atom\% excess of soybean})/(\text{atom\% excess of reference})] \quad [1]$$

The quantity of N_2 fixed by the crop is represented by the product of the total N content per square meter multiplied by NDA. Rates of N_2 fixation are calculated from the increase in the quantity of N_2 fixed by the crop between two sampling dates divided by the number of days between samplings.

An alternative method for estimating NDA has been developed for soybean (Herridge and Peoples, 1990) and other legumes (Herridge and Peoples, 2002) that use allantoin and allantoate (collectively referred to as ureides) as their primary N-export product from nodules. In this procedure, the concentration of ureides from petiole-tissue extracts is expressed as the RAU, which is the fraction of the N from ureides relative to the sum of the N found in ureides and NO_3^-:

$$\text{RAU} = (4 \times \text{ureide conc.})/[(4 \times \text{ureide conc.}) + NO_3^- \text{ conc.}] \quad [2]$$

In Eq. [2], ureide concentration is multiplied by four because there are four N atoms in the ureide molecules. A similar ratio of RAU may also be calculated for xylem sap. In this case, the denominator of Eq. [2] includes

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Abbreviations: DOY, Day of Year; NDA, N derived from atmosphere; NN-Hardee, nonnodulating 'Hardee'; PNDA, proportion of N derived from atmosphere between harvests; RAU, relative abundance of ureides.

the sum of total amino acids found in the extract (Herridge and Peoples, 1990).

The relationship between RAU and N_2 fixation (as determined by ^{15}N dilution) in soybean has been established in greenhouse-grown soybean plants using RAU values from both xylem sap and stem extracts from plants sampled during vegetative and reproductive development (Herridge and Peoples, 1990). By using this relationship between RAU and ^{15}N dilution, Herridge et al. (1990) were able to predict accurately N_2 fixation rates in field-grown soybean. This technique was also shown to be an effective tool in a breeding program for selecting lines capable of nodulating and fixing N_2 in the presence of soil NO_3^- (Herridge and Rose, 1994).

One possible source of error for the RAU technique that has not been evaluated is whether or not the relationship between RAU and N_2 fixation changes under drought conditions. Because ureide concentration in xylem sap (Serraj and Sinclair, 1996) and stem extracts (de Silva et al., 1996; Serraj and Sinclair, 1996) increases greatly under drought conditions, it is important to establish the relationship between RAU and N_2 fixation under water-limited conditions.

The objectives of our research were to: (i) evaluate the sensitivity of N accumulation from inorganic soil-N and of N_2 fixation under well-watered and drought conditions in the field; (ii) compare the relationship between RAU and NDA from ^{15}N dilution data for well-watered and drought conditions; and (iii) assess previous reports that N fertilization increased grain yield under drought conditions. These objectives were pursued in a series of field experiments in Florida, where we compared ^{15}N-dilution and RAU techniques for estimating N_2 fixation for well-watered and drought treatments, and in Arkansas, where we evaluated RAU and yield responses to N fertilization for well-watered and drought conditions.

MATERIALS AND METHODS

Florida, 1996

The experiment was conducted at the Irrigation Research and Education Park, University of Florida, Gainesville, FL (29°4′ N, 82°21′ W). The soil is a Kendrick fine sand (loamy, siliceous, semiactive, hyperthermic Arenic Paleudults) that has little organic matter or residual soil N. The site had a previous cropping history with soybean that was well nodulated, and rhizobial inoculant was not applied in this experiment.

Nodulating and nonnodulating near isolines of 'Hardee' were sown on 4 Apr. 1996. Each plot consisted of seven rows, spaced 0.25 m apart that were 5.5 m in length. Nineteen and 20 d after sowing, plots were thinned to an intrarow spacing of 0.10 m, resulting in a population density of 40 plants m^{-2}.

On 25 Apr. 1996, 25 mm of irrigation was applied to all plots at 0700 h. Beginning at 1100 h, appropriate amounts of $^{15}NH_4NO_3$ were applied in 2 L of water over the top of the crop canopy with a pressurized backpack sprayer to deliver 5, 50, or 100 kg N ha^{-1}. The ^{15}N atom excess for the respective N treatments was 10, 2, and 2%. Immediately after completing the N applications, all plots received an additional 8 mm of irrigation, which served to rinse fertilizer from leaves and move it into the rooting zone. On 2 and 17 May 1996, one-half the amount of $^{15}NH_4NO_3$ was applied (in 2 L of water) to the same plots as applied on the first N-application date. Methods of N application were similar to those described for the first date, but 12 mm of irrigation water was applied after N treatment.

Beginning on 8 May 1996, differential water treatments were begun for the well-watered and drought treatments (Table 1). On this date, plots of the well-watered treatment received 25 mm of irrigation water while there was no water applied to plots of the drought treatment. During the subsequent 20 d, well-watered plots received a total of 180 mm of rainfall and irrigation. Plots of the drought treatment received small amounts of irrigation when the first symptoms of water deficit were visible. The drought treatment received a total of 90 mm of irrigation and rainfall during this period. On 28 May 1996, there was 36 mm of rain, which terminated the drought treatment.

One day after the drought treatment was initiated (9 May 1996), plants were harvested at the soil surface from a 1-m^2 section of plot, dried, weighed, and ground to pass a 2-mm sieve. A subsample of the ground plant material was ground to pass a 0.425-mm sieve. At the end of the drought period, on 29 May 1996, a second 1-m^2 biomass sample was harvested and prepared similarly to the first sample.

Total N in the finely ground subsample was determined by combustion analysis by the Soil Testing and Plant Analysis Laboratory at the University of Arkansas. The ^{15}N concentration in the finely ground subsamples was determined by Isotope Services, Inc. (Los Alamos, NM). Samples were first combusted with a N analyzer (model NA 1500, Carlo-Erba, Milan, Italy), and combustion products were processed through a chemical train. The dinitrogen gas from the combusted sample was then passed through a VG Isotech Iomass Spectrometer (model Sira Series II, Cheshire, UK) that was calibrated with acetanilide standards and which quantified molecular mass peaks of 28, 29, and 30.

The NDA was determined by the ^{15}N-dilution method (Eq. [1]; Fried and Middleboe, 1977; Herridge and Peoples, 1990). The ^{15}N-dilution method determines N_2 fixation by the dilution of N in plant tissues that occurs when plants fix their own N relative to that of a reference crop. In this case, the reference

Table 1. Irrigation and rainfall amounts by date and dates of plant sampling for well-watered and drought treatments in Gainesville, FL, in 1996.

Date	Irrigation or rainfall		N application	Petiole sampling	Biomass sampling
	Well-watered	Drought			
	mm				
23 April	12	12	–	–	–
25 April	33	33	X	–	–
2 May	12	12	X	–	–
5 May	25	25	–	–	–
8 May	25	–	–	–	–
9 May	–	–	–	X	X
11 May	10	10	–	–	–
12 May	12	–	–	–	–
13 May	–	–	–	X	–
14 May	10	10	–	–	–
15 May	12	–	–	–	–
16 May	–	–	–	X	–
17 May	25	12	X	–	–
20 May	8	8	–	X	–
21 May	10	–	–	–	–
22 May	4	4	–	–	–
24 May	18	–	–	–	–
25 May	10	10	–	–	–
28 May	36	36	–	–	–
29 May	–	–	–	–	X

crop was the nonnodulated isoline of Hardee (NN-Hardee) at the same level of N application as the nodulated line. For each date when biomass was sampled, NDA was determined, and the proportion of NDA between harvests (^{P}NDA) was calculated as (Herridge and Peoples, 1990):

$$^{P}\mathrm{NDA} = [(\mathrm{NDA}_{H2} \times \mathrm{TN}_{H2}) - (\mathrm{NDA}_{H1} \times \mathrm{TN}_{H1})]/(\mathrm{TN}_{H2} - \mathrm{TN}_{H1}). \quad [3]$$

In Eq. [3], TN_{H1} and TN_{H2} refer to the total N (g N m^{-2}) at Harvests 1 and 2, respectively, and NDA_{H1} and NDA_{H2} refer to NDA from Harvests 1 and 2, respectively.

At four dates during the experimental period (Table 1), three petioles from fully expanded leaves at the top of the canopy were removed from each plot between 1100 and 1300 h. Petioles were dried at 80°C, bulked, finely chopped, and approximately 35 mg of dried tissue was placed in microfuge tubes. Ureides and NO_3^- were extracted from petioles in 1.25 mL of 0.2 *M* NaOH at 100°C for 30 min (de Silva et al., 1996). Ureides and NO_3^- in the extract were quantified using an autoanalyzer (model San System Plus, Skalar, Atlanta, GA) with the respective procedures described by Van Berkum and Sloger (1983) and Jackson et al. (1975). Relative abundance of ureide (RAU) was calculated from ureide and NO_3^- concentrations as an estimate of the proportion of N derived from N_2 fixation (Eq. [2]; Herridge and Peoples, 1990).

The average RAU for the four sampling dates of each plot of Hardee was used as an integrated estimate of the RAU between the two biomass-sampling dates. The average RAU was compared with estimates of ^{P}NDA from ^{15}N-dilution measurements and to the calibrated relationship of RAU and ^{15}N-dilution for vegetative soybean (Herridge and Peoples, 1990).

The experiment was a multiple split-plot arrangement of treatments in a randomized complete block design with four replications. The main plots were irrigation (well watered and drought stressed), subplots were three levels of N fertilization, and the final split was genotype.

Arkansas, 1995

Seeds of 'Hutcheson' were sown at a density of 28 m^{-2} on 22 May 1995 at the University of Arkansas Main Experiment Station in Fayetteville, AR (36°5′ N, 94°7′ W). The soil is a Captina silt loam (fine-silty, siliceous, active, mesic Typic Fragiudults).

Rows were 1-m apart and placed on raised beds that were 0.15 m high. Each plot consisted of eight rows that were 6 m in length. There were two yield rows, two sample rows, and four border rows per plot. The field had been previously cropped with soybean that was well nodulated, and no rhizobial inoculant was applied.

The experiment was a randomized complete block design with four replications and a split-plot arrangement of treatments. Irrigation was the main plot, and N treatments were subplots. All plots were well-watered with furrow irrigation until the R2 developmental stage (Fehr and Caviness, 1977) whenever the estimated soil-water deficit (Cahoon et al., 1990) reached 32 mm. After R2, the drought-stressed treatments were dependent solely on rainfall.

Within each main plot, four N treatments were established: (i) no N fertilizer applied, (ii) 112 kg N ha^{-1} applied at V6, (iii) 112 kg N ha^{-1} applied at R2, (iv) 112 kg N ha^{-1} applied at V6 and an additional 112 kg N ha^{-1} applied at R2. This last treatment will be referred to subsequently as the V6&R2 treatment. Ammonium nitrate fertilizer was used as the N-fertilizer source. The appropriate amount of fertilizer was weighed for each row, and this was distributed evenly by hand on the soil surface. Immediately after N application, 12 mm of water was applied to all treatments with sprinkle irrigation.

Three petioles from mature leaves at the top of the canopy were removed from each plot approximately every 2 wk during the season, and they were dried and analyzed for ureide and NO_3^- using the same procedures described for the Florida experiment. At maturity, seed yield was determined by combining two end-trimmed rows that were 4.2 m in length.

Arkansas, 1996

The experiment conducted in 1995 in Fayetteville was repeated in 1996 on a similar soil. For 1996, the sowing date was 7 June 1996. Experimental design was similar to the 1995 experiment except that a randomized complete block design with a factorial arrangement of water and N treatments was used rather than a split-plot arrangement of treatments. The irrigation method in 1996 was drip irrigation.

RESULTS

Florida, 1996

The first N application resulted in some leaf necrosis, particularly for the highest N concentration. Two weeks later, however, at the beginning of the drought treatment and first biomass sampling, the plants had largely recovered from the N additions. Subsequent N applications were made using one-half of the amount as the first application, which eliminated any visible effects of leaf damage.

The rate of N accumulation between the two biomass sampling dates had significant interactions among factors of irrigation, N treatment, and genotype. For NN-Hardee, N accumulation was similar between drought and well-watered treatments within each level of N treatment (Fig. 1). For the drought and well-watered treatments of NN-Hardee, N accumulation rate increased approximately eight-fold as the N application was increased from 10 to 200 kg N ha^{-1}.

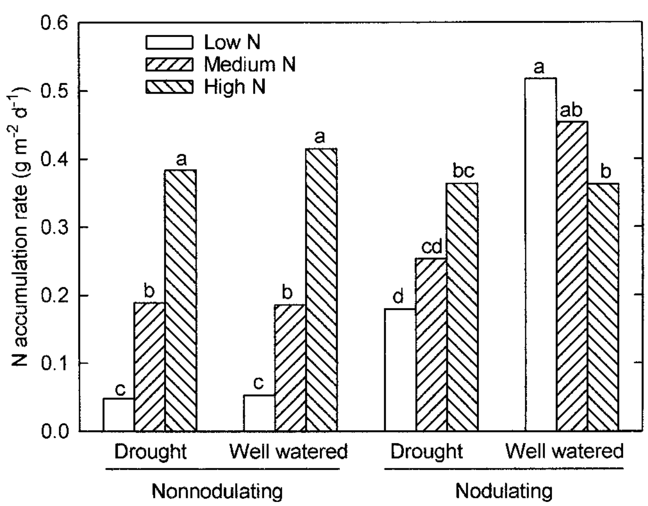

Fig. 1. Nitrogen accumulation rate for nonnodulating and nodulating near isolines of 'Hardee' under well-watered and drought conditions at Gainesville, FL. Nitrogen was applied as NH_4NO_3 in three split applications totaling 10, 100, and 200 kg N ha^{-1} for the low-N, medium-N, and high-N treatments, respectively. Within a genotype, different letters above bars indicate significant differences among means as determined by an LSD (0.05).

The N accumulation rate of the drought treatment of Hardee at the low-N level was approximately four times as great as the N accumulation rate of the low-N treatments for NN-Hardee (Fig. 1), indicating that N_2 fixation was not completely inhibited by drought. Under drought stress, the N accumulation rate of Hardee was significantly increased as the amount of N applied increased. For the well-watered treatment of Hardee, there was a decrease in N accumulation rate at the highest level of N application that may have been because of fertilizer-induced leaf necrosis. For the low-N and medium-N treatments of Hardee, the drought treatment had significantly lower N accumulation rates than did the respective N treatments for the well-watered treatment. At the high-N treatment level, however, N accumulation was similar among the drought and well-watered treatments for both genotypes. These data clearly indicate that N_2 fixation was more sensitive to drought stress than was the uptake and assimilation of inorganic N.

Analysis of variance of petiole NO_3^-, ureides, and RAU for the four sampling dates indicated a complex response to the main effects of irrigation, N, and genotype and to their two-way and three-way interactions (Table 2). The three-way interaction was significant on 16 May for NO_3^-, 13 and 16 May for ureide, and 9 and 20 May for RAU. For measurements made on 9 and 13 May, if the three-way interaction for these variables was nonsignificant, then one or more of the two-way interactions were significant. Conversely, on 16 and 20 May, if the three-way interaction was nonsignificant, then the two-way interactions were also nonsignificant, but one or more of the main effects were significant. To simplify data presentation and to provide a complete profile response of petiole NO_3^-, ureides, and RAU to irrigation, N, and genotype, we have chosen to present means of the three-way interactions (Table 3).

For the four sampling dates, petiole NO_3^- concentration generally increased in response to the quantity of

Table 2. Sources of variation from treatment effects of irrigation (I), nitrogen (N), genotype (G), and their interactions on petiole NO_3^-, petiole ureide, and relative abundance of ureide (RAU) at three different dates in field experiments in Gainesville, FL, in 1996.

		Source of variation						
Date	Variable	I†	N‡	I × N‡	G§	I × G§	N × G§	I × N × G§
9 May	NO_3^-	ns¶	**	ns	ns	ns	**	ns
	ureide	ns	ns	ns	**	ns	*	ns
	RAU	ns	**	*	ns	*	**	*
13 May	NO_3^-	*	**	**	ns	ns	*	ns
	ureide	ns	ns	*	**	**	*	*
	RAU	*	**	**	ns	ns	ns	ns
16 May	NO_3^-	**	**	**	ns	ns	*	*
	ureide	**	ns	ns	**	**	**	**
	RAU	**	**	ns	ns	ns	ns	ns
20 May	NO_3^-	*	**	ns	*	ns	ns	ns
	ureide	ns	ns	ns	**	ns	ns	ns
	RAU	*	**	ns	ns	ns	**	**

† Significance tested using Type III MS of Rep × I as error term.
‡ Significance tested using Type III MS of Rep × I × N as error term.
§ Significance tested using Type III MS of model as error term.
¶ ns, not significant.

Table 3. Response of petiole NO_3^-, ureide, and relative abundance of ureide (RAU) to irrigation (Irrig.) treatment, N fertilization, and measurement date for near isolines of 'Hardee' (Hard.) and nonnodulating (NN) Hardee at Gainesville, FL, in 1996.

			Petiole NO_3^-		Petiole ureide		RAU	
Date	Irrig. treat.†	N treat.‡	Hard.	NN Hard.	Hard.	NN Hard.	Hard.	NN Hard.
			μmol g^{-1} DW§					
9 May	WW	L	34	0	71	4	0.82	1.00
		M	155	26	23	4	0.36	0.50
		H	331	438	21	11	0.21	0.09
	DR	L	16	0	40	4	0.91	1.00
		M	164	107	21	7	0.37	0.18
		H	430	476	35	21	0.25	0.14
LSD (0.05)¶			96		35		0.34	
13 May	WW	L	9	22	11	4	0.86	0.82
		M	40	1	17	3	0.69	0.92
		H	94	164	6	5	0.22	0.35
	DR	L	46	7	54	6	0.82	0.92
		M	194	32	26	4	0.34	0.45
		H	298	337	20	12	0.20	0.13
LSD (0.05)¶			114		15		0.31	
16 May	WW	L	0	0	6	1	0.99	1.00
		M	0	2	5	2	0.97	1.00
		H	5	2	2	2	0.72	0.90
	DR	L	11	0	42	1	0.87	0.25
		M	40	2	13	2	0.63	0.65
		H	125	214	19	19	0.35	0.26
LSD (0.05)¶			46		13		0.37	
20 May	WW	L	13	0	32	1	0.91	1.00
		M	60	4	31	3	0.66	0.79
		H	102	83	24	4	0.54	0.21
	DR	L	55	2	47	7	0.76	0.98
		M	124	86	41	4	0.52	0.30
		H	204	207	32	18	0.38	0.25
LSD (0.05)¶			94		22		0.24	

† Irrigation treatments were well watered (WW) or drought (DR) stressed.
‡ N treatments were low (L, 10 kg N ha^{-1}), medium (M, 100 kg N ha^{-1}), and high (H, 200 kg N ha^{-1}) applied as NH_4NO_3 in three split applications.
§ DW, dry weight.
¶ LSD values are for the comparison among N treatments within a genotype and irrigation treatment.

N applied, and this occurred for both genotypes and irrigation treatments (Table 3). In general, within a measurement date for a given irrigation and N-treatment level, Hardee and NN-Hardee had similar petiole NO_3^- concentrations. The exception to this occurred for the high-N drought treatment of 16 May in which the petioles of the NN-Hardee had approximately 1.7 times the concentration of NO_3^- as the petioles of the high-N drought treatment of Hardee. On the same date, the petiole NO_3^- concentration of NN-Hardee for the high-N, drought treatment was many times greater than the concentration of petiole NO_3^- from the high-N treatment for either genotype of the well-watered treatment.

Petiole ureide concentration was generally not affected by N treatment in either genotype, or it was higher for the low-N treatment than for the medium- and high-N treatments (Table 3). The petiole ureide concentration for the low-N treatment of Hardee was particularly high on 9 May for the well-watered treatment in which it was three-fold greater than the ureide concentration for plants of the medium- and high-N treatments.

Petiole ureide concentration of Hardee increased on 13 and 16 May in response to drought (Table 3), and these dates corresponded to the dates with the most severe stress. The accumulation of ureides in response to drought has been noted in previous reports (de Silva et al., 1996; Serraj and Sinclair, 1996). Despite a complete lack of nodules, NN-Hardee had measurable ureides at all dates. Although the ureide concentration was generally lower in NN-Hardee than in Hardee, ureide concentration tended to increase for the high-N plots of the drought treatment.

Petiole NO_3^- and ureide concentrations were used to calculate RAU, and differences in RAU among treatments were generally dominated by the effect of N treatment on petiole NO_3^- (Table 3). Increased amounts of applied N resulted in decreased RAU. The decrease in

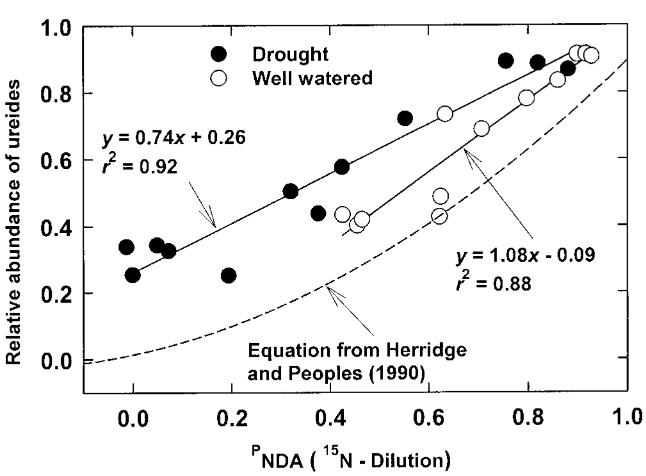

Fig. 2. Relative abundance of ureides (RAU) in petioles averaged across four sampling dates vs. the proportion of N derived from the atmosphere (^{P}NDA) between harvest dates, as determined by ^{15}N-dilution for well-watered and drought treatments at Gainesville, FL. The dashed line is the relationship found by Herridge and Peoples (1990) between RAU and NDA for well-watered, vegetative soybean ($y = 1.38 + 0.311x + 0.0057x^2$).

petiole NO_3^- concentration for Hardee at the two later sampling dates, particularly for the well-watered treatment, resulted in higher RAU values than those observed at the two earlier dates. Despite the fact that NN-Hardee did not have nodules and did not produce ureides from N_2 fixation, RAU values for NN-Hardee were generally similar to the values found for Hardee for the same irrigation and N treatments.

At the same level of N treatment, petiole NO_3^- concentration was generally higher for the drought treatment than for the irrigated treatment, which may have been due to less NO_3^- leaching from plots of the drought treatment, or to a decreased assimilation and utilization of NO_3^- for the drought treatment, or to a combination of these two factors. The parallel increases in both NO_3^- and ureide concentrations for the drought treatment tended to offset one another such that there were generally not large differences in RAU for irrigated and drought treatments within a N treatment on a given date.

The RAU values were calculated (Eq. [2]) from ureide and NO_3^- data from each experimental unit and then averaged across replications for presentation in Table 3. Consequently, values of RAU in Table 3 may differ from calculations of RAU using the mean values of ureide and NO_3^-. This difference was particularly evident for the low-N treatments, in which the NO_3^- concentration was 0 for some observations and >0 for other observations of the same treatment.

The ^{P}NDA for the irrigated treatment, calculated using the ^{15}N-dilution method, ranged from approximately 0.4 for the high-N treatment to 0.95 for the low-N treatment (Fig. 2). For the drought treatment, the ^{P}NDA covered a much greater range: from approximately 0.0 for the high-N treatment to 0.9 for the low-N treatment (Fig. 2). Although the total N accumulation rate for the high-N treatment under drought was approximately the same as for the high-N, well-watered treatment (Fig. 1), <25% of this N was from N_2 fixation (Fig. 2). Therefore, the greater N accumulation rates for the high-N treatment under drought shown in Fig. 1 were due to uptake and assimilation of inorganic N rather than stimulation of N_2 fixation.

For both well-watered and drought treatments, there was a linear relationship between the average RAU values and the ^{P}NDA, as determined by ^{15}N dilution (Table 4, Fig. 2). Although this relationship was linear for both well-watered and drought treatments, covariate analysis indicated that the slopes ($P = 0.024$) and inter-

Table 4. Covariate analysis of relative abundance of ureides to the proportion of N derived from the atmosphere (^{P}NDA) for irrigated and nonirrigated treatments at Gainesville, FL, in 1996.

Source	df	MS	P value†	R^2	CV
Model	4	2.436	<0.0001	0.91	12.5
Error	20	0.0056	–	–	–
Irrigation	2	0.172	<0.0001	–	–
^{P}NDA (irrigation)	2	0.536	<0.0001	–	–
Contrast intercepts	1	0.078	0.001	–	–
Contrast slopes	1	0.033	0.024	–	–

† Probability values were determined using Type III sums of squares.

cepts (P = 0.001) for well-watered and drought treatments were significantly different.

Over the RAU range from 0.4 to 1.0 for the well-watered treatment, the predicted NDA was approximately 0.14 greater for the curvilinear equation reported by Herridge and Peoples (1990) than for the linear relationship predicted from our data (Fig. 2). For the drought treatment at a RAU of 0.10, the predicted ^{P}NDA was 0.43 greater for the curvilinear relationship of Herridge and Peoples (1990) than for the linear relationship that we found. The difference in ^{P}NDA between the curvilinear relationship of Herridge and Peoples (1990) and the linear relationship for the drought treatment from our experiment decreased as RAU increased and was 0.13 at a RAU of 0.90.

Arkansas, 1995

From Day of Year (DOY) 210 to 243, total rainfall was 4.5 mm (Fig. 3A) and maximum temperatures averaged 34°C, which resulted in substantial drought stress during the podfill stages. During peak stress, the soil water potential at a depth of 15 cm was <−80 kPa (data not shown).

Analysis of variance of RAU indicated that there was no interaction between irrigation and N treatments at any of the measurement dates (data not shown). Therefore, the response of RAU to N treatment at the different measurement dates was averaged across irrigation treatments. Before R2, the RAU from petiole samples was approximately 0.20 (Fig. 4A). For the control treatment receiving no N fertilizer, RAU increased to approximately 0.70 at R4 and to 0.90 at R5, indicating an increasing dependence upon N_2 fixation as the season progressed. Application of N fertilizer at the V6 and R2 development stages delayed the increase in RAU, but by the end of the season all treatments had similar RAU values of approximately 0.95.

Drought stress resulted in decreased yield for all N treatments compared with the well-watered treatment (Table 5), and there was a significant interaction between irrigation and N treatments. For the drought treatment, N applications at R2 increased yield 25% relative to plots receiving no fertilizer N, and N treatment at V6&R2 had 15% greater yield than plots receiving no N application. Compared with plots receiving no supplemental N, the application of N fertilizer to the well-watered treatments increased yield 12% when applied at V6 and 16% when applied at V6&R2.

In general, seed protein concentration was greater

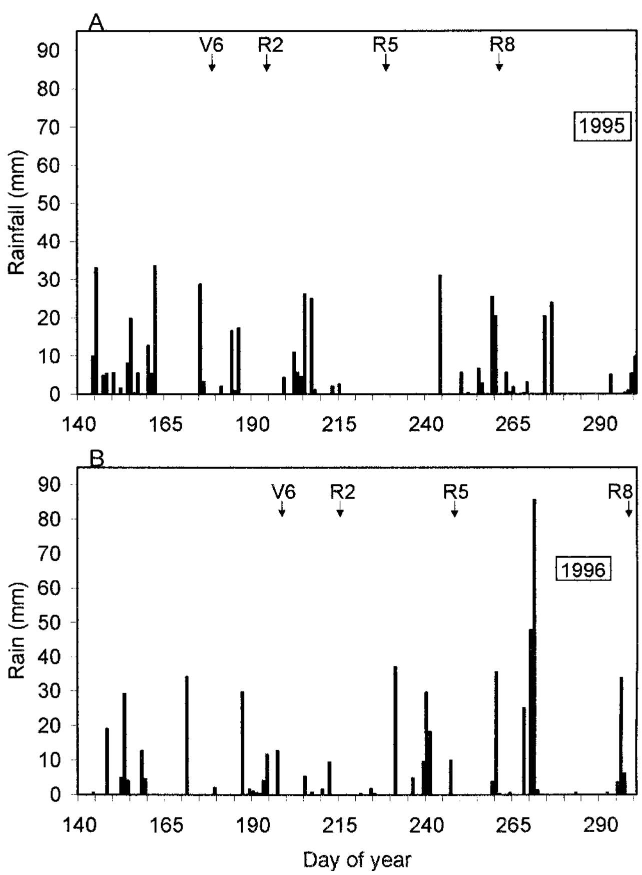

Fig. 3. Rainfall amounts and distribution at Fayetteville, AR, in (A) 1995 and (B) 1996. Crop developmental stages (Fehr and Caviness, 1977) are indicated near the top of each figure.

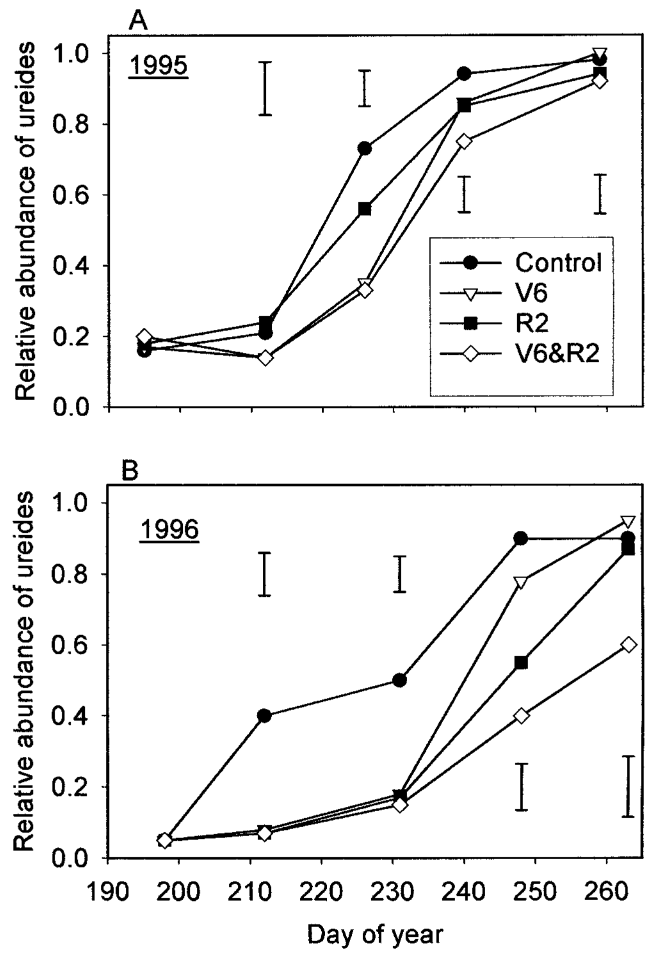

Fig. 4. Relative abundance of ureides in petiole tissues during (A) 1995 and (B) 1996 at Fayetteville, AR. Treatments were the following: control, no N fertilizer applied; V6, 112 kg N ha^{-1} at V6; R2, 112 kg N ha^{-1} at R2; V6&R2, 112 kg N ha^{-1} at both V6 and at R2. At each measurement date, LSD values (0.05) are shown. Values were averaged across irrigation treatments in both years (irrigation and N-treatment interaction was nonsignificant).

Table 5. Response of yield and seed protein and oil concentrations (conc.) to irrigation treatment (treat.) and N applications at Fayetteville, AR, in 1995.

Irrigation treat.	N treat.	Yield	Seed protein conc.	Seed oil conc.
		kg ha^{-1}	g (100 g)$^{-1}$	
Drought	0†	1941d‡	42.3b	20.3c
Drought	V6	2172cd	42.3b	20.9b
Drought	R2	2428c	42.5ab	20.3c
Drought	V6 & R2	2238c	43.2a	20.2c
Irrigated	0	2785b	40.4c	20.7b
Irrigated	V6	3117a	40.1c	21.3a
Irrigated	R2	2828b	40.1c	21.0ab
Irrigated	V6 & R2	3221a	39.9c	21.4a

† No N was applied (0) or 112 kg N ha^{-1} as NH_4NO_3 was applied at development stages V6 or R2, or 112 kg N ha^{-1} was applied at both V6 and at R2.
‡ Means within a column followed by the same letter are not significantly different as determined by an LSD (P = 0.05).

for the drought treatment than for the well-watered treatment, and there were no significant differences in protein among N treatments for the well-watered treatment (Table 5). Within the drought treatment, N treatment at V6&R2 had higher protein than the control treatment. Under drought stress, N treatment at V6 produced the greatest oil concentration compared with the other N treatments. For the well-watered treatment, seed from plants of the N treatment at V6 or at V6& R2 had a greater oil concentration than seed from plants of the control treatment.

Arkansas, 1996

In general, 1996 was a more favorable growing season than 1995. From sowing to R6, rainfall totals were 396 mm in 1995 and 443 mm in 1996. Moreover, rainfall was more evenly distributed in 1996 than in 1995 (Fig. 3A and 3B), and drought stress was probably less severe.

Relative abundance of ureide in petioles generally increased throughout the 1996 season, similar to the 1995 season (Fig. 4B). After the initial application of N at V6, there were significant main effects of N treatment on RAU at subsequent sampling dates. There was no significant interaction between irrigation and N treatment on RAU, however, and the response of RAU to N treatment has been averaged across irrigation treatments (Fig. 4B). Application of N fertilizer at V6 and at R2 delayed the increase in RAU, indicating that for these treatments, the proportion of N derived from N_2 fixation was delayed (Herridge and Peoples, 1990). When plants were sampled on DOY 248 (90 d after planting), there was also a significant main effect of irrigation with drought treatments having less RAU (0.62) than the well-watered treatments (0.72). This difference in RAU might reflect a higher N_2 fixation rate for the well-watered treatment. Alternatively, the higher RAU for the irrigated treatment may be due to a greater depletion of N fertilizer from the soil due to crop utilization and/or leaching.

Although there was no significant interaction between irrigation and N treatments for yield, there were appreciable main effects of N and irrigation treatments

Table 6. Response of yield and seed protein and oil concentrations (conc.) to main effects of irrigation treatment (treat.) and N applications at Fayetteville, AR, in 1996. Interaction of irrigation and N treatments was nonsignificant.

Irrigation treat.	N treat.	Yield	Seed protein conc.	Seed oil conc.
		kg ha^{-1}	g (100 g)$^{-1}$	
Drought	average†	2835**	40.7 ns‡	21.0ns
Irrigated	average	3090	40.7	20.7
Average	0	2889b§	41.5a	20.2b
Average	V6	2957ab	40.2b	21.0a
Average	R2	2829b	40.8ab	20.9a
Average	V6 & R2	3174a	40.2b	21.0a

** Irrigation treatment means are significantly different (P = 0.01), as determined by an F test.
† No N was applied (0) or 112 kg N ha^{-1} as NH_4NO_3 was applied at development stages, V6, or R2 or 112 kg N ha^{-1} was applied at both V6 and at R2.
‡ ns, not significantly different, as determined by an F test.
§ Means within a column followed by the same letter are not significantly different as determined by an LSD (P = 0.05).

(Table 6). The main effect of irrigation on yield was significant with well-watered treatment having 9% greater yield than the drought treatment. Averaged across irrigation treatments, N treatment at V6&R2 gave higher yields compared with the control treatment receiving no N fertilizer; however, in 1996 there was no yield response to N treatment applied only at R2, unlike the 1995 results.

In 1996 there was a significant effect of N treatment on seed protein and oil, but there was no effect of irrigation treatment (Table 6). The general trend was that with the application of N fertilizer, protein concentration decreased and oil concentration increased compared with plants receiving no N fertilizer.

DISCUSSION

The response of crop N accumulation rate to N fertilization under drought stress in the Florida experiment agrees with previous results from greenhouse and field experiments indicating that N_2 fixation was more sensitive to drought stress than was the uptake and assimilation of soil N (Purcell and King, 1996). At increasing levels of N fertilization, the N accumulation rate for Hardee increased for the drought-stressed treatment, and for the high N-level treatment, N accumulation was similar between well-watered and drought-stressed treatments.

Because ureides are generally assumed to be closely linked to N_2 fixation, it is somewhat surprising that RAU values were similar between nodulating and nonnodulating isolines of Hardee at Gainesville (Table 3). Nonnodulated bean (*Phaseolus vulgaris* L.) also contained relatively high concentrations of ureides in leaf and stem tissues (Thomas et al., 1980), and inhibitor experiments indicated that the likely source of ureides was from purine catabolism. The enzyme that breaks down allantoate, allantoate amidohydrolase, requires Mn^{2+} as a cofactor, and in many soybean cultivars, Mn^{2+} appears to be limiting or is unavailable for optimum ureide breakdown (Purcell et al., 2000). Although the NN-Hardee was completely dependent upon soil N, ureides in petiole tissues were readily detectable. Furthermore, the increase in petiole ureides that occurs in response to drought stress (de Silva et al., 1996; Serraj and Sinclair, 1996) was also observed in NN-Hardee for the high-N treatment (Table 3). These results indicate that caution should be exercised when using RAU as a proxy for N_2 fixation in soybean genotypes or in *Bradyrhizobium japonicum* strains that have different efficiencies of N_2 fixation. Our RAU data for the irrigated treatment agreed within 15% of the published calibration of RAU and ^{P}NDA from ^{15}N dilution (Herridge and Peoples, 1990). For the drought treatment, however, there were large differences between the relationship of RAU and ^{P}NDA that we observed and with the published calibration. Further work is required to define the interactions of RAU with symbiotic effectiveness and with drought stress before this approach can be broadly applied for quantifying N_2 fixation under drought conditions.

For the Fayetteville location in both years, RAU was $<$0.20 during vegetative development in the absence of N fertilization (Fig. 4A, 4B). In contrast, RAU in the Gainesville experiment was $>$0.80 for vegetative Hardee plants (Table 3), indicating a greater dependence on N_2 fixation at Gainesville than at Fayetteville (Herridge and Peoples, 1990; Fig. 2), which is not unexpected given the low soil organic matter and propensity for leaching on the sandy soil in Florida.

A greater N accumulation rate for N-fertilized treatments may have resulted in the increased yield that was observed in the experiments conducted in Arkansas. In 1995, N fertilization increased yield 15 to 25% in drought treatments and 12 to 15% in the well-watered treatment. In 1996, averaged across irrigation treatments, N fertilization at V6&R2 increased yield 10% more than the control treatment. Previous reports on yield responses to N fertilization when water supply was adequate have been conflicting. In some cases, relatively low rates of N fertilizer (50 to 100 kg N ha^{-1}, Yinbo et al., 1997) have resulted in large yield increases (64%), but in other cases low rates of N fertilizer (35 kg N ha^{-1}, Heatherly et al., 2003) have had no effect on yield. Likewise, high rates of N fertilizer have had conflicting results. Brevedan et al. (1978) found yield increases of approximately 800 kg ha^{-1} when 168 kg N ha^{-1} was applied at R2, whereas Weber (1966) found no yield response in a nodulating soybean line with fertilizer application as high as 672 kg N ha^{-1}.

There are several possible reasons for the different yield responses to N fertilizer in the absence of obvious drought stress that have been reported. The first is that water-deficit stress is not easily quantified, and a mild water deficit, sufficient to inhibit N_2 fixation, may not be readily recognized. Under these conditions, a greater tolerance to drought of uptake and assimilation of inorganic N would restore crop growth and increase yield relative to that of a crop dependent primarily upon N_2 fixation. A second possible explanation for different responses of yield to N fertilizer under well-watered conditions is that the soybean crop may have been nodulated by strains of *B. japonicum* with different symbiotic efficiencies. For inefficient (but highly competitive) strains, supplemental N would likely benefit the crop regardless of irrigation treatment. Responses of soybean yield to N fertilizer may also differ, depending upon soil characteristics including residual soil N and soil organic matter.

This research confirmed previous reports that N_2 fixation in soybean was more sensitive to drought stress than was the uptake and assimilation of soil N and that fertilizing soybean with large amounts of N fertilizer could increase yield under drought conditions (Purcell and King, 1996). Although application of high rates of N fertilizer may not be an economical means of increasing drought tolerance in soybean, it illustrates the potential gain in yield that perhaps could be achieved if the tolerance of N_2 fixation to drought was genetically enhanced to reach the same level as that of the uptake and assimilation of soil N. In that genotypes have been discovered with increased tolerance of N_2 fixation to drought (Sall and Sinclair, 1991; Sinclair et al., 2000; Purcell and Specht, 2004), selection for drought-tolerant N_2 fixation appears to be an important avenue for future cultivar improvement.

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