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COWPEA RHIZOBIA: COMPARISON OF PLANT INFECTION AND PLATE COUNTS

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Summary—Enumeration of *Rhizobium* in soil is usually accomplished by the most probable number (MPN) plant infection method. The accuracy of MPN counts as compared to plate counts was determined for five strains of cowpea rhizobia. Host plants included cowpea (*Vigna unguiculata* (L.) Walp.), siratro (*Macroptilium atropurpureum* (DC.) Urb.) and peanut (*Arachis hypogaea* L.). Plastic growth pouches were used primarily for cowpea and siratro while plastic cups containing vermiculite were used for peanut. The number of rhizobia determined by the MPN method using cowpea, siratro and peanut underestimated the population from 10- to 100-fold. A control experiment using soybeans (*Glycine max* (L.) Merrill) indicated that the MPN method was accurate for *R. japonicum*. Experimentators using the MPN method should be aware of its accuracy for particular *Rhizobium*-legume combinations.

INTRODUCTION

The plant-infection dilution assay (MPN) is the most frequently used technique to enumerate rhizobia in soil and inoculants when other microorganisms are present. It has proven accurate for counting *Rhizobium japonicum*, *R. meliloti*, *R. trifolii* and *R. leguminosarum* (Date and Vincent, 1962; Brockwell, 1963; Weaver and Frederick, 1972) but the accuracy of the MPN plant-infection method for enumerating cowpea rhizobia has not been established. Date and Vincent (1962) presented evidence, using one cowpea strain of rhizobia, that the method is accurate but Wilson and Trang (1980) reported that the population of cowpea rhizobia in inoculants was grossly underestimated. Wilson and Trang (1980) speculated that a decline in cell vigor during inoculant storage may have caused the underestimation.

While determining survival of cowpea rhizobia in soil, it became apparent to us that populations were underestimated by the MPN plant-infection method. Several other investigators (Herridge and Roughley, 1974; Day *et al.*, 1978; Philpotts, 1977) have used the MPN method to enumerate cowpea rhizobia but they did not report on its accuracy. Our purpose is to present evidence that the MPN plant-infection method is not reliable for cowpea rhizobia and that different indicator plants provided similar results.

MATERIALS AND METHODS

Five strains of cowpea rhizobia were used. Strains Thai-201 and T-1 were isolated by us from field grown peanuts. The other three strains; TAL309, CB756 and 3281 (316a15) were obtained from the NifTal culture collection (Paia, Hawaii). The Nitragin Company (Milwaukee, Wisconsin) and the USDA *Rhizobium* culture collection (Beltsville, Maryland) respectively and were known to be effective on cowpeas. *R. japonicum* strain 110 was obtained from the USDA *Rhizobium* culture collection. Six day-old cultures grown in yeast mannitol broth were used for inoculations.

Different plant hosts were used and included; six varieties of cowpea (*Vigna unguiculata* (L.) Walp.), "Black eye", "Bush", "Calico", "Brawder", "Queen Anne" and "Pink eye", siratro (*Macroptilium atropurpureum* (DC.) Urb.), peanuts (*Arachis hypogaea* L.) var. "Tamnut 74" and soybeans (*Glycine max* (L.) Merrill) var. "Coker 338".

Plastic pouches (Weaver and Frederick, 1972) were primarily used for cowpea, siratro and soybeans. Agar (Vincent, 1970) and vermiculite in small bottles were also compared with plastic pouches. Plastic drinking cups (250 ml) filled with vermiculite were used for growing peanuts. Seed were germinated and only healthy seedlings planted.

Plate counts of broth cultures were made within 2 h of inoculating plants. Duplicate plates of yeast-extract mannitol agar (Vincent, 1970) were used for plate counts. The diluent for MPN plant-infection counts was N-depleted plant nutrient solution (Munns, 1979) which was also used for watering plants. Ten-fold serial dilutions and four replications per dilution were used for MPN counts. Plants were maintained for at least 3 weeks before considering them as negative (nonnodulating).

The plants were grown in a light room equipped with fluorescent lamps which provided a photon flux density 650 $\mu E\ m^{-2}\ s^{-1}$ at the top of the plants. The temperature of the room was maintained at 29°C during 14 h of light and at 21°C during 10 h darkness.

RESULTS AND DISCUSSION

The populations of cowpea rhizobia were measured by MPN plant-infection and plate counts using different strains of cowpea rhizobia and different indicator plants. Using cowpea and siratro as in-

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Table 1. Log_{10} of rhizobia measured by MPN and plate counts using different strains of *Rhizobium* and legumes

Strains	Plate count[a]	MPN[b]-plant infection Soybeans	Cowpea	Siratro
110	8.69	7.90	ND[c]	6.48
110	7.00	6.49	ND	ND
201	8.88	ND	6.46	6.81
309	8.54	ND	6.05	6.64
T-1	8.78	ND	6.49	6.97
3281	8.93	ND	6.49	6.00

[a] Average SD ± 0.03.
[b] Factor for 95% fiducial limits ± 0.58.
[c] ND—Not determined.

Table 3. Comparison of MPN and plate counts using different strains of *Rhizobium* and varieties of cowpeas

Strains	Cowpea varieties	Plate[a] log_{10} No/l	MPN[b] log_{10} No/l
201	Black Eye	8.75	6.49
201	Queen Anne	8.75	6.76
3281	Black Eye	8.95	7.23
3281	Queen Anne	8.95	6.76
3281	Bush	8.95	7.23
3281	Calico	8.95	6.49
309	Black Eye	8.47	7.23
309	Queen Anne	8.47	6.49
309	Bush	8.47	6.76
309	Calico	8.47	6.49
309	Brawder	8.47	7.23
309	Pink eye	8.47	6.76

[a] Average SD ± 0.02.
[b] Factor for 95% fiducial limits ± 0.58.

dicator plants resulted in MPN counts approximately 2 log_{10} units lower than plate counts for all rhizobial strains (Table 1). *R. japonicum* strain 110 and soybean were included as a control and indicated close agreement between counting methods. When siratro was used for the MPN-count of strain 110, the number obtained was 2 log_{10} units lower than the plate count.

Use of plastic pouches was not the cause for the low counts because the results of another experiment in which vermiculite and agar were used as the support media provided similar results (Table 2). The MPN values for T-1 using the different plant support media were similar but for CB756, the MPN obtained from using agar was 1 log_{10} unit higher than for vermiculite and plastic pouches but was 1 log_{10} lower than plate counts.

To be certain the low MPN counts were not due to the particular variety of cowpea, five additional varieties were used as indicator plants. The results indicated the MPN's obtained from using different varieties were not significantly different and populations of all rhizobial strains were underestimated (Table 3).

Peanut was used as a third plant nodulated by cowpea rhizobia. Peanuts were grown for MPN counts in 250 ml cups containing vermiculite because healthy root systems do not always develop when peanuts are grown in plastic pouches. Plate and MPN counts of strains T-1 and 201 were made. The plate counts were respectively 7.66 and 7.43 and the MPN's were respectively 6.49 and 5.49 log_{10} units. Both strains were highly effective on peanuts (Weaver, 1974; Sen and Weaver, 1981). Brockwell (1963) reported that when plants were grown in vermiculite, the MPN underestimated the population of *R. trifolii* by approximately 40%.

The MPN method using plant infection to enumerate cowpea rhizobia was found to be inaccurate. The results indicate the basic assumption, implicit in the use of the MPN plant-infection method, that a single *Rhizobium* cell can multiply and result in nodule formation on cowpea and siratro is invalid. Wilson and Trang (1980) using the MPN plant-infection method to enumerate cowpea rhizobia in inoculants also reported that the MPN count was lower than the plate count. They speculated that the lower MPN might be due to loss of infectiveness of some cells during storage or to poor survival in the plant nutrient solution after inoculation of seedlings. We used more than 100 single colony isolates from plates to inoculate cowpeas and without exception all formed nodules on the plant indicating no loss of infectiveness. The rhizobia not only were capable of surviving in the plant nutrient solution but the population increased with time (Table 4).

We found that prolonging the time for growing plants from 3 to 6 weeks after inoculation did not increase the accuracy of the method because the roots of plants grown in plastic pouches and in small containers do not remain vigorous and healthy beyond 4 weeks. Inoculation of seedlings of peanuts, cowpeas or siratro with large populations of rhizobia resulted in nodulation within 7–10 days. From these considerations it appears that 3 weeks is a reasonable period to expect responsive plants to nodulate.

The results obtained for rhizobial strains used in our investigations may not be representative for other strains. Nevertheless, since the method was inaccurate for some strains, investigators should be cautious about using it to enumerate cowpea rhizobia. When it

Table 2. Log_{10} number of rhizobia measured by MPN plant-infection using siratro grown in different media as compared to plate counts

Strains	Plate count[a]	MPN[b]-plant infection Vermiculite	Pouches	Agar
T-1	8.27	6.76	6.76	7.00
CB756	7.57	5.23	5.49	6.49

[a] Average SD ± 0.02.
[b] Factor for 95% fiducial limits ± 0.58.

Table 4. Growth of cowpea rhizobial strain T-1 in sterile nutrient solution and 0.1% peptone

Days	Nutrient solution (cells ml⁻¹)	Peptone (cells ml⁻¹)
0	70	70
1	71	93
2	200	257
3	732	1200

is used, evidence should be provided that it is accurate for the particular rhizobial strains being used. Future research needs to be undertaken to determine why the population estimates of cowpea rhizobia using the MPN plant-infection method were so low.

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