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A Simple Technique of Studying Water Deficit Effects on Nitrogen Fixation in Nodules without Influencing the Whole Plant

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ABSTRACT

Cowpea (*Vigna unguiculata* L. Walp cv C-152) plants were grown in a system in which watering was withheld from the soil zone containing nodules, while the plants were able to maintain normal water status. The system was developed in a pot by making two soil zones, an upper and a lower separated by a gravel column between these two zones. Plants extended their roots into the lower layer of soil and were able to absorb water. The dry matter accumulation, photosynthesis rate, and leaf area development of the plant were not affected when the upper soil zone was dried, but the water potential of the nodules was lower than in the nodules in fully irrigated pots. Nitrogenase activity in the nodules obtained from plants stressed in the upper zone only was lower than in nodules obtained from fully irrigated plants. The present technique is helpful in distinguishing the direct water stress effects on nitrogen fixation compared to those mediated via photosynthate availability.

Water deficit influences most metabolic processes depending upon the severity of the stress (4, 6). Most grain legumes (pulses) in tropical regions grow in an environment where they experience drought of varying intensity and duration (11). The root nodules are mostly confined to the upper 5 to 15 cm of soil which often dries out during spells of drought. However, within certain limits the plant does not necessarily display water stress effects because roots can tap water from lower zones of the soil.

The effects of water deficit on nitrogen fixation have been studied by exposing either the whole plant or excised nodules to water deficit (3, 7, 8, 15). The main conclusion of such studies is that nitrogen fixation in nodules is reduced because the rate of photosynthesis and translocation of photosynthates are impaired (7, 8). Is nitrogen fixation by nodules exposed to drying soil affected when photosynthesis is not influenced? The available methodology does not offer the possibility of distinguishing these effects. We describe a simple technique to achieve this objective which may prove valuable also for studying other processes.

MATERIALS AND METHODS

Pots of 30 × 30 × 30 cm were used. The system developed for the experiment is described in Figure 1. The lower 18 cm of the pot was filled with the sandy loam soil. On the surface of this soil, a 4-cm layer of gravel (0.5–1.5 cm in size) was placed. A plastic pipe of 2.5 cm diameter was kept vertically on the surface of gravel for irrigating the lower part of the pot. The portion above the gravel layer was filled with the soil mixed with sand in 3 to 1 ratio.

Seeds of cowpea (*Vigna unguiculata* L.) Walp cv C-152 were planted in the upper layer of soil and this layer was irrigated until germination. Seeds were coated with a specific *Rhizobium* strain No. 109 supplied by the Division of Microbiology, Indian Agricultural Research Institute, New Delhi, before planting. The pots were placed out of doors in the natural environment. The daylength, mean maximum temperature, light intensity, and humidity were 12 h, 33 ± 1°C, 1300 $\mu E\ m^{-2}\ s^{-1}$, and 70 to 85%, respectively.

Thirty-five d after germination, the pots were divided into three groups. In one set of pots, 500 ml of water was given to the top soil and 500 ml of water was given through the pipe daily. This constituted the control of irrigated treatment. In a second set of pots, 1000 ml of water was given to the soil zone through the pipe daily while the upper soil layers were not supplied with any water. This constituted the stress in the upper zone treat-

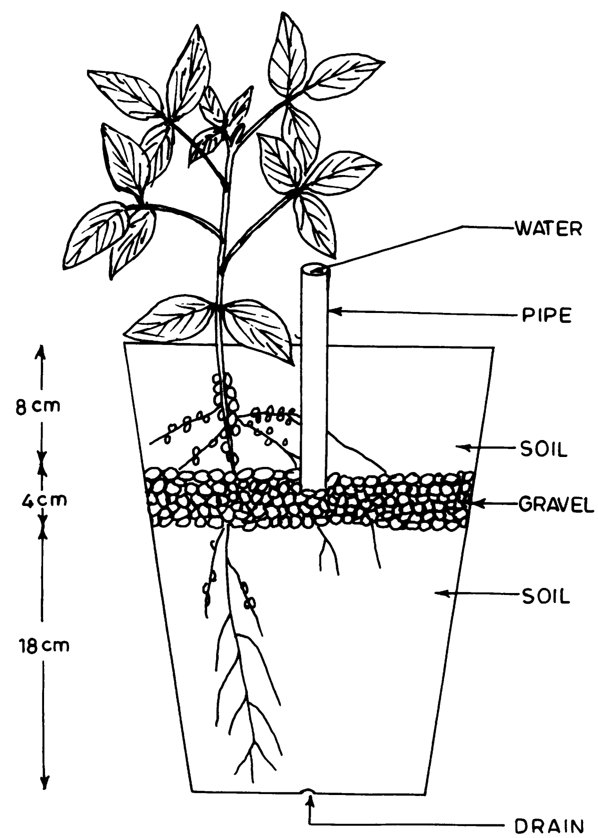

FIG. 1. A system to create water deficit in nodule-containing soil zone, while maintaining adequate supply of water to the root system.

Table I. *Water Potential of Soil and Different Plant Parts in Cowpea Plants Subjected to Water Stress Treatment*

	Water Potential				
	Upper soil layer	Lower soil layer	Leaf	Root	Nodule
			bars		
Control	−0.33 ± 0.1	−0.33 ± 0.1	−6.2 ± 1.0	−3.7 ± 0.4	−6.2 ± 0.4
Stress in upper soil zone only	−10.0 ± 1.0	−0.30 ± 0.1	−5.2 ± 0.3	−4.4 ± 0.6	−10.4 ± 0.5
Stress in both zones	−1.50 ± 0.5	−10.0 ± 1.0	−21.5 ± 1.5	−10.0 ± 0.8	−16.5 ± 1.0

Table II. *Nodulation Characteristics of Cowpea Plants Subjected to Water Stress*

	Upper Soil Layers		Lower Soil Layers	
	Nodules	Nodule dry wt	Nodules	Nodule dry wt
	no./plant	*mg/plant*	*no./plant*	*mg/plant*
Control	77 ± 7	140 ± 13	14 ± 3	16 ± 3
Stress in upper zone only	65 ± 5	125 ± 14	26 ± 3	32 ± 6
Stress in both zones	59 ± 8	70 ± 5	4 ± 1	4 ± 2

Table III. *Effect of Water Stress on Dry Weight and Leaf Area of Plants Using Different Techniques*

	Dry Wt		Leaf Area		
				After stress	
	Before stress	After stress	Before stress	Green	Senescent leaves
	g/plant		*cm²/plant*		
Control	2.5 ± 0.2	3.97 ± 0.6	256 ± 14	353 ± 12	
Stress in upper zone only	2.4 ± 0.4	4.04 ± 0.4	245 ± 20	408 ± 8	
Stress in both zones		3.42 ± 0.4		371 ± 15	67 ± 5

ment. In the third set of pots, watering was stopped completely to the upper and lower soil zone after an initial application of 500 ml of water each to the upper and lower soil zone, respectively. This constituted the stress in the whole plant treatment. There were ten pots in each treatment and six plants were maintained in each pot. Five d after stress, plants were used for determining growth parameters and various aspects of nitrogen fixation such as nodule water potential, leghaemoglobin content, and nitrogenase activity. The soil samples were taken for measuring the soil moisture content by gravimetric method. Water potential *versus* moisture content curves were prepared by using pressure plates for a similar soil (1).

The root and nodule water potential were measured by a thermocouple psychrometer (HR-33(T) Dew point microvoltmeter, Wescor Inc.) using the C-52 sample chamber. The nodules were selected randomly from the tap root and the laternal roots. The fully expanded third leaf from the top was selected for water potential measurements using a Pressure Chamber (model 3005, Soil Moisture Equipment Corp.) following Scholander *et al.* (12).

Leghaemoglobin content and sugar content in the nodules were determined following the method of Proctor (10) and Dubois *et al.* (2). Nitrogenase activity was determined by the method of Hardy *et al.* (5). To measure the acetylene reduction, plants were carefully uprooted and, after gently removing soil, nodulated roots were placed in 100-ml assay bottles (Arthur H. Thomas and Co.). Acetylene was injected to a concentration of 10% (v/v) net volume of the container. After 60 min, gas samples were withdrawn and immediately chromatographed. Acetylene and ethylene were separated with a Shimadzu model GC-4CPF gas chromatograph equipped with a hydrogen flame ionization detector. The stainless steel column was 2 m long and 6 mm in diameter and was packed with Porapak R of 80 to 100 mesh. Nitrogen gas was used as the carrier gas at a flow rate of 60 ml/min. The column temperature was 90°C and the temperatures of the injector and detector were 100°C and 110°C, respectively. After the assay, nodules were removed and washed and the fresh weight was recorded. The dry weight of the nodules was also recorded after drying in an oven at 80°C for 48 h. Specific nitrogenase activity was calculated as μmol ethylene produced/g dry weight nodule·h. At least three replicates were used for each determination.

Photosynthesis rate was determined following the method of Shanthakumari and Sinha (13). Fully expanded leaves (approximately 1 dm^2) were allowed to assimilate $^{14}CO_2$ generated from 5 μCi $Na_2{}^{14}CO_3$ (47 mCi/mmol) by adding 1 N HCl for 1 min in a closed plexiglass chamber (36 × 18 × 12 cm). Each feeding was done in triplicate at 1100 h. The light intensity was 1400 μE m^{-2} s^{-1} and the leaf temperature was 32 ± 1°C. Further processing was done as described in the above method (13). Stomatal conductance and transpiration rate of the fully expanded leaf were determined using a LI-1600 steady state porometer (Li-Cor Inc.)

RESULTS AND DISCUSSION

Withholding the water supply to pots for 5 d reduced the soil water potential to −15.0 bars (Table I). In the pots where water supply was maintained to the lower part through the tube, the soil above the gravel layer dried and had a water potential of

Table IV. *Photosynthesis Rate, Stomatal Resistance, and Transpiration Rate of Cowpea Leaves Subjected to Water Stress by Different Techniques*

	Photosynthesis Rate	Stomatal Resistance	Transpiration Rate
	μmol CO_2 dm^{-2} h^{-1}	*s cm^{-1}*	*μg cm^{-2} s^{-1}*
Control	30.7 ± 1.5	6.7 ± 0.6	3.6 ± 0.5
Stress in upper zone only	28.2 ± 2.0	5.0 ± 0.8	4.5 ± 0.6
Stress in both zones	2.0 ± 0.4	28.4 ± 4.2	0.9 ± 0.1

Table V. *Nitrogenase Activity, Leghaemoglobin Content, and Sugar Content in Cowpea Nodules Subjected to Water Stress by Different Techniques*

	Nitrogenase Activity	Sugar Content	Leghaemoglobin Content
	μmol ethylene·g⁻¹ dry wt·h⁻¹	*mg·g⁻¹ dry wt*	
Control	144.5 ± 8.2	140 ± 7.2	5.52 ± 0.8
Stress in upper zone only	47.5 ± 3.5	104 ± 7.0	3.2 ± 0.4
Complete stress	4.9 ± 0.5	50.5 ± 4.5	3.20 ± 0.30

−10.0 bars. At this stage, the soil water potential of the control pots was −0.33 bars.

The water potential of plants grown with stress in the upper zone only and of control plants was −5 to −6 bars as against −21.5 bars recorded for completely stressed plants (Table I). Thus, the new system was able to create dry conditions around the nodules without any effect on the water status of the plants in comparison with the control. This was further confirmed by examining the water potential of nodules which showed −6.2, −10.4, and −16.5 bars water potential in control, upper zone-stressed, and completely stressed plants, respectively (Table I). Root water potential was quite similar in the irrigated plants and plants with stress in the upper zone only, while it was considerably lower in completely stressed plants.

The roots in the lower zone contained about 1/5th of nodules as compared with the upper zone in control plants (Table II). Nodule number and dry weight were reduced appreciably in the lower zone in completely stressed plants but were enhanced in plants with stress in the upper zone only as compared with control. Complete stress, and stress in the upper zone reduced the nodule dry weight but had no effect on the nodule number in the upper soil zone compared to control plants.

The total dry weight and leaf area of the plant showed no difference between the control and the upper zone-stressed plants (Table III). Reduction in dry weight and senescence of leaves was observed in completely stressed plants after the onset of stress treatment (Table III). The effect of the new system on water status of the plant was measured in terms of some sensitive metabolic processes like photosynthesis, stomatal resistance, and transpiration rate. Photosynthesis rate, transpiration rate, and stomatal resistance were similar in control plants and in those with stress in the upper zone only (Table IV). In completely stressed plants, stomatal closure occurred resulting in drastic reduction in transpiration rate and photosynthesis rate, compared to the control plants (Table IV).

Nitrogenase activity in nodules was reduced by 96%in completely stressed plants and 67% in plants stressed in upper soil zone only compared to control (Table V). Sugar content in the nodules was reduced by 65% and 25% in completely stressed plants and those stressed in upper soil zone only compared to control. The leghaemoglobin content of nodules was reduced by 40% in both the stress treatments compared to control (Table V).

The above results demonstrate that the nodule-containing soil zone could be water stressed without stressing the whole plant as exhibited by water potential of the above ground parts and nodules (Table I). The drying of the soil above the gravel layer is achieved because of the discontinuous column of water.

Our present results clearly demonstrate that considerable loss in activity of nitrogenase can occur if the nodules are under stress but the remaining plant has a normal water status. The photosynthate supply is maintained to the nodules as revealed by sugar content in the nodules (Table V) as well as photosynthesis rate in the leaves (Table IV). The decline in the specific activity of nitrogenase could be partly due to the decline in photosynthate availability and partly the direct effect on oxygen flux, leghaemoglobin content (Table V), ATP levels, and hydrogenase activity. Earlier studies have demonstrated that water stress reduces the activity of nitrogenase in root nodules (9, 11). They suggested that this was possibly due to reduced oxygen availability to nodule cortex. The importance of lenticels both in reduced permeability to oxygen and water loss from nodules was proposed. The structure of lenticels was not examined in the present study, therefore, it is difficult to comment on the mechanism of induction of water deficit in nodules. However, it appears that root nodules lost more water to their surrounding soil than they received from the roots to which they were attached.

The methodology presented here would help in determining the relationship between photosynthesis and nitrogen fixation in a water limiting environment and for studying nodule metabolism.

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