Nodule occupancy determination and \textit{Rhizobium} strain quantification by immunoblot assay

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Received 13 January 1998; revised 28 April 1999

Dot immunoblot assay was used for determination of nodules produced on soybean, frenchbean, pigeonpea and urdbean by \textit{Bradyrhizobium japonicum} USDA-110; \textit{R. leguminosarum} bv. phaseoli FB-77 and N-3; \textit{Rhizobium} sp. A-3 and U-1, respectively. Nodule occupancy by inoculated strains as determined by the test ranged between 73 to 93%. Replica immunoblot assay reduced the time required for enumeration of rhizobia and was suitable for strain specific enumeration of \textit{Rhizobium} strains in nodules. Correlation between immunoblot and traditional plate counts was $r=0.96$ for five rhizobial strains tested.

Strain identification is a major problem in field experimentation with \textit{Rhizobium}. Where legumes are regularly grown, soil invariably contain some rhizobia capable of nodulating the particular host. It becomes difficult to distinguish them since the inoculated rhizobia resemble the native rhizobia. Also, it is not possible to readily determine which legume nodule is formed by which strain except in rare cases where rhizobial strains produce melanin\textsuperscript{1,2}. Thus, to study inoculum establishment and survival, it is necessary to have a sensitive and reliable means of specifically detecting and quantifying the inoculated strains of \textit{Rhizobium}.

A variety of immunological tests are available for the detection of specific \textit{Rhizobium} strains\textsuperscript{3,5}. For large scale screening of \textit{Rhizobium} strains, agglutination and immunodiffusion test have commonly been used\textsuperscript{1}. Both the techniques require isolation and subculturing of rhizobia from the nodules. Also, the specificity of these tests is low and the read out of the test is not fully objective. Immunofluorescent (IF) technique has been used successfully in serotyping rhizobial antigens from nodules and soils and rhizosphere\textsuperscript{4,5}. But this technique requires considerable skill in interpretation of results and is expensive. The enzyme linked immunosorbent assay (ELISA) was used for the first time in \textit{Rhizobium} identification in nodules of \textit{Arachis hypogea}\textsuperscript{6}. Though this method is most sensitive but needs specialized immunoassay equipment. Other marker techniques for recognizing rhizobia are the use of genetic marker by antibiotic resistance and auxotrophy\textsuperscript{7,8}. These markers not only alter the symbiotic ability of \textit{Rhizobium} strains but also gives little information concerning the indigenous population of \textit{Rhizobium}. The attempt was therefore, made to determine (i) the per cent nodule occupancy by inoculant strains using dot immunoblot assay (DIBA) and (ii) the occurrence of introduced strains in nodules by replica immunoblot assay (RIBA).

Materials and Methods

Organisms—Five different \textit{Rhizobium} strains obtained from the Department of Soil Sciences, G B Pant University of Agriculture and Technology, Pantnagar, India were used in the present study (Table 1). These were maintained on the yeast extract mannitol (YEM) medium.

Preparation of antigens and antisera—Rhizobial cultures grown for 5 days in YEM broth were harvested in normal saline as described by Vincent\textsuperscript{7}. Pellet was resuspended in normal saline to obtain a thick suspension containing $-10^{9}$ cells/ml and subjected to heat treatment in boiling water bath for one hour to inactivate flagellar antigens. The suspension was then preserved at 4°C by adding 1% merthiolate till use. One ml culture suspension of each strain emulsified with an equal volume of Freund’s adjuvant was injected subcutaneously at weekly intervals into Newzealand white rabbits. Seven days
after the last injection, the rabbits were bled and antisera was stored in 1% merthiolate solution at 4°C.

* Nodulation under pot house conditions—Surface sterilized seeds of soybean (Glycine max L.) Merr.; french bean (Phaseolus vulgaris L.), pigeon pea (Cajanus cajan (L.) Mill sp.) and urd bean (Phaseolus mungo L.) were inoculated with *Bradyrhizobium japonicum* USDA-110, *Rhizobium leguminosarum* bv. phaseoli FB-77 and N-3, *Rhizobium* sp. A-3 and U-1. Charcoal was used as carrier. A 10% pharmaceutical grade of gum arabic was used to serve as a sticker of *Rhizobium* cells to seed. Seeds (10⁸ cells/seed) were sown immediately in pots (20 cm high and 20 cm internal diameter). Also, uninoculated control was kept with each treatment. Nodules were collected after 8 weeks of incubation from the root systems of each plants. Adhering soil particles were carefully removed by gentle shaking. Nodule rating was determined by the method as outlined by Burton and Curley⁹.

* Dot immunoblot assay—*Nitrocellulose membranes were soaked in sterile distilled water and dried. Samples (10 µl crushed nodule suspension) and rhizobial cultures were spotted on the nitrocellulose membrane. Spots were dried and the membranes were immersed in sterile water, washed (2X) with phosphate buffered saline (PBS) and soaked in acidic PBS (pH 2.6) to denature bound alkaline phosphatase produced by the strains.¹⁰ Membranes were then washed to neutrality (PBS, pH 7.4), blotted lightly on tissue paper and soaked in a solution of 3% skimmed milk (prepared in PBS), for 45 min at 37°C. Membranes were washed (3X) with 250 µl of PBS plus 0.05% tween-20 (PBST, pH 7.2). The spots were covered with 1:1000 dilution of desired antiserum for 3 hr at room temperature. Membranes were again washed with PBST (5X) and transferred to the solution of goat antirabbit IgG alkaline phosphatase conjugate for 2 hr. Membranes were washed again with gentle agitation and immersed in 0.5 M carbonate buffer (pH 9.5) for 5 min and transferred to freshly prepared substrate (5'-bromo-4-chloro-3-indolyl phosphate; BCIP). Distinct purple spot on the membrane was taken as the positive reaction and nodule occupancy was calculated as:

\[
\text{% nodule occupancy} = \frac{\text{No. of purple spots}}{\text{Total No. of nodule tested}} \times 100
\]

*Replica immunoblot assay—*Nitrocellulose membranes were laid on a single selected plate of an appropriate dilution containing 30 to 300 colonies and allowed to adsorb the colonies for 5 min. Membranes were then removed from the plates and washed thoroughly with a stream of water to remove loosely attached rhizobial cells. Membranes were air dried (colony side up) and processed in similar way as discussed under dot immunoblot assay.

**Results and Discussion**

*Inoculation effects on nodulation—*Number of nodules produced on each plant was used as an index for assessing the occupancy of *Rhizobium* spp. However, this does not reflect the true occupancy since the nodule count may include nodules formed by indigenous population also. Hence, the present study was undertaken to evaluate the effect of *Rhizobium* strains on nodulation of their homologous hosts and to assess the true occupancy by the inoculated strains using immunoprobe available against *B. japonicum* USDA-110, *R. leguminosarum* bv. phaseoli FB-77 and N-3, *Rhizobium* sp. A-3 and U-1.

Results presented in Table 1 showed that all of the inoculant strains produced nodules on their respective hosts. In all the treatments, nodule number, nodule dry weight and nodule rating varied considerably (Table 2). Increase in nodule number over its control was maximum in urdbean (194.5%) followed by frenchbean inoculated with *R. leguminosarum* bv. phaseoli FB-77 (180%). While minimum was recorded in frenchbean inoculated with *R. leguminosarum* bv. phaseoli N-3 (11.0%). The production of low number of nodules in soybean and frenchbean compared to pigeon pea and urdbean under the identical set of conditions could either be due to inefficient release of bacteria from the infection thread or due to competition from indigenous rhizobial population. Also, the host plant differed significantly with regard to their nodule dry weight (Table 2). The correlation between nodule number

<table>
<thead>
<tr>
<th>Table 1—<em>Rhizobium</em> strains used in the present study</th>
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<tr>
<td><strong>Rhizobium strain</strong></td>
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<tr>
<td><em>Bradyrhizobium japonicum</em> USDA-110</td>
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<tr>
<td><em>R. leguminosarum</em> bv. phaseoli FB-77</td>
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<tr>
<td><em>R. leguminosarum</em> bv. phaseoli N-3</td>
</tr>
<tr>
<td><em>Rhizobium</em> sp. A-3</td>
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<tr>
<td><em>Rhizobium</em> sp. U-1</td>
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and nodule dry weight was highly significant \( (r = 0.73) \). Nodule rating on the other hand seems to be a better qualitative procedure for differentiating *Rhizobium* sp. varying in degree of effectiveness. Nodule rating was between 4.86 and 8.40 among legume hosts (Table 2). A high nodule rating was observed in soybean PK-416 (8.06) which indicated a tap root nodulation. Since it was the tap root that developed at the earliest, the presence of nodules on tap root indicated the ability of rhizobia to form nodules at early stage of plant growth. This finding is in agreement with the findings as reported earlier11,12.

**Nodule occupancy**—Nodule occupancy was determined using single strain nodule samples that were collected in bulk. Nodules from each of five inoculated plants were individually tested by dot immunoblot assay with five homologous antisera, USDA-I10, FB-77, N-3, A-3 and U-1, and the results of occupant strains are shown in Fig. 1.

Nodules formed by inoculated strains varied between 73 and 93 per cent, maximum being produced by soybean. (Table 2). Between *R. leguminosarum* bv. phaseoli FB-77 and N-3 infecting the same crop, N-3 strains showed greater occupancy. All the nodules from a single plant, however, never showed 100% immunoreactivity with their homologous antisera in DIBA test (Fig.1). Thus, it can be seen that 7 to 27% nodules were formed by serologically unrelated strains. Inoculation thus, markedly increased the number of nodules by reducing the non-competitive indigenous rhizobial population. Similar nodule occupancy determination have been reported by Leung et al.13 for clover plants using immunofluorescence technique where they observed 60-73% of serologically positive nodules. In *Arachis hypogea*, 92% of nodules were reported to be formed by inoculant strains as determined by ELISA test6.

To study inoculum establishment and survival, it is necessary to have a sensitive and reliable means of specifically detecting and quantifying the inoculated strains. The quantitative analysis of rhizobial population involves the determination of viable counts by plating crushed nodule suspension on YEMA plates. The result presented in Table 3 shows that the visible colonies of slow growing (*B. japonicum* USDA-I10) and fast growing (*Rhizobium* spp.) rhizobia appeared in 56 and 48 hr respectively, which not only delays the observation but may also encourage contamination and thus, may cause difficulty in interpretation. In situ replica immunoblot assay (RIBA) provides a rapid, strain specific and highly reliable alternative for quantitative estimation of root nodule bacteria using specific antiserum. Testing of rhizobial colonies using conventional agglutination, immunodiffusion and IF technique would require handling of individual colonies.14 Replica immunoblot assay, however, is an easy and rapid method which revealed the strain specificity of each colony easily. Micro-colonies (< 0.6mm) which are even difficult to visualise can be picked up on nitrocellulose membrane and can be immunostained by specific antiserum and alkaline phosphatase conjugated second antibody to yield a positive purple spots on the membrane, which is the mirror-image of live rhizobial cells. The rhizobial population count obtained by plate counts and RIBA method at different time intervals were fairly close to each other and the immunoblot estimates were highly correlated with the plate count method \( (r = 0.96) \) (Table 3). Surprisingly, nodules from each plant showed 3 to 6% serologically negative spot after 56 hr growth suggesting appearance of serologically unrelated indigenous rhizobia within a single nodule. Our finding is in close agreement to the findings of Galiana et al.14 who have also found some indigenous

| Table 3—*Rhizobium* population analysis by replica immunoblot assay (RIBA) at different time intervals |
|--------------|--------------|-----------|
| Nodules formed by | Time (hr) | Nodules suspension (\( \times 10^5/\text{ml} \)) | Plate count | RIBA count |
| *B. japonicum* | USDA-I10 | 24 | — | — | 0.17 |
| | | 48 | — | 1.2 | 1.2 |
| | | 56 | 1.2 | 1.2 |
| *R. leguminosarum* bv. phaseoli | FB-77 | 24 | — | — | 25 |
| | | 48 | 66 | 62 |
| | 56 | 89 | 86 |
| *R. leguminosarum* bv. phaseoli | N-3 | 24 | — | 29 |
| | | 48 | 45 | 41 |
| | | 56 | 65 | 62 |
| *Rhizobium* sp. | A-3 | 24 | — | 31 |
| | | 48 | 60 | 56 |
| | | 56 | 68 | 65 |
| *Rhizobium* sp. | U-1 | 24 | — | 10 |
| | | 48 | 65 | 62 |
| | 56 | 85 | 75 |

*Correlation between plate count and RIBA count, \( r=0.96 \)
Table 2—Effects of Rhizobium spp. on nodulation and nodule occupancy

<table>
<thead>
<tr>
<th>Rhizobial species</th>
<th>Nodule no/plant</th>
<th>Nodule dry wt/plant (g)</th>
<th>Nodule rating</th>
<th>Nodule occupancy by inoculant rhizobia (%)</th>
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<tbody>
<tr>
<td>B. japonicum</td>
<td></td>
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<tr>
<td>USDA-110</td>
<td>20(150)</td>
<td>0.21(75)</td>
<td>8.06</td>
<td>93.0</td>
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<tr>
<td>Control</td>
<td>8</td>
<td>0.12</td>
<td>3.13</td>
<td>—</td>
</tr>
<tr>
<td>R. leguminosarum bv. phaseoli</td>
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<td></td>
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<tr>
<td>FB-77</td>
<td>11.2(180)</td>
<td>0.11(87)</td>
<td>8.40</td>
<td>73.0</td>
</tr>
<tr>
<td>Control</td>
<td>4</td>
<td>0.06</td>
<td>3.46</td>
<td>—</td>
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<tr>
<td>R. leguminosarum bv. phaseoli</td>
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<tr>
<td>N-3</td>
<td>10.5(111)</td>
<td>0.10(75)</td>
<td>7.0</td>
<td>77.0</td>
</tr>
<tr>
<td>Control</td>
<td>5</td>
<td>0.06</td>
<td>1.33</td>
<td>—</td>
</tr>
<tr>
<td>Rhizobium sp.</td>
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<tr>
<td>A-3</td>
<td>37.0(164)</td>
<td>0.32(174)</td>
<td>4.86</td>
<td>80.0</td>
</tr>
<tr>
<td>Control</td>
<td>14</td>
<td>0.12</td>
<td>2.53</td>
<td>—</td>
</tr>
<tr>
<td>Rhizobium sp.</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>U-I</td>
<td>59.0(195)</td>
<td>0.23(135)</td>
<td>5.53</td>
<td>73.0</td>
</tr>
<tr>
<td>Control</td>
<td>20</td>
<td>0.10</td>
<td>2.65</td>
<td>—</td>
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*Nodules were analyzed by dot immunoblot assay technique. Twenty nodules per plant were tested, and each nodule was analyzed with each of the five homologous antisera. Values in parenthesis indicate % increase over control.*

Fig. 1—Nodule typing by dot immunoblot assay (DIBA) using homologous antisera. Wells- (1-5) Crushed nodule suspension; (6) Cultured cell suspension and (7) Negative control.[Lane a—Soybean nodules; b—Frenchbean (FB-77) nodules; c—Frenchbean (N-3) nodules; d—Pigeonpea nodules and e—Urdbean nodules].

Fig. 2—Immunoblot count of Rhizobial cells using RIBA test. [(a)—Control plate after being imprinted with cells and run directly with substrate; (b)—Immunoblot prepared from soybean crushed nodule and (c)—Immunoblot prepared from pigeonpea crushed nodule].
rhizobia in the nodules of *Acacia mangium* inoculated with *Bradyrhizobium* strains. *B. japonicum* USDA-110 however, showed 100% immunoreaction with its homologous antisera. Further, using RIBA, specific rhizobial population can be enumerated somewhat earlier than normal plate counting method as presented in Fig. 2 and Table 3.

The applied inoculum in all the crops improved nodulation and thus reduced the competition from indigenous rhizobial population. The immunoblot procedure discussed is rapid, specific and reliable. It requires only 6 hr for identification and enumeration of fast and slow growing rhizobia as compared to 5 to 7 days required for plate count method. Also, the plate count method does not determine strains specificity, whereas, the membrane immunoassay checks strains specificity of all colonies on a single plate.

Acknowledgement

The author thanks UGC, New Delhi, India for providing fellowship during the course of investigation.

References