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Evaluation of Two Methods to Estimate the Population of Indigenous N-fixers Associated with Tropical Legumes

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Abstract—Growth pouch and test tube methods were compared to estimate the populations of two indigenous nitrogen fixing rhizobia, *Bradyrhizobium* spp. associated with *Macroptilium atropurpureum* (DC) Urb. and *Rhizobium* spp. hosted by *Leucaena leucocephala* (Lam.) de Wit on Guam. Indicator plants required less nutrient solution in test tube method than in growth pouch method. Generally plant growth and nodule formation were better with the pouch method. Because *L. leucocephala* had developed very fine white fibrous structures on roots instead of nodules when it was incubated in a test tube, the detection of nodules was difficult. *Bradyrhizobium* spp. nodulated *M. atropurpureum* were more numerous in both Pulantat soil (Udic Haplustalf) and Akina soil (Oxic Haplustalfs) while *Rhizobium* spp. associated with *L. leucocephala* were predominant in the Guam cobbly clay soil (Lithic Ustorthents). Although a difference in population density was found between the two methods, the distribution pattern of the two groups of rhizobia was consistent at each locality.

Introduction

Biological nitrogen fixation (BNF) in legumes plays an important role in agroecosystems in the tropics. Nitrogen derived from BNF offers an alternative fertilizer source for subsistence farming and sustainable agriculture in areas where fossil fuel-derived N fertilizer is not readily available (Singleton et al. 1992). Indigenous rhizobia are adapted to their local soil and climatic environments, and are good indicators of nutrient status (Woomer et al. 1988). One of the common methods for estimating populations of native rhizobia from soil is determination of the most probable number (MPN) using the "growth pouch" (Somasegaran & Hoben 1985). In this method, legume seeds are aseptically germinated and transferred to a "growth pouch" containing N-free nutrient solution, and inoculated with a dilution series of soil solution. It is a simple and easy method, but it requires frequent replenishment of nutrient solution when plants are maintained in a greenhouse or in an open area without the high humidity of an enclosed incubation chamber. In addition, occasionally microbial contamination may occur at the initial stage of plant development, resulting in poor

growth and death of seedlings. An alternative method is the use of test tubes as growing containers (Somasegaran, NifTAL, University of Hawai'i, personal comunication). The small volume of test tubes reduce the amount of N-free solution used during the incubation period and also reduce the amount of contamination. In this study, the growth pouch method and the test tube method were compared to estimate indigenous rhizobia populations from three different soil regimes on Guam.

Materials and Methods

Plant Materials

Two tropical legumes, siratro, *Macroptilium atropurpureum* (DC.) Urb. and tangantangan, *Leucaena leucocephala* (Lam.) de Wit, were used as host plants of two nitrogen fixing rhizobia, *Bradyrhizobium* spp. and *Rhizobium* spp., respectively (Somasegaran & Hoben 1985). Siratro seeds were obtained from USDA Natural Resources and Conservation Services in Molokai, Hawai'i, while leucaena seeds were locally collected from the field. Seeds were surface sterilized with 0.5% sodium hypochlorite (NaOC1) for 15 min and were rinsed 3 times with sterile distilled water. Leucaena seeds were scarified using concentrated sulfuric acid for 5 to 10 min and were rinsed thoroughly with sterile distilled water. Seeds imbibed water overnight. On the following day, they were pre-germinated in moist, sterile paper towels kept in ziploc bags or petri-dishes. Seedlings with radicle of about 4 mm were used for this study.

Culture container preparation

For the test tube method, glass culture tubes with the size of 25 x 125 mm (for Experiment 1) and 25 x 100 mm (for Experiment 2) were used. Paper towels were folded to fit a tube and were soaked overnight in distilled water to rinse off any bleaching materials. Water in the test tube was replaced by 20 mL of N-free plant nutrient solution (Somasegaran & Hoben 1985). Tubes were capped and autoclaved at 15 psi for 20 min. A small rectangular opening was made to each cap to allow gas exchange.

For the growth pouch method, 40 mL of the same N-free plant nutrient was added to each pouch (Mega Co. Minneapolis, MN) after they were autoclaved at 15 psi for 5 min.

Soil sampling and inoculum preparation

Three soil types were examined: Akina soil (Oxic Haplustalfs) from Ija, Pulantat soil (Udic Haplustalfs) from Barrigada and Guam cobbly clay soil (Lithic Ustorthents) from Yigo. At each locality, twenty soil sub-samples for Experiment 1 and ten sub-samples for Experiment 2 were collected from a 100 m^2 area. They were pooled together to make a composite soil sample per site. Samples were stored at 4ºC prior to use and they were used within 24 hours. Ten grams of each soil sample (oven dry basis at 65ºC) were mixed with 90 mL ster-

ile distilled water to make the 1:9 soil solution. This soil solution was then mixed thoroughly using a multi-wrist shaker for 15 min. A series of soil dilutions from 10^{-1} to 10^{-9} was prepared as inoculum. Approximately 10 grams of soil sample were used to measure soil pH. For pH determination, soil samples were air-dried, ground and mixed with distilled water (1:1) on a volume.

Plant inoculation

Each legume seedling with a radicle was placed individually in a culture container (growth pouch or test tube). A paper wick was placed in each pouch and a small hole was made near the top into which a germinated seedling was placed. The radicle was inserted into the paper wick with the cotyledons above. For test tubes, the radicle of a plant was inserted in a space between the tube and a paper towel. Starting with 10^{-9} soil dilution, inoculation was done aseptically by adding one mL of the soil solution into each culture container. Sterilized distilled water was used as a control. Each soil dilution was mixed well using a vortex mixer prior to inoculation. There were four replications of each soil dilution to inoculate each plant to determine the most probable number of bacteria in soil sample (Somasegaran & Hoben 1985). For Experiment 1, inoculation of both *L. leucocephala* and *M. atropurpureum* was done on March 16, 1995. In Experiment 2, *L. leucocephala* was inoculated on September 7, 1995 to estimate the population of *Rhizobium* spp. and *M. atropurpureum* for *Bradyrhizobium* spp. on September 15, 1995.

Incubation and plant development

Both pouches and test tubes were placed in a screenhouse. The N-free nutrient solution was replenished as needed, and the date and the amount of solution added were recorded. Caps of tubes were removed when seedlings grew and reached the caps.

The date of initial nodule formation was recorded and further nodule development was observed for at least 30 days afterwards. MPN was determined according to the procedure described by Somasegaran & Hoben (1985) on April 3, 1995 and May 1, 1995 for Experiment 1, and on October 11 and November 18, 1995 for Experiment 2.

During the plant incubation period, leaf greenness was examined by using a SPAD 501 chlorophyll meter (Minolta Co. Ramsey, NJ) for *M. atropurpureum*. Chlorophyll readings were not possible for leucaena plants because of their small leaf and leaflet size. On the termination date, wet biomass of shoots and roots were measured to compare the effect of nodulation on plant growth. Chlorophyll contents and biomass data were taken from four uninoculated control plants and four plants nodulated by soil inoculum from each site and from each culture container. Data were analyzed using a General Linear Model (Data Desk, 1988).

Table 1. Populations of native *Rhizobium* spp. and *Bradyrhizobium* spp. at three sites on Guam.[a] (Experiment 1)

Site	*Rhizobium* ssp.				*Bradyrhizobium* spp.			
	4/3/95		5/1/95		4/3/95		5/1/95	
	Tube	Pouch	Tube	Pouch	Tube	Pouch	Tube	Pouch
	No. of bacteria in one gram soil sample							
Yigo	A[b]	31	ND[c]	170	A	17	170	1,700
Barigada	A	6	ND	6	58	31	17,000	170
Ija	A	10	ND	31	17,000	1,000	10,000,000	58,000

[a]Plant inoculation with soil samples was done on 3/16/95. *Leucaena leucocephala* (Lam.) de Wit was used as the host plant to determine the population of *Rhizobium* spp. and *Macroptilium atropurpureum* (DC.) Urb. as the host plant for *Bradyrhizobium* spp.

[b]A = No nodules were observed.

[c]ND = Not determined due to formation of cottony structures on roots.

Results and Discussion

Two indicator plants, *L. leucocephala* and *M. atropurpureum*, nodulated when they were inoculated with soil samples collected from three different classes of soils on Guam. It indicated that two types of nitrogen fixing bacteria, *Rhizobium* spp. which only nodulated *L. leucocephala* and *Bradyrhizobium* spp. that nodulated *M. atropurpureum*, existed at all three locations. Estimated populations of those native rhizobia varied among sampling dates, types of culture containers and soil types. The soils of the March sampling in Experiment 1 had more bradyrhizobia than rhizobia at all three sites (Table 1). From the soils sampled in September in Experiment 2, however, more rhizobia were found in Guam cobbly clay soil with pH of 7.5 from Yigo site while bradyrhizobia were predominant in both Pulantat soil from Barrigada with pH of 6.9 and Akina soil from Ija with pH of 6.2 (Table 2). The population density of bradyrhizobia was highest in Ija soil in both experiments.

The type of containers also affected the determination of MPN. Both methods had advantages and disadvantages. One advantage of the test tube method was having less contamination problems, especially at the beginning of the incubation period. Covering the growing container during the early stage of plant development kept contamination minimal. Secondly, the test tube method needed less volume of N-free nutrient solution (Table 3) and gave better contact between rhizobia and roots. It was also easier to inoculate germinating seeds in test tubes. However, the development of root systems inside the paper wick made it difficult to observe nodules with the test tube method. With the pouch method, on the other hand, plants had visible and well-spread root systems in later stages of plant growth. When a plant culture container was contaminated, fungal and algal growth was more prominent in the tubes to interfere examination of nodule

Table 2. Populations of native *Rhizobium* spp. and *Bradyrhizobium* spp. at three sites on Guam.[a] (Experiment 2)

Site	*Rhizobium* ssp.				*Bradyrhizobium* spp.			
	10/11/95		11/18/95		10/11/95		11/18/95	
	Tube	Pouch	Tube	Pouch	Tube	Pouch	Tube	Pouch
	No. of bacteria in one gram soil sample							
Yigo	< 6	6	170	580	< 6	6	< 6	6
Barigada	17	31	31	31	6	580	5800	1000
Ija	< 6	< 6	< 6	6	170	5800	3100	10000

[a]The inoculation of the host plant, *Leucaena leucocephala* (Lam.) de Wit, with soil samples was done on 9/15/95 to determine the population of *Rhizobium* spp. and the inoculation of *Macroptilium atropurpureum* (DC.) Urb. for *Bradyrhizobium* spp. was done on 9/7/95.

formation. In the pouch method, contamination was limited to the surface of the paper wick and did not affect root systems.

Host plants, in general, grew more vigorously in pouches than in test tubes (Table 3). The solution in the test tube method appeared to be stagnant and poor aeration may have reduced plant growth. Conversely, in the pouch method, the root zone of legumes were well aerated and regular replenishment of N-free nutrient solution seemed to promote better plant development. The effect of growing methods was more evident with *M. atropurpureum*. At the initial nodulation stage,

Table 3. Maintenance of culture containers to determine indigenous rhizobia populations.

Characteristics	*Rhizobium* ssp.		*Bradyrhizobium* spp.	
	Pouch	Tube	Pouch	Tube
Total amount of N-free solution used (mL):				
Experiment 1	240	70	240	70
Experiment 2	520	140	360	120
Presence of cottony structure on roots:				
Experiment 1	Absent	Present	Absent	Absent
Experiment 2	Absent (present at late stages)	Present	Absent	Absent
Plant growth:				
Experiment 1	Good	Poor	Good	Poor
Experiment 1	Good	Poor	Good	Poor

L. leucocephala and *M. atropurpureum* were host plants of *Rhizobium* spp. *Bradyrhizobium* spp., respectively.

Table 4. Biomass of shoot and root of *M. atropurpureum* at 47 days after inoculation in Exp. 1.

Soil sample site	Method	Nodulation[a]	Wet biomass		Chlorophyll
			Shoot	Root	(SPAD reading)
			mg plant^{-1}		
Barrigada	Tube	+	360	188	28
		–	142	285	9
	Pouch	+	713	268	30
		–	120	190	7
Ija	Tube	+	340	205	29
		–	110	228	10
	Pouch	+	1265	465	28
		–	59	183	6
Yigo	Tube	+	213	113	12
		–	148	248	8
	Pouch	+	498	168	25
		–	70	220	8
ANOVA:					
Soil sample site (S)			***	*	**
Method (M)			***	NS	NS
Nodulation (N)			***	NS	***
S x M			***	NS	**
S x N			***	**	**
M x N			***	**	**
S x M x N			***	NS	NS

[a]Four uninoculated control plants and four plants nodulated by soil inoculum were examined.

*, **, *** Significant at the 0.05, 0.01 and 0.001 probability levels, respectively.

the size of nodules was larger and more nodules were formed on *M. atropurpureum* in pouches. Wet biomass of shoots of *M. atropurpureum* was also greater when grown in pouches than in tubes (Table 4). Distinct differences in greeness of leaves became evident after a month from the date of the first nodule observation. Plants without nodules were pale green although no visual distinction was detected among plants with different number of nodules. The readings of the SPAD chlorophyll meter indicated that *M. atropurpureum* with nodules became greener as they possibly obtained nitrogen through the BNF system while plants without nodules were chlorotic due to the nitrogen deficiency. The shoot

biomass and chlorophyll readings were lower in plants inoculated with Guam cobbly clay soil of Yigo than in plants nodulated with soil inoculants from the other two sites, Barrigada and Ija. Shoot biomass was affected by interactions among the type of method, soil sample location, and the presence or absence of nodulation (Table 4).

White, very fine thread-like structures resembling cotton fibers were formed on roots of *L. leucocephala* (Table 3) instead of distinct nodules. This interfered with counting nodules to estimate the rhizobia population, especially in Experiment 1. Furthermore root tissues of *L. leucocephala* were often split when grown in test tubes. It was not possible to use the SPAD chlorophyll meter for *L. leucocephala* since their compound leaves were too small. Leaves of *L. leucocephala* grown in both tube and pouch remained green for about one month. Compared to *M. atropurpureum*, no distinct differences in the greenness of the leaves were observed until close to the termination date of the experiment. White cottony structure formed on the roots in the test tube culture seemed to help leucaena leaves green. Leucaena grew taller with larger leaves in the pouch than in the tubes. In both methods, the foliage of all plants was infested lightly with leaf miner, *Liriomyza* sp. and broad mite, *Polyphagotarsonemus latus* (Banks).

In summary, the test tube method can be used for determination of *Bradyrhizobium* spp. with *M. atropurpureum* as a test plant. However, the test tube method is not recommended to estimate *Rhizobium* spp. using *L. leucocephala* due to formation of cotton-like fibrous structures instead of normal nodules. Although the pouch method requires more N-free solution, it is easier to view root development and nodule formation of host plants, and to count the number of nodules. Initial preparation for setting up the experiment was easier with the pouch method.

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