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Reliability of the Most-Probable-Number Technique for Enumerating Rhizobia in Tropical Soils†

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We used six rhizobium-legume systems to test the reliability of the most-probable-number (MPN) technique for enumerating rhizobia introduced into 14 sites representing four soil orders. The range-of-transition values (the number of dilution steps between the first not-entirely-positive and the last not-entirely-negative growth units) were compared for each species and for each soil. The probability that the observed data were significantly different from theoretical values varied with the species. The acceptability of MPN codes (P > 0.99) was the highest (97 to 99%) with *Vicia saliva, Trifolium repens,* and Glycine near and lowest (72%) with *Leucaena leucocephala. Medicago saliva* and *Macroptilium atropurpureum* yielded 87 and 75% acceptable MPN codes, respectively. The acceptability of the MPN data obtained for a host species was related to rooting habit and time to nodulation. Comparison of data for each soil indicated that, despite large differences in characteristics, the soil was not a major source of variability in the MPN counts. There was no significant interaction of the range of transition of rhizobium-legume plant infection count data between species and site.

The most-probable-number (MPN) technique has been used to estimate microbial populations in natural samples. It is based on probability theory and relies on determination of the presence or absence of the organism of interest in several consecutive dilutions of the sample being tested. For enumeration of rhizobia, the appropriate host plant serves as a selective agent. The sum of nodulated growth units (5), or the pattern of nodulated and nonnodulated units (1), is then used to derive a population estimate, the MPN. Two primary assumptions underlying this technique are that the organisms are randomly distributed and that one or more rhizobia are capable of producing a root nodule (3). Failure to meet these assumptions results in extended range-of-transition (ROT; the number of dilution steps between the first notentirely-positive to the last not-entirely-negative dilution) values. Thus, the smaller the ROT, the higher the probability that the data are not significantly different from theoretical values. Stevens (14) described ROT analysis in detail and produced tables of predicted frequency distributions, as well as exp ected means and variances for ROT as a function of both replication number and dilution ratio. He cautioned that these tests should not be applied to few observations of MPN data until a bulk of data has been compiled with which a single code may be compared.

Additional tables for the ROT were presented by Scott and Porter (9), who applied the technique specifically to legume infection counts. They found that the observed mean ROT values differed significantly from those predicted by Stevens (14) when *Medicago saliva* and *Trifolium pratense* were used as test hosts to enumerate rhizobia in peat inoculants. Mean ROT values that did not differ significantly from the theoretical values were described as having internal cohesion. Their study indicates that many factors (i.e., legume cross-inoculation groups, growth systems, and source of inoculum) may affect the reliability of the MPN technique.

In this study, we examined the interaction of six legume species and 14 sites representing four soil orders on the reliability of MPN estimates of introduced populations of rhizobia. The approach of Stevens (14) has general applicability in microbiology, for it establishes quantitative criteria for the validity and acceptability of MPN data.

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MATERIALS AND METHODS

Site selection and preparation. The 14 sites for this study were chosen from the Maui Soil, Climate, and Land Use Network (Table 1). Soil characterizations were conducted by the U.S. Soil Conservation Service (11, 12) and the University of Hawaii Department of Agronomy and Soil Science (6). At each site, an area (2 by 2 m) was cleared of vegetation and rocks and tilled to a 25-cm depth. Peat inoculant was prepared for each of 18 Rhizobium strains by injecting 35 ml of broth into bags containing 50 g of sterile peat. Inoculants were enumerated by the drop plate method (13) and applied at adjusted rates to produce a final soil population of 1.5×10^5 rhizobia g of soil^{-1} for each strain. The inoculants were incorporated into the soil with repeated passes of a tiller. The rhizobium species and strains used in this study are presented in Table 2.

Host legume preparation. The following legumes were grown in a growth room: Glycine max (L.) Merr. cv. Lee, Macroptilium atropurpureum (D.C.) Urb. cv. Siratro, T. repens L. cv. Regal Ladino, Medicago saliva L. cv. Florida 77, Vicia saliva L., and Leucaena leucocephala (Lam.) deWit cv. K8. Seeds of G. max were surface sterilized in 2% NaOCI, repeatedly rinsed, and planted one per growth pouch (15). All other species were grown in sterile culture slants containing 1.5% agar and N-free plant nutrient solution (10). Seeds of *M.* atropurpureum and L. leucocephala required scarification in concentrated H_2S0_4 (5 and 25 min, respectively), followed by repeated rinses. V. saliva was surface sterilized with 2% NaOCI for 2 min, followed by repeated rinses. Seeds were pregerminated in sterile wateragar in petri dishes and planted in agar slants (25 by 250 mm) with plexiglass covers. one per tube.

Seeds of T. repens and *M.* sativa were shown to be rhizobium free by absence of root nodule formation in

TABLE 1. Classification and characteristics of the soils used in this study

Soil subgroup	Order	Organic carbon (%)	Clay (%)	pH
Cumulic Haplustoll	Mollisols	1.2	25.2	7.4
Torroxic Haplustolls[a]	Mollisols	2.0	38.2	7.1
Typic Haplustoll	Mollisols	2.4	51.8	6.8
Typic Eutrandept	Inceptisols	8.7	2.9	6.8
Typic Dystrandepts[b]	Inceptisol	12.8	3.7	6.2
Entic Dystrandept	Inceptisols	7.5	12.9	5.2
Humoxic Tropohumult	Ultisols	2.4	26.8	5.3
Humoxic Tropohumult	Ultisols	2.9	47.8	4.9
Typic Torrox	Oxisols	1.5	26.2	6.8

[a] Data for the Torroxic Haplustolls are the means of five sites.
[b] Data for the Typic Dystrandepts are the means of two sites; all other data are for single sites.

otherwise sterile plant culture. Seeds (6 to 10) were placed directly onto the tops of the agar slants with a small spatula. *M. saliva* was grown in tubes (25 by 200 mm) with plexiglass covers; T. *repens* was grown in capped tubes (16 by 100 mm). Plant units were grown at 28°C in 16-h day lengths under 1,000-W metal halide lamps that produced 350 microeinsteins m^{-2} s^{-1} at plant height. After 7 to 10 days, plants were selected for uniformity and inoculated.

Soil collection and inoculation. Soil samples were collected 0, 2, 8, 14, 27, 41, and 52 weeks after incorporation of the peat inoculant. Twenty-five subsamples were taken to a depth of 25 cm with a 25-mm-diameter soil core sampler and combined. The samples were mixed thoroughly, rocks and large roots were removed, and the samples were refrigerated overnight. A subsample was oven dried (110°C) to determine the moisture content. Soil was diluted on a dry-weight basis as described by Somasegaran and Hoben (13). Serial 10-fold

TABLE 2. Sources of rhizobium cultures and designations of rhizobia

NifTAL no.	Original designation[a]	Species (host legume)
102	USDA 110	*Bradyrhizobium japonicum*
377	USDA 138	*Bradyrhizobium japonicum*
379	USDA 1366 (CB 1809)	*Bradyrhizobium japonicum*
169	Nit 176 A22	*Bradyrhizobium* sp. (*Macroptilium* sp.)
209	TAL 209	*Bradyrhizobium* sp. (*Vigna* sp.)
658	CIAT 71	*Bradyrhizobium* sp. (*Macroptilium* sp.)
1826	S11-6	*Rhizobium leguminosarum* biovar *trifolii*
1827	S11-16	*Rhizobium leguminosarum* biovar *trifolii*
1828	AR21	*Rhizobium leguminosarum* biovar *trifolii*
380	SU47	*Rhizobium meliloti*
1372	POA 116	*Rhizobium meliloti*
1373	POA 135	*Rhizobium meliloti*
1397	Nit 175 F9	*Rhizobium leguminosarum* biovar *viceae*
1399	Nit 175 F12	*Rhizobium leguminosarum* biovar *viceae*
1400	Nit 175 F16	*Rhizobium leguminosarum* biovar *viceae*
82	TAL 82	*Rhizobium* sp. (*Leucaena* sp.)
582	CB 81	*Rhizobium* sp. (*Leucaena* sp.)
1145	CIAT 1967	*Rhizobium* sp. (*Leucaena* sp.)

[a] Laboratory sources were as follows: USDA, U.S. Department of Agriculture, Beltsville, Md.; Nit, Nitragin Co., Milwaukee, Wis.; TAL, NifTAL Project, University of Hawaii, Honolulu; CIAT, Cali, Columbia; S and AR, received from P. Bottomly, Oregon State University, Corvallis; SU, University of Sydney, Sydney, Australia; POA, Universidade Federal do Rio Grande do Sul, Pôrto Alegre, Brazil; CB, Commonwealth Scientific and Industrial Research Organisation, Queensland, Australia.

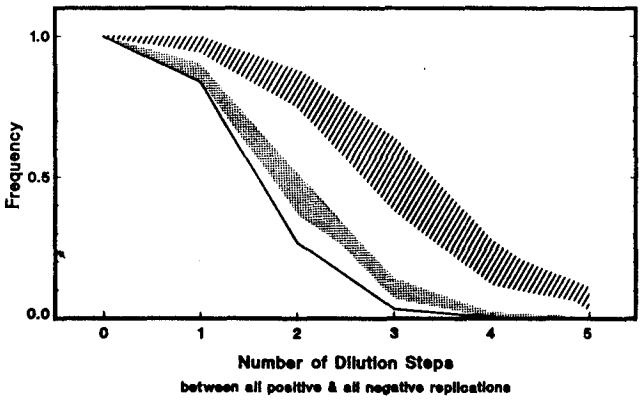

FIG. 1. Frequency of equaling or exceeding the ROT. ——, Predicted; ▩, *G. max*, *T. repens*, and *V. sativa*; ▨, *M. sativa*, *L. leucocephala*, and *M. atropurpureum*.

dilutions (10^{-3} to 10^{-7} at week 0 and 10^{-1} to 10^{-5} or 10^{-2} to 10^{-6} thereafter) were prepared, and 1 ml of the appropriate dilution was applied directly to the roots of the test legumes with a repeating pipette. There were four replications of each dilution.

Enumeration and analysis. Daily observations were made for root nodules, and MPN codes were recorded after 17 to 21 days. Population estimates were assigned by using a recently developed computer program (unpublished data). Chi-square analysis was conducted to compare the mean frequencies of the *ROT* with predicted values (14). ROT values were compared by host species and site by two-way analysis of variance. Since the ROT data consisted of counts ranging from 0 to 5, square root transformations served to normalize the data (8). Simple analysis of variance compared the transformed ROT values by soil order. Linear regression was conducted which compared the rhizobial population with transformed ROT values.

RESULTS

The ROT values for 569 MPN codes generated from this study were compiled by legume host, site, and soil order. The predicted frequency distribution for 10-fold dilutions replicated four times was compared with the observed frequencies in ROT for six legume species (Fig. 1). Chisquare analysis indicated that the legume species fall into two categories on the basis of their agreement with predicted values. T. *repens*, *V. saliva*, and C. *max* were not significantly different from the predicted values of Stevens (14). *M. saliva*, *L. leucocephala*, and M. *atropurpureum* all differed significantly from predicted values ($P < 0.01$).

The mean values of the ROT of the MPN code differed significantly among legume species ($P < 0.001$) but not among sites or soil orders (Table 3). The number of observations for each comparison and the percentages of unacceptable codes ($P > 0.99$) are also presented. Lower mean ROT values (greater internal cohesion of MPN codes) resulted in a decreased percentage of unacceptable codes.

The rhizobial populations ranged from 106 to not detectable over the course of 52 weeks. Many of the MPN estimates were too low for *ROT* values to be assigned. The regression between the rhizobial population of a sample and the *ROT* value was not significant ($r = 0.07$).

The order in which nodules were first observed after inoculation varied from 5 days for T. *repens* and V. *saliva* to

TABLE 3. Mean ROT and percentage of unacceptable codes as a function of host legume and soil order

Host[a] or soil order (no.)	Mean range[b]	% of unacceptable codes[c]
G. max (103)	1.18 (a)	1.9
T. repens (99)	1.27 (a)	1.0
V. sativa (81)	1.41 (a)	2.5
M. sativa (83)	2.09 (b)	13.2
M. atropurpureum (99)	2.58 (c)	25.2
L. leucocephala (104)	2.73 (c)	27.9
Mollisol (299)	1.73 (a)	10.0
Inceptisol (135)	1.94 (a)	12.6
Ultisol (103)	1.95 (a)	15.5
Oxisol (32)	2.01 (a)	9.4

[a] Plant infection data codes from 10-fold dilutions with four replications.
[b] Mean values and analysis of variance conducted with square root transformation ($\sqrt{x + 0.1}$), mean comparisons with Duncan multiple-range analysis ($P = 0.01$ for host; $P = 0.05$ for soil order). Predicted mean value, 1.15 (14). Values followed by the same letter do not differ significantly.
[c] Indicates percent failure of technique ($P > 0.99$).

7 days for G. max and *M.* sativa to 10 or more days for *L.* leucocephala and *M.* atropurpureum.

DISCUSSION

Extended ROT values in plant infection count data indicate failure to satisfy the principal assumptions of the MPN procedure (14). It is difficult to identify which assumption has not been satisfied, although some speculations may be made. For example, a code of 434310, which results from a 10-fold dilution series of four replications, has a ROT value of 4 and a probability of 0.0036 that the underlying assumptions have been satisfied. The failure of one replicate of the second dilution to nodulate is probably a failure of the rhizobia applied to produce nodules. On the other hand, a code of 430010 produced under the same circumstances is probably a failure to disperse the rhizobia during dilution or the result of contamination. Further, it is logically difcult to reject the former code and accept the latter, despite the identical probabilities of observing these extended ROT values. Examples of MPN data from this study, their ROT, and their predicted frequency distribution are given in Table 4. Note that ROT values of 4 and 5 may not have been taken to dilution extinction because the infection count procedure in this experiment consisted of five serial dilutions.

Effect of host. In the legume infection technique, the host acts as an extremely selective agent for the rhizobia. Infectable root surfaces must be placed in close proximity to a relatively small volume of inoculum. Extremely uniform, infectable host legumes are an important prerequisite to meeting the assumption of the MPN technique that one or more rhizobia are capable of producing nodulation. Growth pouches and agar slants allow for direct observation of the location of actively growing roots. These observations may be used in two ways to protect against experimental failure due to faulty technique: (i) in selecting for uniformity of plant material and (ii) by placing the inoculum directly onto infectable root surfaces.

Although infection mechanisms may vary (4), rhizobia must come into contact with root surfaces to initiate nodulation. Growth pouches offer an advantage in this respect, since all roots which develop in these pouches are on the surface of the rooting medium and available to rhizobia for infection. Disadvantages of this growth system include the need for frequent maintenance, the large size of growth pouch racks, and difficulties in excluding contamination.

Culture of legumes for plant infection counts in N-free agar slants overcomes many of these disadvantages. Slants need not be watered, tube size may be adjusted to plant size, tubes may be placed in conventional culture tube racks, and tubes may be stoppered to prevent contamination. A serious disadvantage of agar slant culture is that roots of some species penetrate the surface of the agar slant, placing some fraction of the root system beyond immediate contact with the inoculum.

The root systems of T. repens, V. sativa, and *M.* sativa tend to remain on the surface of the agar slant or reemerge from beneath the plane of the slant. The strong tap root of *L.* leucocephala tends to grow beneath the surface of the agar. The rooting habit of M. atropurpureum is intermediate between the two. The result of this disadvantage is that many more *L.* leucocephala tube units than needed must be planted and units with nonuniform roots must be removed.

Delayed time to nodulation, combined with rapid root growth, presents a problem with *M.* atropurpureum. Whereas some roots follow the surface of the agar to the bottom of the slant and the inoculum may be applied to these roots, the roots are quickly buried beneath the base of the slant and are less infectable. Generally, speed to nodulation resulted in greater internal cohesion in the MPN code in that species which nodulate within 5 to 7 days (V. saliva, G. max, and T. repens) had lower mean ROT values than did those which require more than 7 days to nodulate.

Regardless of the particular failure of technique, the species used in this study differed widely in the internal cohesion of their MPN data. These findings provide the bulk of data which Stevens (14) required before single or few codes could be subjected to ROT analysis.

After repeated plant infection counts, the ROT values in the MPN codes of G. max, T. repens, and V. saliva agreed with the predicted values. This was further demonstrated in two-way analysis of variance (host species x soil order), which demonstrated that these three species were not significantly different from one another. In comparison, unacceptable codes ($P > 0.99$) for M. sativa, M. atropurpureum, and L. leucocephala constituted 13, 25, and 28% of the observations, respectively. This indicated that at least one of the principal assumptions in MPN was not met.

It should be noted that G. max was the only legume species in this study that was grown in growth pouches and that comparison of G. max with other legume species is confounded by differences between the growth systems. G. max is a large-seeded, fast-growing legume which is not suited to culture in 25-mm-diameter agar slants.

Effect of soil. When the 14 sites were grouped by soil

TABLE 4. Examples of MPN codes, the ROT of the codes, and the probability to equal or exceed the ROT

MPN code	Range of transition[a]	Probability[b]
44000	0	1.000
44200	1	0.838
43100	2	0.271
43310	3	0.035
43301	4	0.0036
34302	5	0.00036

[a] Five serial 10-fold dilutions were replicated four times.
[b] Described by Stevens (14).

order, there was no significant difference among the mean ROT values of these diverse soils (Table 3). In contrast, researchers using immunofluorescence techniques have found that there are significant differences among tropical soils in release of rhizobia recovered by filtration (7). The plant infection procedure allows for direct recovery by the -host legume root system. Masking of the rhizobia through adsorption by soil particles interferes with immunofluorescence enumeration. That recovery of rhizobia through dilution extinction appears to be less sensitive to soil characteristics is encouraging but should not be extrapolated to all soils. The clay fractions of the soils in this study were kaolinitic, oxidic, and amorphous but did not include montmorillanite, which is known to interact with rhizobia in soils (2).

The high percentage of unacceptable MPN data for all of the soils (Table 3) was due to the high incidence of unacceptable codes for certain plant species that were inoculated with all of the soils. The effects of site, soil order, and site-species interaction were not significant.

In conclusion, we were able to test the internal cohesion of legume MPN counts of rhizobia by using large numbers of samples to satisfy the Stevens (14) requirements. With two-way analysis of variance, significant differences among species were observed. With chi-square analysis, significant differences were observed between some species and the predicted values of the ROT of MPN data. Despite large differences in soil characteristics, there was no effect on the ROT of MPN codes. Researchers using the MPN technique should verify that the host species and growth system used result in reliable code data. Furthermore, researchers should be aware that there are statistical methods which allow for testing of experimental technique and, when possible, collect the data which allow for these tests to be used.

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