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COUNTING *CICER–RHIZOBIUM* USING A PLANT INFECTION TECHNIQUE

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Summary—Chickpea (*Cicer arietinum* L.), a large seeded pulse legume, is nodulated only by a very specific group of *Rhizobium* strains which do not nodulate plants other than *Cicer* spp. If the cotyledons are removed just after germination, subsequent seedling growth is dwarfed, and plants will reliably grow and nodulate in large test tubes (25 × 200 mm) under axenic conditions with either sand or sand + vermiculite as the root medium. The dwarfed seedlings of some selected chickpea lines can be used as a "trap host" for counting *Cicer–Rhizobium* in pure cultures and contaminated materials by a most probable number, serial dilution–plant infection technique. The value of such a plantlet as a "trap host" for studying *Cicer–Rhizobium* ecology, strain authentication and inoculum quality control is demonstrated.

INTRODUCTION

One of the causes of poor nodulation of chickpea (*Cicer arietinum* L.) in farmers' fields is the paucity in the soil of the very specific *Rhizobium* strains that nodulate chickpea. Although media have been developed which reduce the growth of many soil organisms other than *Rhizobium* (Graham, 1969; Pattison and Skinner, 1974), there are no truly selective media for counting rhizobia directly except when antibiotic resistant strains are used (Bushby, 1981), hence indirect most probable number methods need to be resorted to.

Wilson (1926) developed a method for estimating the *Rhizobium* population in a sample based on the nodulation pattern of plants grown in otherwise sterile conditions and inoculated by serial dilutions of the sample. Such a method has been widely used and modified (see Brockwell, 1980). Small-seeded species usually grow and nodulate well in test tubes with root media of agar, sand or vermiculite. For rhizobia nodulating large seeded legumes, the counts are usually made using a small seeded legume which nodulates readily with the same *Rhizobium* strains e.g. siratro (*Macroptylium atropurpureum* L.) for counting the cowpea group of *Rhizobium*, *Glycine ussuriensis* Regel and Maack (*Glycine soja* Sieb. and Zucc.) for counting populations of *Rhizobium japonicum* (Brockwell *et al.*, 1975). Rhizobia for soybean can also be counted by an MPN method using soybean grown in growth pouches (Weaver and Frederick, 1972). Alternatively, Leonard jar assemblies (Vincent, 1970) can be used for growing the plants but these are cumbersome, and require a large amount of labour to set up and maintain.

Cicer–Rhizobium are very specific for *Cicer* spp, and although some strains have been reported to nodulate *Sesbania* ineffectively (Gaur and Sen, 1979), *Rhizobium* normally nodulating *Sesbania* spp do not nodulate chickpea. Chickpeas are large seeded and no suitable alternative host exists for the plant infection (MPN) method of counting rhizobia for *Cicer*. Chickpeas nodulated sporadically when grown in test tubes and inconsistently in growth pouches where we had difficulty preventing contamination. We report here a method for counting *Cicer–Rhizobium* using chickpea plants which are dwarfed by excising their cotyledons.

MATERIALS AND METHODS

Culture of chickpea in test tubes

Seeds of cv. K-850 were surface sterilized with 0.2% $HgCl_2$ for 3 min and then washed thoroughly 8–10 times with sterilized tapwater. The seeds were then placed on sterilized 1.2% plain agar in Petri plates (9 cm dia), 30 seeds per plate and kept for about 3 days at 28°C until the emergent radicle was 1–2 cm long. The cotyledons of the germinating seedlings were then aseptically excised using a scalpel and forceps, and one seedling transferred into each 2.5 cm dia × 20 cm test tube containing either 30 ml washed coarse sand, a 1:1 or 2:1 (v/v) mixture of sand and vermiculite moistened with 9 ml of N-free nutrient solution, pH 6.8 (Summerfield *et al.*, 1977). Before inoculation, the test tube sown seedlings were placed in plant growth units for 3–4 days to ensure their establishment. Over 90% of the seedlings usually established as indicated by their growth and were selected for use. After inoculation plants were brought back to the same plant growth units where ambient temperature was 19 ± 2°C. The temperature inside the plant tubes was usually 5–7°C higher than the ambient temperature due to the proximity of plant tubes to the cool daylight fluorescent tubes

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Table 1. Effect of cotyledon removal on the estimation of *Cicer–Rhizobium* numbers (strain IC-2046) in broth culture counted by plant infection dilution (MPN) method

Treatments	Plate count ($\log_{10}$ ml^{-1} broth)	Proportion of plants nodulating	MPN count ($\log_{10}$ ml^{-1} broth)[a]
Whole seed	9.55	6/36	5.24
1 cotyledon removed	9.58	18/36	7.24
$1\frac{1}{2}$ cotyledons removed	9.53	30/36	9.26
Both cotyledons removed	9.48	31/36	9.43
95% fiducial limits			±0.47

[a]Six dilution steps (10^{-5}–10^{-10}) and 6 replicate tubes per dilution were used for these plant counts.

which were used as source of light (Rupela *et al.*, 1984). The plant tube temperature was always kept below 30°C.

Inoculation procedure

Rhizobium cultures were grown in yeast extract-mannitol broth (Vincent, 1970) on a rotary shaker. 2-, 4- or 10-fold dilution series were prepared with sterilized tapwater. Dilution series need to be made to the level where no rhizobia remain. Plants were inoculated with 1 ml of the last six dilutions in the series. The number of replicate tubes per dilution varied from 3 to 12 depending on the experiment. Uninoculated controls were also included. The plants were watered 3–4 weeks after inoculation by adding 3–4 ml of sterilized $\frac{1}{4}$ strength N-free nutrient solution per tube, and harvested at 6 weeks except when harvesting time was at test (Table 3).

The recovery of chickpea rhizobia from unsterilized soil was tested as follows: 5-day old broth cultures of *Rhizobium* strains IC-2091 and IC-59 were used to inoculate two Vertisol soils (1 ml per 100 gram soil) which were then thoroughly mixed in a plastic bag. These broths were counted by direct plating and by the plant infection method. Four hours after inoculation both the inoculated and uninoculated soils were counted by the dilution plant infection method. Twenty g of soil was added to 180 ml water and shaken by wrist action shaker for 15 min. This was considered the 10^{-1} dilution. A 10-fold dilution series was made and 1 ml of the last six dilutions in the series used to inoculate 3 replicate plant tubes.

For counting rhizobia in peat inocula, 10 g of peat was added to a 90 ml water and shaken for 15 min. This was considered the 10^{-1} dilution. The MPN were determined from a 10-fold dilution series as before.

Plate counts

Three successive dilutions were counted by the spread plate method with 0.1 ml aliquots and 3 replicate plates of Congo Red, Mannitol Agar (Brockwell, 1980). The plates were incubated at 28°C for 5–8 days.

Calculation of the most probable number

Plants were harvested at 6 weeks after inoculation and scored for presence or absence of nodules. The total number of positive and negative tubes were then used to calculate the estimates and fiducial limits for the number of *Rhizobium* (Fisher and Yates, 1963).

RESULTS AND DISCUSSION

Cotyledon removal, plant growth and nodulation

It is generally believed that cotyledons supply factors that are necessary for nodulation of seedlings, but that these substances are transferred to the radicle soon after germination (Dart, 1975). Apparently, the three days after seed imbibition before we excised the cotyledons were sufficient for transfer of such substances in chickpea. Removing parts of the cotyledons reduced plant growth and it took about 6 weeks for seedlings with both cotyledons removed to reach the test tube plug.

Table 1 shows the effect of removing different proportions of the cotyledons on the MPN count of rhizobia. As more of the cotyledons were removed, the accuracy of the plant infection count increased. The whole seed and removal of one cotyledon gave significantly lower counts than the plate counts but the removal of one and a half or both cotyledons resulted in close agreement of the MPN of the plant method and plate count. We do not know why chickpea seedlings with cotyledons intact nodulate poorly in test tubes.

The number of nodules per dwarfed chickpea plant grown in a test tube ranged from 1 to 7. This technique is not useful for testing the effectiveness of *Rhizobium* strains in fixing nitrogen. However, it is very useful for *Rhizobium* strain authentication as demonstrated by the capability of forming nodules.

Rooting medium, harvest date and plant cultivar

A preliminary experiment showed that agar, and unwashed sand and vermiculite were not suitable root media. Washed sand either alone or mixed with washed vermiculite gave good plant growth and reliable MPN counts (Table 2). Washed sand gave

Table 2. Effect of root medium on the estimation of *Cicer–Rhizobium* numbers in broth cultures (strain IC-128 and IC-2046) by plant infection dilution (MPN) method

	$\log_{10}$ *Rhizobium* ml^{-1} broth			
	IC-128		IC-2046	
	Plate count	MPN count[a]	Plate count	MPN count[a]
Washed sand	9.68	9.43	9.44	9.63
1:1 (v/v) sand: vermiculite	9.64	9.63	9.51	9.63
2:1 (v/v) sand: vermiculite	9.61	9.84	9.49	9.09
95% fiducial limits		±0.47		±0.47

[a]Six dilution steps (10^{-5}–10^{-10}) with 6 replicate tubes were used in the plate counts.

Table 3. The effect of harvest time on the apparent numbers of *Cicer–Rhizobium* strain IC-128 in a broth culture as determined by plant infection dilution (MPN) method

Time after inoculation	Rooting medium: 1:1 sand:vermiculite[a] Proportion of plants nodulated	MPN count[c] (log_{10} ml^{-1})	Sand[b] Proportion of plants nodulated	MPN count[c] (log_{10} ml^{-1})
3½ weeks	5/36	5.08	27/36	7.77
4 weeks	23/36	8.08	30/36	9.26
5 weeks	28/36	8.94	34/36	9.94
6 weeks	31/36	9.34	33/36	9.84
95% fiducial limits		±0.47		±0.47

[a]Inoculum contained log_{10} 9.48 *Rhizobium* ml^{-1} by plate count.
[b]Inoculum contained log_{10} 9.64 *Rhizobium* ml^{-1} by plate count.
[c]Six dilution steps (10^{-5}–10^{-10}) and 6 replicate tubes were used in the plant counts.

Table 4. Comparison of recovery of *Rhizobium* strain IC-128 by plate, and by plant infection dilution (MPN) method using different cultivars of chickpea

Cultivar	Dilution level 10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}	MPN[b]
	Plate count–*Rhizobium* ml^{-1} (50000)	(5000)	(500)	50 ± 3.06	(5)	(0.5)	5.0×10^{6a}
	MPN count expected positive tubes 12	12	12	12	11.9	4.8	
	Observed positive tubes						
K-850	12	12	12	12	12	8	8.67×10^{9}
JG-62	12	12	12	12	12	3	3.37×10^{9}
Annigeri	12	10	12	12	12	6	4.23×10^{9}
BEG-482	10	8	12	12	11	9	2.71×10^{9}
G-130	9	9	12	10	9	6	5.90×10^{8}
Rabat	7	6	6	6	6	3	1.44×10^{7}
95% fiducial limits × or ÷							1.47

[a]Estimates extrapolated from counts at $1:10^{8}$ dilution.
[b]Six dilution steps (10^{-5}–10^{-10}) using 12 tubes per dilution.

Table 5. Relationship between observed and theoretical positive plant tube numbers for a 10-, 4- and 2-fold dilution series of chickpea *Rhizobium* strain IC-2091. Appropriate dilutions were plated and inoculated to the plants

10-fold dilution series	Dilution level 10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}	10^{-11}	10^{-12}
Plate count	32,000	3200	320	32 ± 3.7	3.2	0.32	0.032	0.0032
Theoretical + tube	12	12	12	12	11.55	3.36	0.35	0
Observed + tube	12	12	12	11	12	3	0	0
4-fold dilution series	Dilution level 4^{-1}	4^{-2}	4^{-3}	4^{-4}	4^{-5}	4^{-6}	4^{-7}	
Plate count	3147	784	197 ± 19	49	12	3	0.8	0.2
Theoretical + tube	12	12	12	12	12	11.49	6.54	2.13
Observed + tube	12	12	12	12	12	11	6	1
2-fold dilution series	Dilution level 2^{0}	2^{-1}	2^{-2}	2^{-3}	2^{-4}	2^{-5}	2^{-6}	2^{-7}	2^{-8}
Plate count	450 ± 67	225	113	56	28	14	7	3.5	1.7
Theoretical + tube	12	12	12	12	12	12	12	12	10
Observed + tube	12	12	12	12	12	12	12	12	7

Table 6. Estimates of *Rhizobium* numbers recovered from soil inoculated with *Rhizobium* strain IC-2091 and IC-59 using the plant infection dilution (MPN) method

	Soil A	Soil B
Rhizobium g^{-1} soil before inoculation	8.61	1.73×10^3
Strain IC-2091[a]		
Rhizobium added g^{-1} soil	2.75×10^7	2.75×10^7
Number recovered g^{-1} soil	1.70×10^7	2.72×10^7
Strain IC-59[a]		
Rhizobium added g^{-1} soil	1.95×10^7	1.95×10^7
Number recovered g^{-1} soil	3.72×10^7	9.33×10^6
95% fiducial limits × or ÷	4.79	

[a]Six dilution steps (10^{-2}–10^{-7}) and 3 replicate tubes per dilution were used for these plant counts.

reliable counts by 4 weeks after inoculation but nodulation in sand:vermiculite was slightly delayed (Table 3). Secondary roots developed faster in sand than in sand:vermiculite and the earlier nodulation may be related to this.

Table 4 shows that the MPN count differed with the cultivar used as the trap host. Using a 10-fold dilution series of a broth culture of strain IC-128, cultivars K-850, JG-62, BEG-482 and Annigeri agreed well with the plate counts. However, MPN counts for the varieties G-130 and Rabat did not agree well with the plate count. Rabat, a kabuli cultivar, did not nodulate well in test tube cultures and is also poorly nodulated in the field at the ICRISAT Centre. BEG-482, a desi cultivar also forms few nodules in the field but nodulated freely in the test tube. However, the reproducibility of its nodulation pattern for a dilution series was poor, with only 10 and 8 out of 12 positive tubes at 10^{-5} and 10^{-6} dilutions (Table 4).

We routinely use cv. K-850 which is well nodulated in field trials over several locations in India, as the trap host. The cultivar chosen should preferably be tolerant to soil-borne fungal diseases such as *Fusarium* wilt (*Fusarium oxysporum* F. sp. *ciceri*).

Accuracy of the plant infection counts

A broth culture of strain IC-2091 containing 3.2×10^9 cells ml^{-1} by plate count was diluted serially (10-, 4- and 2-fold) and these dilutions were used to inoculate chickpea plants in tubes. For the 10-fold dilution series, the theoretical and observed number of positive tubes agreed well. In the 4-fold dilution series, theoretical and observed positive tubes agreed until the dilution 4^{-7} when the plate count suggested there were fewer than 0.2 cells added per test tube. In the 2-fold dilution series, the theoretical and observed positive tubes agreed well until the calculated number of rhizobia added per tube at the 2^{-8} dilution was less than 3.5 (Table 5).

Counting rhizobia in soils and inoculants

Table 6 shows the recovery of *Rhizobium* after adding broth cultures to two Vertisol soils with different background populations of native rhizobia. For both *Rhizobium* strains, the recovery from the soil and subsequent MPN count agreed well with the plate count of the broth added.

Inoculants of chickpea rhizobia received from various places were checked for *Rhizobium* number using both plate and plant infection count.

Table 7 shows that when the inoculants contained few or no contaminants the plate and plant counts agreed well. For inoculants which were heavily contaminated, the numbers of *Rhizobium* estimates from plate counts were always much higher than the plate count. This is obviously due to the difficulty of distinguishing *Rhizobium* colonies from other commonly occurring contaminants that also did not take up Congo Red stain. These results indicate that plate counts should not be used for counting *Rhizobium* in contaminated backgrounds.

We have used the technique for counting *Cicer–Rhizobium* in a range of soils and contaminated inoculants. At present this is the only reliable technique for such a count.

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Table 7. Chickpea *Rhizobium* populations in various peat inoculants estimated by plate and plant infection dilution (MPN) methods. Numbers expressed as $\log_{10}$

	Plate count		Plant infection dilution method	
Sample	Contaminant bacteria g^{-1} peat	*Rhizobium*-like colonies g^{-1} peat	MPN count g^{-1} peat	95% fiducial limits (±)
1	Nil	10.02	9.94	0.44
2	Nil	9.82	9.26	0.44
3	Nil	9.48	10.26	0.62
4	Nil	9.57	9.94	0.62
5	Nil	9.66	9.94	0.62
6	8.25	8.32	7.08	0.44
7	9.63	10.26	3.40	0.44
8	9.41	9.35	7.58	0.44
9	8.26	9.01	8.57	0.62
10	9.56	8.40	5.94	0.44
11	8.63	8.03	4.24	0.62
12	8.41	8.44	7.65	0.44
13	7.59	9.25	8.58	0.62
14	7.67	8.56	5.24	0.62

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