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A Split-Root Growth System for Evaluating the Effect of Salinity on Components of the Soybean *Rhizobium japonicum* Symbiosis[1]

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ABSTRACT

Study of the effects of salinity on symbiotic nitrogen fixation is complicated by the high degree of integration between shoot and nodules. Shoot photosynthetic capacity determines not only the amount of photosynthate available to the nodule, but the strength of the sink for nodule products. Salinity may also directly affect functional processes within the nodule. Two experiments were undertaken which employed a split-root solution culture assembly that confined N_2 fixation to one-half of soybean [*Glycine max* (L.) Merr.] root systems. In one experiment, one-half of the root system was inoculated with non-N_2 fixing strain (ineffective) of *Rhizobium japonicum*, SM-5, 48 hours prior to inoculating the other side with effective strain USDA 110. Nodulation and N_2 fixation by USDA 110 when in combination with SM-5 was significantly suppressed compared to a second treatment where one-half of the root system was inoculated with USDA 110 and the other half remained uninoculated. Root development was significantly greater on the side producing effective nodules than that of the uninoculated or SM-5 nodulated half-root systems. In another experiment, one-half of the root system was nodulated with USDA 110 and the opposite side with SM-5. After nodule development, NaCl (120 m*M*) was added to the nutrient solutions of: 1) neither half, 2) effective nodule (USDA 110) half, 3) ineffective nodule (SM-5) half, or 4) both halves of the root systems. Osmotic potentials ;($\Psi\pi$) of roots, nodules, and leaves were measured. Leaves of plants with only half their roots salinized had $\Psi\pi$(-10.3 bar and -11.1 bar treatments 2 and 3 respectively) comparable to the non-salinized control (-9.3 bar). Leaf $\Psi\pi$ of plants with both root halves in salt solutions was -17.9 bar. Nodules exposed to salt solutions had $\Psi\pi$ values ranging from -9.3 to -12.2 bar while $\Psi\pi$ of nodules in normal solutions ranged from -6.1 to -7.8 bar. Ineffective nodules always had a lower $\Psi\pi$ than similarly treated effective nodules. These results demonstrate that functional components of the soybean *Rhizobium japonicum* symbiosis can be independently subjected to salinity stress. This methodology can be used to evaluate the relative sensitivity of the components of the symbiosis.

Additional index words: Glycine max, Nitrogenanse activity, Nodule mass, Ineffective nodule, Root development.

THE legume-*Rhizobium* symbiosis is a highly integrated system. Soil stress may act on the symbiosis indirectly by reducing plant growth and available photosynthate, or act directly on nodule function and/or the infection process. In order to increase stress tolerance of the symbiotic system, the most stress-sensitive component(s) need to be identified. This can be done by subjecting shoot photosynthetic capacity and nodule functioning to differential levels of stress and measuring the effects of stress on the components of the symbiosis.

Split-root growth systems have been used to study the effects of various stresses on plant growth and root development: salinity (Kirkham et al., 1972); moisture tension (Volk, 1947); nutrient availability (Gile and Carrero, 1917; Hackett, 1972). Hinson (1975) inoculated half the root system of soybeans [*Glycine max* (L.) Merr.] grown in vermiculite to study the localized effect of mineral N on nodule development. Such a system is not, however, suitable for studying the effect of salinity on the symbiosis because migration of salts in the rooting medium may create zones of variable osmotic potential, so that nodules and roots will not be exposed to a uniform stress.

This paper describes two experiments using a solution culture, split-root growth system to examine the effects of various stresses on components of the legume-*Rhizobium* symbiosis. The first experiment examined two methods of confining N_2-fixing root nodules to one-half of soybean root systems and the effect of nodulation on root development, water uptake, and N_2 fixation. The second experiment examined the effect of applying NaCl to either half or whole root systems on leaf, nodule and root osmotic potential ($\Psi\pi$).

MATERIALS AND METHODS

Growth System and Inoculum Preparation

Steel containers (20 liters) were coated inside and out with an elastomeric protective coating the USDA has approved for potable water (Vabar 792, United Coatings)[3]. The top of each container was removed except for an 8.0 cm strip along one side (Fig. 1). This strip had eight 2.54 cm holes to receive one end of a polyvinylchloride (PVC) elbow (21.0 mm O.D., 6.0 cm sides), packed with sterile vermiculite. A planting hole (10 mm diam) was drilled on the joint of each elbow. Planting holes were designed to receive seedling radicles. The ends of elbows were fitted with a rubber washer to stabilize the elbows which spanned two containers. A sight glass was fitted to each container and calibrated volumetrically to the nearest 0.5 liter. Each container had an aquarium airstone connected to a flexible plastic lateral air line to create a constant splash of solution on the ends of the PVC elbows. The nutrient solutions were saturated with oxygen as measured with a YSI oxygen meter. Lids for the containers were made from 2.54 cm sheets of polystyrene. The bottom lid was covered with black polyethylene.

Seeds of the soybean cultivar Davis were treated for 5 min with 70% ethanol, rinsed, planted hilum down in trays containing sterile vermiculite and maintained at 28 C for 48 hours. The tips of the seedling radicles were removed to induce branching and seedlings were then inserted into the planting holes of the PVC elbows. Sterile water was applied to the seedling to ensure capillarity between the cut radicle and vermiculite. The sides of the elbows were covered with foil to maintain the same temperature on both sides.

A N-free nutrient solution was used consisting of: 0.5 m*M* P; 0.96 m*M* K; 1.56 m*M* S; 0.82 m*M* Mg; 0.75 m*M* Ca. Sources were: K_2HPO_4; $MgSO_4 \cdot 7H_2O$; $CaSO_4$. Micronutrients were added according to Broughton and Dilworth (1971).

Cultures of *Rhizobium japonicum*, ineffective strain SM-5 (W. J. Brill, Univ. of Wisconsin, Madison) and an effective strain USDA 110 (USDA, Beltsville, Md.) were grown in yeast extract mannitol broth and counted by the drop plate method (Vincent, 1970) then centrifuged for 10 min at 12,100 × g and 4 C. The

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[3] Mention of a trademark does not constitute an endorsement by the author, Univ. of Hawaii, or the United States Agency for International Development.

cells were re-suspended in water and inoculated into the appropriate containers.

Effective vs. Ineffective *Rhizobium*

Five days after planting roots emerged from elbows into the containers. At this time, designated containers were inoculated with SM-5 at the rate of 1.5×10^6 viable cells/ml of plant nutrient solution. Forty-eight hours later USDA 110 was inoculated at 4.4×10^4 cells/ml in containers to institute as two treatment combinations: 1) USDA 110 on one side and no inoculation on the other, 2) USDA 110 on one side with ineffective SM-5 on the other. Inoculation with SM-5 preceded that of USDA 110 so that the early processes of infection would be complete and reduce the chance of forming contaminant effective nodules on the non-N_2 fixing (SM-5) side. Containers were arranged in a completely randomized design and replicated three times. Both experiments were carried out in a glasshouse during the months of June and July. Solution pH was monitored but never declined below 5.8 from an initial pH of 6.5. Solution temperatures ranged from 26 to 28 C at 1400 hours. Nutrient solutions were changed at 20 and 40 days from planting. Containers were inoculated to the original cell density with the appropriate *Rhizobium*, after each solution change.

At harvest (60 days after planting), the containers were drained, shoots were cut, and dried at 60 C. Roots were removed and immediately incubated in 2.0 liter plastic vessels containing 5% acetylene. Ethylene production was determined by gas chromatography. Nodules were removed from roots, oven dried, counted, and weighed.

Salinization of Half-Root Systems

The planting procedure was identical to that of the first experiment. Solutions were changed at 21 and 34 days from planting. Each container of a pair was inoculated with either SM-5 or USDA 110.

Salt treatments were instituted at Day 34 by adding NaCl to designated containers during a 48-hour period to obtain a final concentration of 120 m*M* NaCl (normal solution osmotic potential ($\Psi\pi$) was -0.15 bar, while $\Psi\pi$ of salinized solutions was -5.5 bar). Four combinations of salt treatments were applied to the half-root systems: 1) No salt added to either side, 2) salt added to the non-N_2 fixing half-root system (SM-5), 3) salt added to N_2-fixing half-root system (USDA 110), and 4) salt added to both half-root systems.

The osmotic potential ($\Psi\pi$) of the most recently expanded trifoliate, nodules, and roots was determined at Day 45. Nodule and root samples were blotted dry and each placed in 8 cm lengths of 12 mm I.D. flexible plastic tubing. Leaves were placed directly into a tube. Tubes were plugged at both ends with rubber stoppers after expressing most of the air and then frozen on dry ice. Samples were thawed for 20 min, pressed at 1,050 kg/cm^2, and the expressed sap was sampled with filter paper discs which were placed in a Wescor Model 5130 vapor pressure osmometer.

RESULTS

Plants with half their roots inoculated with ineffective strain SM5 had lower shoot, root, and nodule dry weights than plants with the effective strain USDA 110 (Table 1).

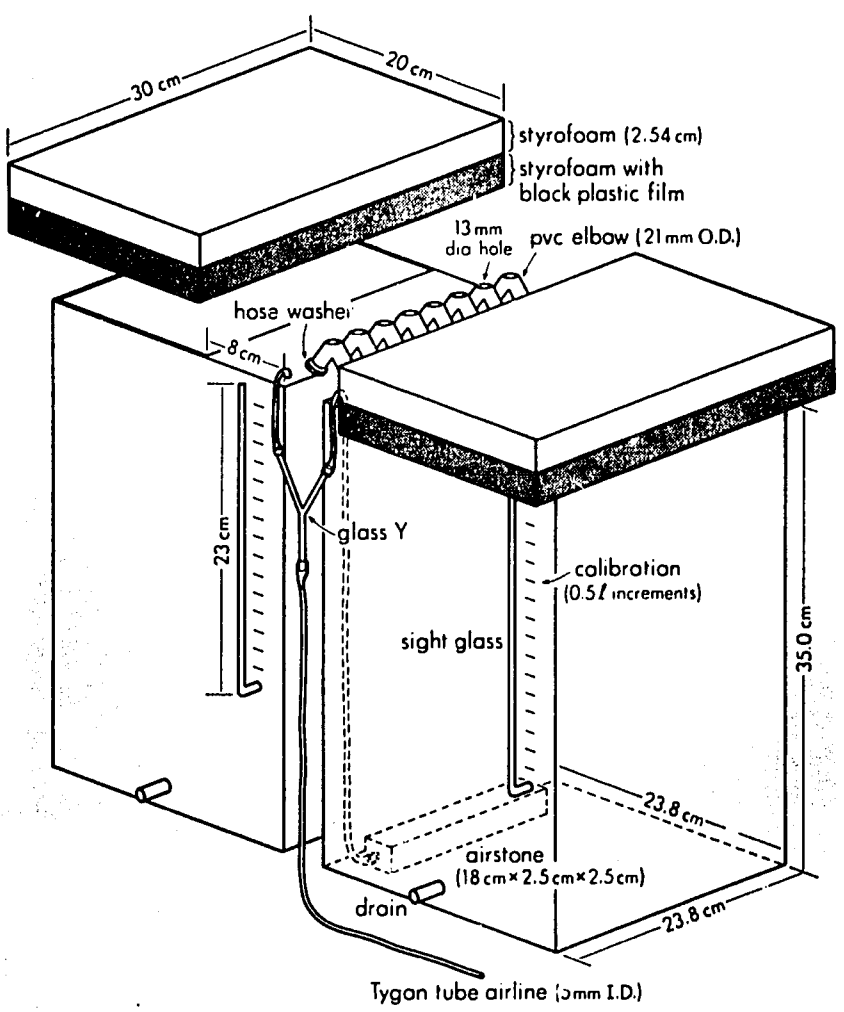

Fig. 1. A split-root growth system used to confine root nodules to one-half the root system.

The number, mass, and nitrogenase activity of nodules formed by effective strain USDA 110 was reduced when the other half of the root system had SM-5 nodules (Table 1). Uninoculated half-root systems remained free of nodules. The growth of half-root systems was stimulated by inoculation with USDA 110. Water consumption was similarly greater by plants which had been inoculated with USDA 110. No nitrogenase activity was detected by the uninoculated or the ineffectively nodulated half-root systems.

Leaf $\Psi\pi$ (−10.3 and −11.1 bar) of plants with half of their root system exposed to 120 m*M* NaCl was not different from the control (−9.3 bar) (Table 2). Leaf $\Psi\pi$ was reduced to −17.9 bar when both root halves were exposed to salt. Nodule $\Psi\pi$ was apparently determined by the solution to which they were exposed. When USDA 110 nodules were exposed to NaCl, nodule $\Psi\pi$ was −10.9 bar and −12.2 bar, respectively, for half and whole-root salt treatments. Ineffective nodules (SM-5) had nodule $\Psi\pi$ of −9.3 bar for both half and whole-root salt treatments. The $\Psi\pi$ of non-salinized USDA 110 and SM-5 nodules was −7.8 bar and −6.1 bar, respectively. Water absorption by half-root systems was reduced by the addition of 120 m*M* NaCl. This reduction was compensated by increased water uptake on the non-salinized side.

DISCUSSION

To test the effects of salinity on the integrated processes of the legume-*Rhizobium* symbiosis, it is necessary to isolate and independently subject photosynthetic and nodule processes to the stress. A growth system suitable for such purposes must meet two requirements. First, all N_2-fixing nodules must be confined to a portion of the root system. Secondly, the salinity stress must be uniformly applied to the nodules with a minimal effect on the photosynthetic apparatus.

Solution culture systems are useful in salinity studies because uniform osmotic potentials in the root environment can be maintained. By employing a split-root technique which confines rhizobial inoculation and subsequent nodulation to one-half of the root system, it is possible to subject the functional processes of the nitrogenase system to salinity stress while maintaining the shoot under less stressful conditions. Kirkham et al. (1969) demonstrated that although leaf water potential of *Phaseolus vulgaris* with half the root system in salinized medium was reduced, the potential more closely resembled the no salt control than the leaf potential of plants with both root halves in salinized media. Using tritiated water, Sprent (1972) concluded that nodules received water enroute from roots to leaves. This would imply that nodules are at a lower water potential than surrounding roots. However, given the large amount of phloem transport taking place from shoot to nodule (Minchin and Pate, 1974), it seems plausible that the osmotic potential of nodules in the localized stress environment of differentially salinized plants may not adjust to their environment.

Our study examines two methods of maintaining N_2-fixing nodules on half the root system of soybean. In addition, we evaluate the osmotic adjustment of roots, nodules, and leaves of plants with differentially salinized root systems.

Table 1. Effect of inoculating soybean half-root systems with effective and ineffective strains of *Rhizobium japonicum* on shoot weight, root and nodule development, nitrogenase activity, and total water use.

Inoculation treatments and comparisons	Dry weight			Nodule number	Nitrogenase activity	Water consumption
	Shoot	Root	Nodule			
	g/pot			no./pot	μM C_2H_4/pot·hour	liter/pot
Inoculated vs. control						
Uninoculated side		1.2	trace	1	0	13.0
USDA 110 side		2.0	1.30	621	56	18.0
Total	18.8	3.2	1.30	622		31.0
Effective vs. ineffective†						
SM-5 side		0.5	0.15	424	0	10.0
USDA 110 side		0.7	0.23	191	11	11.0
Total	5.1	1.2	0.33	615		21.0
$LSD_{0.05}$	13.0	0.6	0.54	233	23	5.0

† The ineffective strain, SM-5, was inoculated into the designated side 48 hours before the application of the effective strain, USDA 110, to the opposite side.

Effective vs. Ineffective Rhizobium

Inoculation of half the root system with an ineffective strain (SM-5) 48 hours before the introduction of the effective strain (USDA 110) on the opposite side caused a reduction in the number, weight, and activity of the resulting effective nodules (Table 1). Total nodule number (effective an ineffective) however, remained the same regardless of the treatment. Weight of effective nodules was reduced when SM-5 was inoculated to the other side. Apparently, total nodule number on a soybean root system is controlled by the host and strains of *Rhizobium* complete for a limited number of nodule sites. Competition for nodule sites exists even when there is spatial separation of the strains on the root system. The competitive advantage of SM-5 when inoculated first indicates that events early in the infection and nodule development processes determine what proportion of the nodules will be formed by each strain. When SM-5 was used as a method to confine effective strain USDA 110 to one-half the root system the proportion of total nodule number (USDA 110 nodules plus

Table 2. Effect of 120 m*M* NaCl added to half-root systems of nodulated soybean on the osmotic potential ($\Psi\pi$) of leaves, nodules, roots, and water consumption subsequent to NaCl addition.

Salt treatment to half-root system growing:		Osmotic potential ($\Psi\pi$)					Water consumption	
			Nodule		Root			
USDA 110 Nodules	SM-5 Nodules	Leaves	USDA 110	SM-5	USDA 110	SM-5	USDA 110	SM-5
m*M* NaCl		bar					liter/day	
0	0	−9.3	−7.8	−6.1	−5.4	−6.0	0.61	0.44
0	120	−10.3	−7.8	−9.3	−5.5	−7.5	1.10	0.23
120	0	−11.1	−10.9	−6.9	−7.1	−5.8	0.32	0.98
120	120	−17.9	−12.2	−9.3	−8.1	−7.1	0.29	0.33
	$LSD_{0.05}$	3.2	1.2		1.3			

SM-5 nodules) that was formed by USDA 110 was less than 30%. Singleton and Stockinger (1982) have shown that although plants may compensate somewhat for ineffective nodulation, effective nodule mass and N_2 fixation is seriously reduced when effective nodules were less than 50% of the total. The design of the growth system maintained the uninoculated half-root system sufficiently free of contaminant nodules so that the source of symbiotic N can be isolated and inoculation with an ineffective strain on one side is not necessary.

Root development was stimulated by effective nodules. Root weight was consistently greater on the side with effective nodules than uninoculated and ineffectively nodulated sides (Table 1). Total water consumption followed the same pattern (Table 1). Increased flow of carbon to active nodules and the consequent increased N from active nodules may have caused increased root development. Drew (1975) showed that application of nitrate and ammonium had a localized stimulatory effect on root development. When the source of symbiotic N is localized there may be a similar stimulation of root development.

Salinization of Half-Root Systems

Application of 120 m*M* NaCl to half-root systems reduced the osmotic potential ($\Psi\pi$) of roots and nodules exposed to these solutions. Nodules were always at a lower potential than their respective roots. Leaf $\Psi\pi$ was more negative than the $\Psi\pi$ of both nodules and roots when only those half-root systems were exposed to NaCl. The potential gradient necessary for xylem transport of fixation products between nodule and shoot was therefore maintained.

Shoots of plants with half their root systems in salinized solutions had leaf $\Psi\pi$ values closer to those of the non-salinized control, than to leaf $\Psi\pi$ of plants with the complete root system in salinized solutions. These results suggest that increased water uptake by the non-salinized half-root system compensated for salinity stress on the opposite half-root system (Table 2). Hence the symbiotic system was subjected to salinity stress while maintaining a relatively unstressed photosynthetic apparatus.

In conclusion, the growth system design described in this paper provides sufficient control of root nodulation to confine symbiotic N_2 fixation to half-root systems of soybean. Osmotic adjustment by roots, nodules, and leaves of differentially salinized soybeans with split-root systems allows observation of nodule processes under salinity stress when the shoot is under relatively normal conditions. The use of this type of growth system may prove useful in the study of the effects of other soil stress factors on symbiotic processes.

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4