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ACCURACY OF MOST-PROBABLE-NUMBER ESTIMATES OF RHIZOBIA FOR TREE LEGUMES

DAN TURK* and HAROLD H. KEYSER†

NifTAL Project, University of Hawaii, 1000 Holomua Road, Paia, HI 96779, U.S.A.

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Summary-Rhizobia that nodulate tree legumes have rarely been enumerated by the most-probable number (MPN) plant infection assay. We compared MPN estimates of pure cultures of rhizobia with plate counts, using as hosts seedlings of 14 tree legume species grown separately in growth pouches or glass tubes. Reasonable agreement was obtained with 11 of the 14 tree species in one or both growth systems, with closer agreement in glass tubes than growth pouches for small-seeded species. Weekly assessment of MPN assays indicated that glass tubes and growth pouches should be scored for nodulation at least 7 and 5 weeks after inoculation. respectively.

INTRODUCTION

Nitrogen-fixing leguminous trees are widely planted for fuelwood, fodder, construction material and biologically-fixed N for associated crops in agroforestry systems. In many tropical soils inadequate rhizobial populations limit N_2 fixation (Singleton *et al.*, 1992). In such soils, inoculation with appropriate rhizobia can improve yield, provided no other constraints limit growth.

Estimating rhizobial population density is a means of predicting whether or not a legume will respond to inoculation (Thies *et al.*, 1991b). Although there is no direct way of measuring the density of indigenous rhizobial populations in soil (Brockwell, 1980), the most-probable-number (MPN) plant-infection assay (Vincent, 1970) provides an indirect estimate. MPN assays for enumerating rhizobia are also used in ecological studies, and for evaluating inoculant quality (Brockwell, 1980).

Although frequently used with grain and forage legumes, the MPN assay has seldom been used with tree legumes as hosts (Table 1). There is, therefore, a need to evaluate the accuracy of the technique when used with trees. Comparisons of pure cultures with plate counts have been recommended as a means of assessing the accuracy of estimates obtained from particular legume-rhizobium growth systems (Brockwell, 1963). Such comparisons have only rarely been made with tree legumes (Table 1).

The main objective of our experiments was to evaluate the accuracy of MPN assays using pure rhizobial cultures by comparing MPN estimates with plate counts. Other objectives were to determine an appropriate MPN growth system for each of the tree species used and to determine how long the assays needed to be maintained before scoring for nodulation.

MATERIALS AND METHODS

Growth systems

Two growth systems were used in this study: plastic growth pouches (Northrup King Co.) and agar slants in 25 x 250 mm glass tubes. *Acacia auriculiformis* A. Cunn. ex Benth., *Acacia mangium* Willd., *Acacia mearnsii* De Wild., *Leucaena diversifolia* (Schlecht.) Benth., *Paraserianthes falcataria (L.)* Nielsen, *Robinia pseudoacacia L.,* and *Sesbania grandii lora* Poir. were grown in pouches and on agar slants. Additional MPN assays were conducted in pouches with *Albizia lebbeck (L.)* Benth., *Albizia saman* (Jacq.) F. Muell., *Calliandra calothyrsus* Meissn., *Enterolobium cyclocarpum* Griseb., *Flemingia macrophylla* (Willd.) Merrill, *Gliricidia sepium* (Jacq.) Steud., *Leucaena leucocephala* (Lam.) de Wit, and S. *sesban (L.)* Merr. Seeds of all species were obtained from either the Nitrogen Fixing Tree Association (Box 680, Waimanalo, HI 96795, U.S.A.) or NifTAL Project seed collections.

Agar slants were prepared using 15 ml per tube of N-free nutrient solution modified from Singleton (1983) and 15 g agar l^{-1}. After autoclaving, the agar was allowed to cool in the tube slanted at an angle of 10° from the horizontal, producing an agar surface *ca* 10 cm long, beginning at the base of the tube. Growth pouches were prepared by filling each pouch with 50 ml of N-free nutrient solution, minus the agar. An additional 50 ml of N-free nutrient solution was added prior to inoculation. After inoculation pouches were provided with sterile deionized water as needed until harvest.

Seedling preparation

Seeds were appropriately scarified and surface sterilized. After imbibition, seeds were germinated on water-agar plates (Somasegaran and Hoben, 1985).

*Present address: Department of Forestry, North Carolina State University, Box 8002, Raleigh, NC 27695, U.S.A.
†Author for correspondence.

Table 1. Summary of studies where MPN assays have been conducted with tree legumes

Species	Growth system	Time to assessment[a]	Reference
Leucaena leucocephala	agar deeps	up to 11 weeks	Davis (1982)[b]
L. leucocephala	not recorded	2–3 weeks[c]	Sanginga *et al.* (1985)
L. leucocephala	not recorded	2–3 weeks[c]	Sanginga *et al.* (1987)
L. leucocephala	growth pouches	2–3 weeks	Singleton and Tavares (1986)
L. leucocephala	growth pouches	not recorded	Singleton *et al.* (1991)
L. leucocephala	growth pouches	3–4 weeks	Thies *et al.* (1991a)
L. leucocephala	agar slants	7 weeks	Woomer *et al.* (1988a)
L. leucocephala	agar slants	2–3 weeks	Woomer *et al.* (1988b)
Prosopis glandulosa	dibble tubes	6 weeks	Virginia *et al.* (1986)
Sesbania sesban	growth pouches	not recorded	Singleton *et al.* (1991)

[a]Weeks after inoculation.
[b]Includes a comparison of a pure culture MPN estimate to a plate count.
[c]According to the reference cited, Vincent (1970).

Two to five days later, when radicles had reached 0.5-1.5 cm in length, the seed coats of uniform seedlings were removed to prevent seed coat hardening and to facilitate examination of tubes for nodulation. In tubes, seedlings were placed on the surface of the agar, with the root collar *ca* 6-7 cm from the bottom of the slant. One and two seedlings were planted in tubes and pouches respectively. After planting, racks of pouches and tubes were placed in a growth room receiving > 300 ìE m^{-2} s^{-1} of photosynthetically-active radiation at plant height for 16 h day^{-1} from 1000 W high-pressure sodium lamps.

Prior to inoculation, growth units with poorly growing plants were eliminated. These included tubes with seedlings whose tap roots penetrated the surface of the agar, in accordance with the observation of Woomer *et al.* (1988b) that agar penetration by roots of L. *leucocephala* resulted in poor nodulation. Enough uniform plant growth units were retained for at least six dilution levels of four replicate growth units each.

Rhizobial cultures, inoculation, and plate counts

Rhizobial strains (Table 2) were grown for 6-10 days in either yeast-extract mannitol broth (Vincent, 1970) or arabinose-gluconate medium (Sadowsky *et al.*, 1987). Cultures were serially diluted using either 4- or 10-fold dilution ratios as outlined in Somasegaran and Hoben (1985). The diluent contained the salts found in yeast-extract mannitol broth (Vincent, 1970) with 0.01 % Tween 80 (Fisher Scientific Co.) added as a surfactant.

Seedlings were inoculated 7-12 days after planting, when root radicles had reached the bottom of the tubes or had begun to differentiate into secondary roots in pouches. Diluted rhizobial culture (1 ml) was applied directly to roots in pouches and to the lower portion of roots in tubes. An average of seven uninoculated growth units were included per MPN assay as checks for contamination within the system. Uninoculated controls received 1 ml of rhizobia-free diluent.

Plate counts were conducted at the time of inoculation using the Miles and Misra drop plate method (Somasegaran and Hoben, 1985). At least four replicate 30 pl aliquots were used from each of three dilution levels from the inoculation dilution series. Prior to data collection, plates were kept at 27°C for 7-10 days for strains of *Bradyrhizobium* Jordan, and for 3-5 days for strains of *Rhizobium* Frank.

Table 2. Rhizobial strains and host species used in the MPN assays

Host species in MPN assay	Strain	Strain synonym	Rhizobial genus[a]	Original host
Acacia auriculiformis	TAL 569	MAR 472	*Bradyrhizobium*	*Desmodium uncinatum*
Acacia auriculiformis	TAL 651	UMKL 44	*Bradyrhizobium*	*Calopogonium mucunoides*
Acacia auriculiformis	TAL 1446		*Bradyrhizobium*	*Acacia auriculiformis*
Acacia mangium	TAL 1867	LB 5	*Bradyrhizobium*	*Acacia mangium*
Acacia mearnsii	TAL 940	Num 777	*Bradyrhizobium*	*Acacia mearnsii*
Acacia mearnsii	TAL 941	Num 778	*Bradyrhizobium*	*Acacia mearnsii*
Acacia mearnsii	TAL 1388		*Bradyrhizobium*	*Acacia mearnsii*
Albizia lebbeck	TAL 1536		*Bradyrhizobium*	*Albizia lebbeck*
Albizia saman	TAL 833	UMKL 27	*Bradyrhizobium*	*Albizia saman*
Calliandra calothyrsus	TAL 1455		*Bradyrhizobium*	*Calliandra surinamensis*
Flemingia macrophylla	TAL 1883	Nit 52A1	*Bradyrhizobium*	*F. macrophylla*
Gliricidia sepium	TAL 1806	BR 8801	*Rhizobium*	*G. sepium*
G. sepium	TAL 1145	CIAT 1967	*Rhizobium*	*L. leucocephala*
L. leucocephala	TAL 1145	CIAT 1967	*Rhizobium*	*L. leucocephala*
Paraserianthes falcataria	TAL 45		*Bradyrhizobium*	*P. falcataria*
Robinia pseudoacacia	TAL 1889	USDA 3436	*Rhizobium*	*R. pseudoacacia*
Sesbania grandiflora	TAL 1114	IC 71	*Rhizobium*	*Sesbania* sp.
S. grandiflora	TAL 1119	IC 91	*Rhizobium*	*Sesbania* sp.
S. sesban	TAL 674		*Rhizobium*	*S. rostrata*
S. sesban	TAL 1042	Nit 145B1	*Rhizobium*	*S. longifolia*

[a]Determined by IPTG XGal assay (Sambrook *et al.*, 1989) in conjunction with growth on sucrose and lactose.

MPN determinations

With two exceptions scored at 4 weeks, growth units of MPN assays were scored for nodulation 5-7 weeks after inoculation. The MPN was determined using a computer program (Woomer *et al.*, 1990), beginning with the lowest dilution step in which all growth units nodulated.

MPN assays in pouches and tubes containing *A. auriculiformis, Acacia mangium, A. mearnsii* and *R. pseudoacacia,* were scored weekly for nodulation from the second to the seventh week after inoculation. Weekly scores were also recorded for S. *grandiflora* and *L. diversifolia,* grown in pouches and tubes respectively.

RESULTS

Plate counts were within the corresponding 95% confidence interval of MPN estimates (Cochran, 1950) for 18 of 53 plate count-MPN comparisons (Table 3). In all but three plate count-MPN comparisons the MPN was lower than the plate count.

Rhizobial density of the original solutions ranged from 3.67 x 10' to 7.55 x 10^9 rhizobia ml^{-1} as measured by the plate count method. In every MPN assay, nodules were found in only 5 of 384 uninoculated control growth units, with never more than one nodulated control unit per assay.

Plate count: MPN estimate (PC: MPN) ratios < 35 were obtained in either tubes or pouches for 11 of the 14 tree species (Table 3). In tubes, all species with seed weights < 25 mg $seed^{-1}$ *(A. auriculiformis, Acacia mangium, A. mearnsii, L. diversifolia, P. falcataria* and *R. pseudoacacia)* had average PC: MPN ratios < 26. In pouches, of six species with seed weight > 25 mg $seed^{-1}$ (S. *grandiora, A. lebbeck, Albizia saman, C. calothyrsus, G. sepium* and *L. leucocephala),* all but two (A. *lebbeck* and *Albizia saman)* had PC: MPN ratios < 35. Of the seven species grown in tubes and pouches, five (A. *auriculiformis, Acacia mangium, A. mearnsii, P. falcataria* and S. *grandi flora)* had PC: MPN ratios > 200 in one of the two growth systems.

Five of 10 MPN estimates from nodulation scores taken weekly from 2 to 7 weeks after inoculation increased after the fourth week (Fig. 1). No nodules were formed in any of the 48 uninoculated control growth units used in these assays. For species grown in tubes and pouches, the average number of weeks to arrive at the final MPN estimate was 6.0 in tubes compared with 4.8 in pouches. In tubes, the MPN estimates of *A. auriculiformis* and *A. mearnsii* increased during the interval from 6 to 7 weeks after inoculation. In pouches, the MPN estimate of only one species, *Acacia mangium,* increased in the interval from 5 to 6 weeks and no species after 6 weeks.

DISCUSSION

Comparing MPN estimates with plate counts of pure rhizobial cultures indicates the degree of compliance of the MPN assay with its major underlying assumption, that the presence of a single rhizobium will result in nodule formation (Scott and Porter, 1986). The comparison provides an indication of a growth system's potential for accurate results using a particular species and set of growth conditions. This is the basis for recommending that pure culture comparisons of MPN estimates with plate counts accompany MPN assays conducted in soil for each growth system-species combination used

Table 3. Comparison of MPN estimates using tree legumes as hosts to plate counts (PC) of viable rhizobia

Growth system and species	Seed weight	MPN assays performed	Plate count within 95% C.I. of MPN[a]	PC:MPN ratio				
				Mean	Standard deviation	Range		
Agar slants	— mg $seed^{-1}$ —	— No. —		— PC:MPN ratio —				
Acacia auriculiformis	24	3	0	25.7	29.2	5.0	—	67.0
Acacia mangium	10	4	0	16.0	6.4	5.0	—	21.0
Acacia mearnsii	17	4	3	2.0	0.9	0.6	—	2.2
L. diversifolia	18	4	2	1.6	1.6	0.3	—	4.2
P. falcataria	22	2	2	1.4	0.4	1.0	—	1.9
R. pseudoacacia	18	3	1	14.7	19.9	0.2	—	42.9
S. grandiflora	36	2	0	> 1428.9[b]	1252.7	176.2	—	> 2682
Growth pouches								
Acacia auriculiformis	24	3	0	8057.7	9305.3	746.4	—	21189.2
Acacia mangium	10	3	0	2370.3	2450.7	517.3	—	5833.3
Acacia mearnsii	17	3	0	234.8	300.2	5.5	—	658.9
L. diversifolia	18	2	2	1.3	0.4	1.0	—	1.7
P. falcataria	22	2	0	548.7	335.6	213.1	—	884.4
R. pseudoacacia	18	3	1	5.9	6.8	0.1	—	15.4
S. grandiflora	36	3	1	5.8	2.9	1.9	—	8.8
Albizia lebbeck	135	1	0	118.8			—	
Albizia saman	221	1	0	12823.5			—	
Calliandra calothyrsus	52	1	1	2.1			—	
F. macrophylla	17	1	0	865.1			—	
G. sepium	131	2	1	7.1	5.9	1.1	—	13.0
L. leucocephala	47	3	1	34.3	26.8	0.7	—	66.3
S. sesban	15	3	3	1.2	0.4	0.9	—	1.8

[a]C.I. of MPN = confidence interval of MPN calculated according to Cochran (1950).
[b]Indicates dilution range of MPN assay was exceeded by population.

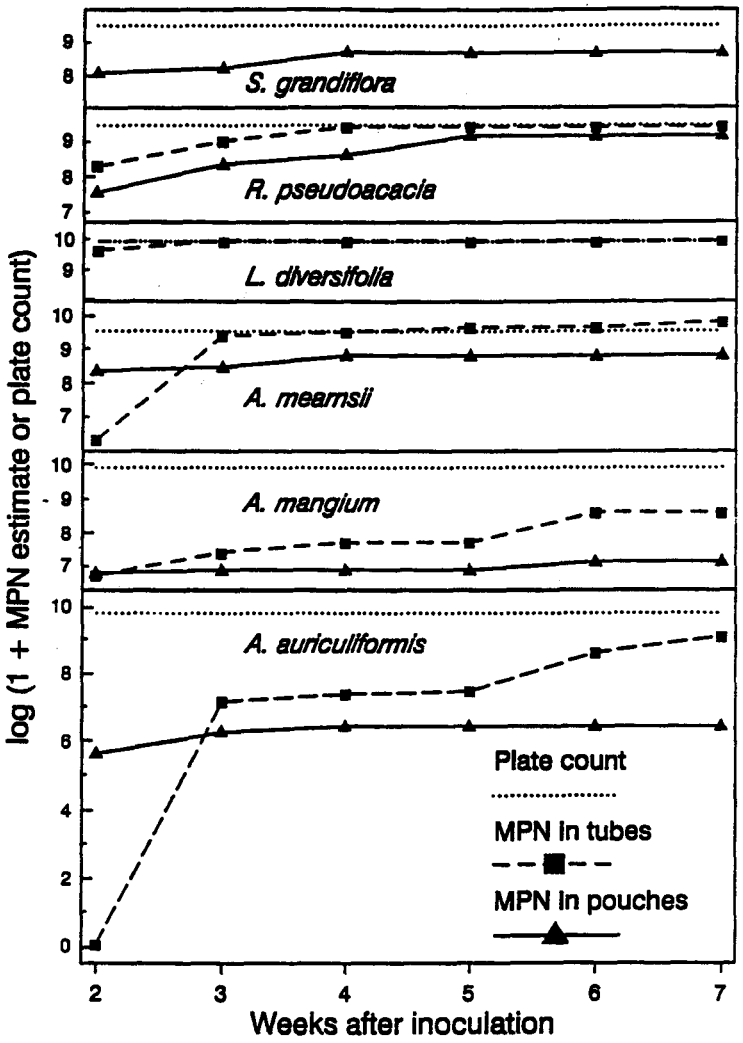

Fig. 1. Weekly assessment of MPN estimates.

(Thompson and Vincent 1967; Scott and Porter, 1986; Singleton et al., 1991).

MPN estimates obtained with a variety of grain and forage legumes indicate that the MPN assay tends to underestimate plate counts, suggesting that one rhizobium is generally not sufficient to cause nodule formation. PC: MPN ratios < 6 have been obtained with many herbaceous legumes including Trifolium spp (Brockwell, 1963; Tuzimura and Watanabe, 1961), Medicago sativa (Weaver and Frederick, 1972; Scott and Porter, 1986), Cicer arietinum (Toomsan et al., 1984) and Glycine spp (Weaver and Frederick, 1972; Brockwell et al., 1975). The discrepancy of < 1.5 log units between MPN estimates and plate counts obtained with A. mearnsii, *L.* diversifolia and P. falcataria in tubes and with *L.* diversifolia, C. calothyrsus and S. sesban in pouches, indicates that reasonable agreement between MPN estimates and plate counts can also be obtained with a variety of tree legumes.

Our results with trees agreed with the recommendation of Vincent (1970) that agar slants are suitable for legumes with seed weight less than that of "vetch", *ca* 25 mg seed^{-1}. Growth pouches were suitable for most larger-seeded tree species, but not for A. lebbeck and Albizia saman. Other growth systems, such as Leonard jars, should be evaluated for these species.

Growth pouches were not suitable growth systems for seven of the 14 tree species and tubes not suitable for S. grandiflora. These combinations had PC: MPN ratios > 100, indicating that over 100 rhizobia were required for nodule formation in these particular systems. Other authors have reported large discrepancies between plate counts and MPN estimates, notably Boonkerd and Weaver (1982), who reported PC: MPN ratios in pouches > 250 for Vigna unguiculata and Macroptilium atropurpureum. Boonkerd and Weaver (1982) suggested that the large ratios obtained were due to the inherent inability of these legumes to nodulate with a single rhizobial cell.

However, large PC: MPN ratios from species grown in only one of the two growth systems used in these experiments imply that problems with nodulation are growth-system related. The range of PC: MPN ratios observed emphasizes the need to evaluate growth system-species combinations prior to performing MPN assays for experimental purposes.

Woomer *et al.* (1988b) noted the importance of placing the diluted inoculant directly on the roots of the plants to get good nodulation. They noticed that the roots of *M. atropurpureum* tended to accumulate at the bottom of the glass tube without growing extensively on the surface of the slanted agar where the inoculant was applied and found that estimating rhizobial populations with this species was less precise than for species with greater root proliferation. We observed that roots of the *Acacia spp,* in particular, did not grow extensively on the agar surface, with most nodulation occurring at the bottom of the tubes. The quantity of agar we used and the angle of the slant, were, therefore, designed to channel both roots and rhizobia directly to the bottom of the tube to ensure maximal contact between them. We found that 15 ml of agar was sufficient to support growth of tree seedlings for the duration of the assays.

Weekly assessment of MPN estimates (Fig.1) indicated the importance of keeping tubes for at least 7 weeks and pouches for at least 5 weeks after inoculation. Delayed nodule formation in tubes relative to pouches appeared to be related to longer retention of photosynthetic capability: seedlings in tubes remained healthy longer than plants in pouches, despite being smaller. These times for scoring nodulated growth units are longer than recommended for other legumes. For grain and forage legumes, 2-4 weeks have been recommended as adequate by Vincent (1970), Brockwell (1980) and Boonkerd and Weaver (1982). Reports of times to assessment of MPN assays with tree legumes (Table 1) indicate a range from 2 to 3 weeks to 11 weeks after inoculation, with nodulation in four of seven reports assessed at S 3 weeks. Our results suggest that scoring at < 5 weeks is likely to underestimate the true population density, at least for species other than *L. leucocephala*

In conclusion, the results from our experiments provide a basis for selecting appropriate growth systems for MPN assays with a range of tree species. They show that MPN assays can provide accurate estimates of rhizobial populations for tree legumes but point to the need for selecting growth systems that are tailored to the characteristics of the particular tree species being used. It is imperative that growth systems be tested for each tree species using pure cultures of rhizobia prior to conducting MPN assays with soils.

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