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Water Stress Effects on Nitrogen Assimilation and Growth of *Trifolium subterraneum* L. Using Dinitrogen or Ammonium Nitrate[1]

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ABSTRACT

The relative effects of water stress on growth parameters of subterranean clover (*Trifolium subterraneum* L. cv. Woogenellup) dependent on either N_2 or 8 millimolar NH_4NO_3 for N were examined. Whole-plant carbon exchange rate (CER), acetylene reduction (AR), dry matter production, and Kjeldahl N accumulation were measured on uniform, intact swards of clover that were maintained under adequately watered conditions or were subjected to three cycles of water stress (leaf water potential ≤ −30 bar) over an 18-day period. In the absence or presence of water stress, growth rate, net N accumulation rate, and total N concentration of plants dependent on N_2 were 25 to 26, 45 to 50, and 20 to 21% less, respectively, than plants supplied with 8 millimolar NH_4NO_3. The water stress treatment produced less than a 50% decrease in CER regardless of plant N source, a 90% inhibition of AR in plants dependent on N_2, and a 41% decline in dry matter production on both N sources. Water stress decreased reduced N accumulation 55% in N_2-dependent plants and 50% in NH_4NO_3-dependent plants. Changes in growth and N accumulation caused a 10 to 11% decrease in total plant N concentration of water-stressed plants compared to adequately irrigated controls, but water stress decreased the N concentration of tissue synthesized during the 18-day treatment period in N_2-grown plants more than in plants supplied 8 millimolar NH_4NO_3. Thus, the relative effect of water stress on growth under the two N regimes was similar, but N accumulation by N_2-dependent clover was inhibited to a slightly greater extent ($P \leq 0.001$) than in NH_4NO_3-dependent plants.

Many recent studies of the *Rhizobium*-legume association have concentrated on the energy costs associated with symbiotic N_2 fixation (2, 14, 17). Although some investigators have reported that the energy costs of N_2 reduction and NO_3^- assimilation are similar (9, 15), data from other studies suggest that metabolic costs associated with N_2 fixation exceed those of combined N utilization (18, 20). Comparisons of legume performance on different N sources, however, must consider more than the energy costs of N assimilation under optimum conditions. One important unknown involves the relative effect of environmental stresses on legumes using different N sources.

It is known that water stress decreases symbiotic N_2 fixation and growth of nodulated legumes (7, 11, 12, 22). Some data indicate that water stress disrupts interactions between *Rhizobium* and the host plant directly by altering nodule fine-structure which leads to changes in either nodule membrane permeability or enzyme activities (22); other evidence suggests that root nodules are affected indirectly after a decrease in photosynthesis (7, 12). It is also known that water stress decreases nitrate reductase activity (23) and negatively affects various other aspects of N metabolism (10). Unfortunately none of the studies that examined the effect of water stress on growth and symbiotic N_2 fixation of legumes included plants supplied with combined N as separate, but related, controls.

The purpose of the present research was to determine if the relative effect of water stress on growth of N_2-dependent plants is greater than that on plants supplied with combined N. If more energy is required to reduce N_2 than to assimilate combined N in legumes (18, 20) and if water stress inhibits both photosynthesis (7, 12), and N_2 reduction (7, 11, 22) in N_2-dependent legumes, then one can hypothesize that water stress has greater effects on growth and N assimilation of N_2-dependent plants than on plants supplied combined N. The present experiments were designed to test that possibility.

MATERIALS AND METHODS

Growth Conditions. Twenty-one, 2-d-old subterranean clover seedlings (*Trifolium subterraneum* L. cv. Woogenellup) were planted at uniform spacings in each of 48, 15-cm diameter plastic pots. Each pot was surrounded by a metal screen 15.5 cm in diameter so that the horizontal surface area of the plant canopy was the same in each pot (21). Each pot contained 150 g gravel on the bottom and 750 g air-dry granulated, montmorillinite clay (Turface, courtesy of IMC, Blue Mountain, MS). The plants were inoculated at planting with *Rhizobium trifolii* strain 162X68 (obtained originally from The Nitragin Co., Milwaukee, WI) and watered for the first 20 days with a standard nutrient solution (5) supplemented to contain 2 mM NH_4NO_3. After 20 d, 24 pots were leached with distilled H_2O and then provided with the standard nutrient solution lacking any NH_4NO_3. The other 24 pots were given the standard nutrient solution supplemented to contain 8 mM NH_4NO_3. When the plants were 34 d old, the 24 pots in each N treatment were separated into eight identical groups of three pots each. One pot from each group was assayed for AR[3] and harvested immediately, one from each group was designated for water stress treatments, and one from each group was designated as a control pot and was watered every other day throughout the experiment to maintain $\psi_l \geq -12$ bar. Plants were grown under controlled conditions with a photon flux density of 650 μE $m^{-2}s^{-1}$ (400–700 nm), a 14:10 h light:dark photoperiod at a constant 20°C

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[3] Abbreviations: AR, acetylene reduction; ψ_l, leaf water potential; CER, carbon exchange rate; E, evapotranspiration.

and 50% RH.

During the 18-d treatment period, the stressed pots were subjected to three sequential dry-down cycles. Each cycle took approximately 5 d. At regular intervals during each cycle the visual stage of water stress was noted. Four visual stages of water stress were established as follows: stage 1, the uppermost leaves of the plant canopy first showed signs of wilting; stage 2, the center of the canopy began to collapse; stage 3, the center of the plant canopy collapsed, and the outer rim of the canopy next to the metal screen also began to fall inward; stage 4, the entire canopy had collapsed, and at the end of this period some leaves began to shrivel. When plants had been in stage 4 for 6 to 8 h, those pots were rewatered. Due to the uniform rate of growth and transpiration maintained by the defined canopy exposure, all the pots in a given treatment generally reached the final stage of water stress within an 8-h period. After the last of the stressed plants had been watered at the end of a given cycle, all the pots were again watered to raise ψ_l to ≥ -12 bar, and the next cycle was started.

Physiological Measurements. Continuous measurements of whole-plant CER were made on selected pots throughout the 18-d water-stress treatment period in each of three Plexiglas assimilation chambers. Assimilation chamber temperatures were maintained constant by using internally mounted tangential blowers that circulated air across water-cooled radiators and past the plants. Each chamber was connected independently to the gas circuitry so that CER of plants in each chamber could be determined by IR gas analysis without disrupting the conditions of the other chambers. Carbon dioxide was monitored with a Beckman 315B differential IR gas analyzer in an open system.

Apparent N_2 fixation was measured on intact plants with the AR assay similar to Fishbeck *et al.* (8). Acetylene was injected into 6-liter, sealed plastic containers to give a final concentration of 0.1 atm, and samples were taken 15 and 45 min later during the linear phase of ethylene production. Acetylene and ethylene were measured by gas chromatography (3).

ψ_l was measured at various times during the light period with a pressure chamber (19). Since petioles tended to collapse at pressures greater than 30 bars, accurate measurements of ψ_l below -30 were not possible. E was calculated by gravimetric determination of water loss from each pot during every stage of water stress.

Harvested plants were separated into leaves, crowns, and roots. Crowns consisted of all materials between 0 and 2 cm above the soil surface. Leaf area was measured with a leaf area meter (LI-3000 Lambda Instruments, Lincoln, NB), and all plant material was dried at 70°C for 48 h before weighing. Total reduced N was determined by Kjeldahl analysis (4) using methods that do not detect NO_3^- or NO_2^-.

RESULTS

The experiment was repeated twice with similar results. Data reported here were collected during the second trial. Plants grown with 8 mM NH_4NO_3 had mean AR activities of <1.5 μmol ethylene pot^{-1} h^{-1} and were considered to be dependent on the combined N because plants grown without combined N had AR activities of 38 μmol ethylene pot^{-1} h^{-1} at the beginning of the treatment period. On day 34, when water treatments were begun, there was no significant difference in total plant dry matter between the two N sources (NH_4NO_3, 4.93 ± 0.18 g/pot; N_2, 4.77 ± 0.16 g/pot).

During the first cycle of water stress, AR in plants grown without combined N was affected earlier and to a much greater extent than was CER in the light (Fig. 1). In the initial stage of water stress, AR declined 56%, whereas photosynthetic CER was relatively unaffected until the third stage of water stress. During the fourth stage of water stress, photosynthetic CER was still greater than 50% of that of adequately watered control plants, but AR was less than 10% of the controls. Although initial decreases in dark respiratory CER corresponded in time with decreases in AR, the total decrease in dark respiration in stressed plants was less than 50% of the rate of the adequately watered control plants (Fig. 1).

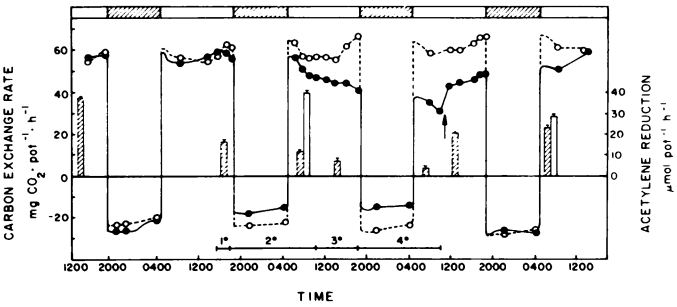

FIG. 1. CER (circles) and AR rate (histograms) for subterranean clover growing without combined N during the first of three dry-down and recovery cycles. ψ_l was maintained above -12 bar in control pots (○, □) or allowed to drop from that value to ≤ -30 bar over approximately 5 days in stressed pots (●, ▨). Symbols above acetylene reduction histograms represent 1 SE. Dark periods are indicated by shaded areas on the top horizontal axis. Various degrees of water stress, defined in the text, are designated on a horizontal bar in the lower part of the figure. The arrow indicates when stressed pots were rewatered.

Within 4 h after rewatering at the end of the first cycle of water stress, both photosynthetic CER and AR recovered to approximately 70% of the rates of adequately watered control plants. Complete recovery of dark respiratory CER occurred within 12 h, whereas complete recovery of photosynthetic CER required approximately 24 h. It was difficult to determine when AR recovered to its original value because plants that were assayed during the recovery period were being assayed for the second time, and neither the control nor the stressed pots had AR rates that were equal to the original AR rates. Subsequent measurements of AR indicated that the plants eventually did achieve rates similar to initial values.

Declines in AR, photosynthetic CER, and E during the first water-stress cycle were not uniform functions of ψ_l (Fig. 2). Decreases in ψ_l from approximately -9 bar to -18 bar were associated with a 56 and 42% inhibition of AR and E, respectively, while canopy CER was not affected significantly. Decreases in ψ_l that occurred between stage 2 and 4 of the dry-down cycle (a change from approximately -21 to ≤ -30 bar) were associated with only 13 and 15% of the total inhibition that occurred in AR and E. Decreases in CER that occurred between stage 2 and 4 accounted for 83% of the total inhibition of CER associated with water stress.

When plants supplied with 8 mM NH_4NO_3 were exposed to water stress, CER showed the same pattern as in the N_2-fixing plants, and photosynthetic CER during the final stage of stress was approximately 67% of the prestress rate (data not shown).

Although the CER of stressed plants dependent on N_2 fixation recovered to the level of the adequately watered control plants at the end of the first dry down cycle, the CER of the stressed plants that were supplied 8 mM NH_4NO_3 did not (Table I). After recovery from subsequent periods of water stress, there were significant negative effects of the stress on CER under both N regimes (Table I).

Water stress and N source treatments had various effects on foliar parameters (Table II). The specific leaf weight of the water-stressed plants was significantly greater than the control plants in N_2-dependent plants, and the N source tended to affect specific leaf weight in the adequately watered plants. Leaf size, leaf area index, and foliar N concentration were affected both by water stress and by N source.

Water stress reduced dry matter accumulated during the 18-d treatment period by approximately 41% under both N treatments

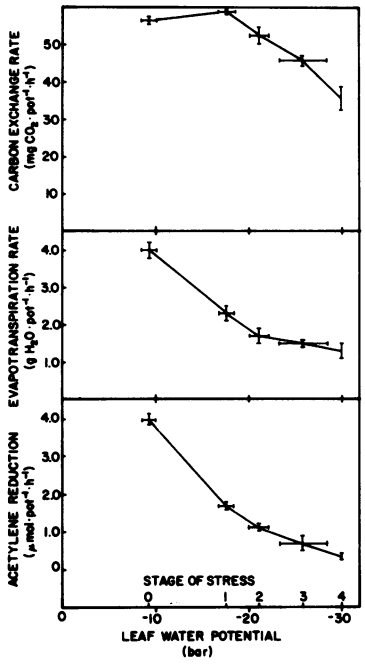

FIG. 2. The relationships between mean ψ_l and CERs, E, or AR at various stages of stress for plants reported in Figure 1. ψ_l at stress stage 4 is given as −30 bar because all of the measurements during that stage were ≤ −30 and it was not possible to measure ψ_l accurately below −30 bar. Mean values ±SE from 3 to 22 measurements are given, depending on the variable measured.

Table I. *The Effects of Water Stress and Nitrogen Source on Photosynthetic Carbon Exchange Rate of Subterranean Clover*

Plants were grown on either N_2 or 8 mM NH_4NO_3 and exposed to three stress/recovery cycles in 18 d prior to harvesting 52 d after planting. All measurements were made within 48 h after providing adequate water to the stressed plants at the end of the stress cycle indicated. Each cycle lasted approximately 5 d.

Water Treatment	Time of Measurement	N Treatment	
		NH_4NO_3	N_2
		mg CO_2/pot·h	
Adequate	Cycle 1	72.1	58.8
	Cycle 2	81.4	74.9
	Cycle 3	79.9	75.8
Stressed	Cycle 1	58.2	58.6
	Cycle 2	61.0	56.4
	Cycle 3	65.5	63.3
$LSD_{(0.05)}$		3.3	

(Table III). Under both water treatments, plants dependent on symbiotic N_2 fixation grew approximately 25% less than the plants that were supplied 8 mM NH_4NO_3, and therefore there was no significant difference in the relative water stress effect on growth between the two N treatments.

The effect of water stress on reduced N accumulation over the 18-d treatment period was greater than the effect on growth, and it differed with N source (Table IV). The 50.6% decrease in N accumulation by stressed clover plants grown with 8 mM NH_4NO_3 was significantly less ($P \leq 0.001$) than the 55.1% decline in that parameter measured in N_2-dependent plants. Plants dependent on N_2 accumulated 45.2% less N under adequate water treatments than plants supplied NH_4NO_3, and there was a significant ($P \leq 0.001$) N source effect of −50.2% under water-stressed conditions.

Whole-plant N concentration of 52-day-old clover following the 18-d treatment period differed significantly ($P \leq 0.001$) among N and water treatments, but there was no difference in the relative water stress effect between N treatments (Table Va). In contrast, for plant tissue formed during the 18-day treatment period, water stress decreased N concentration more in N_2-dependent plants than in clover grown with 8 mM NH_4NO_3 (Table Vb).

DISCUSSION

Data from this study show that, for the biological materials and environmental conditions used, water stress decreased N accumulation only slightly more on a relative basis in plants dependent on N_2 than in those supplied NH_4NO_3 (Table IV). Because dry matter accumulation was decreased to a similar extent on a percentage basis in both N treatments (Table III), the overall effect of water stress was to decrease the N concentration of new tissue more in N_2-dependent plants (Table Vb). These seemingly modest results are of interest because considerable work has been done on the inhibitory effect of water stress on root nodule activity in legumes without considering the effect of similar stresses on plants using combined N (6, 7, 11, 12, 22). Those valid studies described interactions between water stress and N_2 fixation, but they provided little information relating to environments where plants could use N_2 and/or soil N.

Previous water stress studies with soybeans have indicated either that the effect of water stress on symbiotic N_2 fixation is closely correlated with the effect of water stress on shoot CER (11, 12) or that shoot CER in the light is affected more rapidly by water stress than is nodule activity (7). The data presented in this paper indicate that apparent N_2 fixation in subterranean clover is affected sooner and to a greater extent during a water-stress cycle than is photosynthetic CER (Figs. 1 and 2). These short-term physiological data are supported by harvest data indicating that the water stress treatments affected net N accumulation more than dry weight accumulation in the N_2-fixing plants (Tables III and IV). These data agree with those reported for white clover (*Trifolium repens*) by Engin and Sprent (6) and support their hypothesis that water stress has direct effects on the N_2-fixation process that are greater than the effects on photosynthesis.

A correlation between decreases in E and AR (Fig. 2) has been noted previously (1, 11). It has been suggested that reduction of transpiration during water stress may reduce the rate at which amino acids are transported away from the nodule in the xylem sap and thus affect N_2-fixation activity (11, 22). Pate (16), however, suggested that it is unlikely that the accumulation of fixation products in the nodule causes any direct inhibitory effect on the fixation rate. Decreases in transpiration rates of unstressed soybean plants have failed to affect AR (12). Thus, the apparent correlation between E and AR shown in Figure 2 may not indicate any causal relationship between the two responses.

The ability of subterranean clover nodules to recover after experiencing ψ_l of less than −30 bar indicates that although there are direct effects of water stress on root nodule functioning, those effects are reversible and do not always represent permanent nodule damage. Studies on white clover indicate that the ability of clover nodules to recover after water stress is related to the duration of the water stress period (6). Previous research indicates that subterranean clover can recover more rapidly than white clover after more than 24 h at a ψ_l of less than −20 bar (1).

The relative insensitivity of CER to water stress in this experiment was remarkable. Even during stage 2 of water stress, when measured ψ_l was less than −20 bar and the center of the canopy was beginning to collapse, the CER for the plant canopy was greater than 90% of the prestress rate. Previous measurements on much less dense ladino clover canopies indicated that photosynthesis declined rapidly at the first sign of wilt (24). However, Johns (13) reported that *T. repens* maintained canopy photosynthetic

Table II. *The Effects of Water Stress and Nitrogen Source on Foliar and Canopy Characteristics of Subterranean Clover*

Plants were grown on either N_2 or 8 mM NH_4NO_3 and exposed to three stress/recovery cycles in 18 d prior to harvesting 52 d after planting.

Water Treatment	Specific Leaf Wt.		Leaf Size		Leaf Area Index		Leaf N	
	NH_4NO_3	N_2	NH_4NO_3	N_2	NH_4NO_3	N_2	NH_4NO_3	N_2
	mg/cm²		*cm²/leaf*		*cm²/cm²*		%	
Adequate	2.79	2.67	7.1	6.0	18.0	14.5	5.36	4.38
Stressed	2.91	2.90	5.4	4.6	10.8	8.4	4.62	3.80
$LSD_{(0.05)}$	0.13		0.4		0.7		0.23	

Table III. *The Effects of Water Stress and Nitrogen Source on Dry Matter Production in Subterranean Clover*

Plants were grown on either N_2 or 8 mM NH_4NO_3 and exposed to three stress/recovery cycles in 18 d prior to harvesting 52 d after planting. Data represent dry matter accumulated during the 18-d treatment period. Total plant dry matter on day 34 was 4.93 ± 0.18 and 4.77 ± 0.16 g dry wt/pot for the NH_4NO_3 and N_2 grown plants, respectively.

Water Treatment	N Treatment		N Source Effect
	NH_4NO_3	N_2	
	g dry wt/pot		%
Adequate	9.43[a]	7.03	−25.4
Stressed	5.60	4.15	−25.9
Water stress effect %	−40.6	−41.0	

[a] $LSD_{(0.001)}$ = 0.96 for net growth values (df = 28).

Table IV. *The Effects of Water Stress and Nitrogen Source on Reduced Nitrogen Accumulation in Subterranean Clover*

Plants were grown on either N_2 or 8 mM NH_4NO_3 and exposed to three stress/recovery cycles in 18 d prior to harvesting 52 d after planting. Data represent reduced N accumulated during the 18-d treatment period. Total N content on day 34 was 218 ± 10 and 189 ± 3.4 mg N/pot for the NH_4NO_3 and N_2-grown plants, respectively.

Water Treatment	N Treatment		N Source Effect
	NH_4NO_3	N_2	
	mg N/pot		%
Adequate	480[a]	263	−45.2[b]
Stressed	237	118	−50.2
Water stress effect %	−50.6[b]	−55.1	

[a] $LSD_{(0.001)}$ = 50 for net N accumulation values (df = 20).

[b] Two-way analysis of variance statistics: $F_{0.001}$ [1, 20] = 14.8; $F_{(N\ source)}$ = 335.7; $F_{(water\ stress)}$ = 448.4; $F_{(interaction)}$ = 27.9.

rates that were >50% of unstressed controls when ψ_l was between −30 and −40 bar. In the present experiments, it may be that initial wilting of upper leaves allowed light penetration farther down into the canopy and thus stimulated CER of lower leaves. The long-term effects of water stress on the canopy CER during the subsequent recovery period of both the N_2-dependent and the 8 mM NH_4NO_3-grown plants (Table I) are probably a result of several factors. Among those factors are smaller leaf size, increased specific leaf weight, lower leaf area index, and lower foliar N concentration (Table II).

The effect of N source on dry matter and N accumulation over the 18-d period in both water treatments (Tables III and IV) and on final tissue N concentration (Table Va) demonstrates that the absolute growth of subterranean clover plants supplied with 8 mM NH_4NO_3 was greater than plants that were dependent on symbiotically fixed N. Those differences in growth and N accumulation probably can be attributed to the greater energy requirement

Table V. *The Effects of Water Stress and Nitrogen Source on Nitrogen Concentration in Subterranean Clover*

Plants were grown on either N_2 or 8 mM NH_4NO_3 and exposed to three stress/recovery cycles in 18 d prior to harvesting 52 d after planting. Plant harvests at the beginning and end of the 18-d stress period allowed calculation of treatment effects on (a) N concentrations for the entire plant over the 52-d growing period, or (b) N concentrations of tissue added during the 18-d treatment period.

Water Treatment	N Treatment		N Source Effect
	NH_4NO_3	N_2	
	%N		%
Part a			
Adequate	4.80[a]	3.80	−20.8
Stressed	4.28	3.40	−20.4
Water stress effect %	−10.8	−10.5	
Part b			
Adequate	5.06[b]	3.82	−24.5
Stressed	4.24	2.93	−30.9
Water stress effect %	−16.2	−23.3	

[a] $LSD_{(0.001)}$ = 0.27 for N concentration values (df = 20).

[b] $LSD_{(0.001)}$ = 0.59 for N concentration values (df = 20).

for symbiotic N_2 fixation in subterranean clover previously reported by Silsbury (20). If the water stress treatments in these experiments had been of long enough duration to prevent rapid recovery of the N_2-fixing system (6), it is anticipated that the differential effect of water stress on N accumulation in the N_2-dependent plants could have become large enough to cause differential effects on plant growth. Thus, subterranean clover dependent on symbiotic N_2 fixation under water stress is at a potential disadvantage relative to stressed plants supplied with combined N, but whether that potential disadvantage is actually realized in nature cannot be determined from the present study. Factors such as the effect of lower N concentration on progeny number and vigor, as well as possible effects on herbivore pressure, would have to be considered in a more comprehensive evaluation.

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