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Short Communication

Replica Immunoblot Assay (RIBA): A New Method for Quantification and Specific Determination of *Rhizobium* and *Bradyrhizobium* Strains Directly in Legume Nodules

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Abstract

Replica Immunoblot Assay (RIBA) was used for quantification and determination of specificity of inoculated rhizobial strains in nodules produced on greengram [*Vigna radiata* (L.) wilczek], chickpea (*Cicer arietinum*, L.), pigeonpea (*Cajanus cajan* (L.) Mill sp.), urdbean (*Phaseolus mungo* L.) and frenchbean (*Phaseolus vulgaris* L.). RIBA reduced the time required for enumeration of rhizobia and was suitable for strain-specific quantification of rhizobial strains in nodules. *Rhizobium* diversity within a single nodule of each legume host plant ranged between 2–16%. Correlation between immunoblot and traditional plate counts was r = 0.95 for six rhizobial strains tested. The method can be used to enumerate the viable cells of *Rhizobium* in biofertilizers and therefore can help to check the quality of *Rhizobium*/*Bradyrhizobium* biofertilizers.

Keywords: *Bradyrhizobium*, legumes, Replica Immunoblot Assay (RIBA), *Rhizobium*

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1. Introduction

Identification of *Rhizobium* strains under field conditions is a major problem. Where legumes are grown regularly, soils invariably contain some rhizobia that can nodulate a particular legume host. It therefore becomes difficult to distinguish the indigenous rhizobia since the inoculated rhizobia resemble physiologically and biochemically the native rhizobia. Also, it is not possible to readily determine which legume nodule is formed by indigenous and which by inoculated rhizobia except in rare cases where rhizobial strains produce melanin (Cubo et al., 1988; Eaglesham et al., 1982). Thus, to determine the specificity of rhizobia in nodules, it is necessary to develop a sensitive and reliable means of specifically detecting and quantifying the inoculated strains of *Rhizobium* in single nodule of each legume host.

A variety of serological tests is available for the identification of *Rhizobium* strains (Vincent, 1970; Khan, 1996). For large scale screening of *Rhizobium* strains, agglutination and immunodiffusion tests have commonly been used (Trinick, 1969; Khan et al., 1997). Both of theses methods, however, require isolation and sub-culturing of rhizobia from nodules and are time consuming. Immunofluorescent (IF) technique has been used successfully in serotyping rhizobial antigens from nodules and soils (Bohlool and Schmidt, 1982), but this technique requires considerable skill in the interpretation of results and is expensive. The enzyme linked immunosorbent assay (ELISA) initially described by Engvall and Perlman (1971; 1972) is an additional serological method used in rhizobium research. The method is simple, flexible and efficient with respect to time, and reagents yet is more sensitive by four to six orders of magnitude than other serological methods. Though this method is most sensitive, it requires specialized and costly immunoassay equipment. Other marker techniques for detecting rhizobia includes the use of antibiotic resistance and auxotrophy (Schwinghamer and Dudman, 1973). These markers not only alter the symbiotic properties of *Rhizobium* strains but also provide little information about the indigenous population of *Rhizobium* inhabiting the nodules. The attempt was, made therefore, to develop a serological method for quantification and specific determination of inoculated strains of rhizobia directly in nodules of legume host plants.

2. Materials and Methods

Bradyrhizobium sp. (*vigna*), *Rhizobium cicerii*, *Rhizobium* sp. A-3, *Rhizobium* sp. U-1, *Rhizobium leguminosarum* bv. *phaseoli* FB-77 and *Rhizobium leguminosarum* bv. *phaseoli* N-3 obtained from Department of Soil Science, Govind Ballabh Pant University of Agriculture and Technology,

Pantnagar, U.P. India were grown for 3–5 d in yeast extract mannitol broth and were harvested in normal saline as described by Vincent (1970). Pellets were resuspended in normal saline to obtain a thick suspension containing $\cong 10^9$ cell/ml and subjected to heat treatment in boiling water bath for 1 h to inactivate flagellar antigens. The suspension was then preserved at 4°C by adding 1% Merthiolate till use. A one-ml culture suspension of each strain emulsified with an equal volume of Freund's complete adjuvant (FCA) was injected subcutaneously at weekly intervals into New Zealand white rabbits. Seven days after the last injection, the rabbits were bled from the marginal veins of ear, and antisera were stored in 1% Merthiolate solution till use. Surface-sterilized seeds of greengram var. T-44 [*Vigna radiata* (L.) wilczek], chickpea (*Cicer arietinum* L.), pigeonpea [*Cajanus cajan* (L.) Mill sp.], urdbean (*Phaseolus mungo* L.), and frenchbean (*Phaseolus vulgaris* L.) were inoculated with *Bradyrhizobium* sp. (*vigna*), *Rhizobium cicerii*, *Rhizobium* sp. A-3, *Rhizobium* sp. U-1 and *Rhizobium leguminosarum* respectively using charcoal as a carrier and a 10% gum arabic to stick rhizobial cells to the seeds. The coated seeds ($\cong 10^8$ cells/ml) were sown immediately in earthen pots. Also, uninoculated plant (3 plants per treatment) was kept with each treatment. Nodules were collected after 8 weeks of incubation from the root system of each plant except greengram where nodules were detached after 45 days of sowing. Adhering soil particles were removed carefully from each root system of legume hosts, and the occurrence of specific *Rhizobium* strains (inoculated strains) in each nodule was determined by direct replica immunoblot assay (RIBA) developed during the present study.

Nitrocellulose membranes were soaked in sterile distilled water and dried. Nitrocellulose membranes were then laid on a single selected plate of an appropriate dilutions of nodule suspension and were allowed to adsorb the colonies for 5 min. Membranes were then removed from the plates gently and washed thoroughly with a stream of water to remove loosely attached rhizobial cells. Membranes were air dried (colony side up) and then immersed in sterile water, washed twice with phosphate buffered saline (PBS) and soaked in acidic PBS (pH 2.6) to denature bound alkaline phosphatase produced endogenously. 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 in humid environment. Membranes were washed five times with 250 µl of PBS plus 0.05% Tween-20 (PBST, pH 7.2). Membranes were covered with 1:1000 dilutions of each specifically raised homologous antiserum separately for 3 h at room temperature. Membranes were again washed with PBST five times and transferred to the solution of goat anti-rabbit IgG alkaline phosphate conjugate for 2 h. Membranes were washed again with gentle agitation and immersed in 0.5 M carbonate buffer (pH 9.2) for 5 min and transferred to freshly prepared substrate solution (Nitro Blue

Tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate; BCIP). Distinct purple spots on the nitrocellulose membrane were considered as the positive immunoreaction representing specific *Rhizobium* and *Bradyrhizobium* strains and rhizobial strains were quantified as:
(number of purple spots on the nitrocellulose membrane) / (dilution factor) × (volume of inoculum).

3. Results and Discussion

The results presented in Table 1 show that the visible colonies of slow growing [*Bradyrhizobium* sp. (*vigna*)] and fast growing rhizobial species i.e. *Rhizobium cicerii, Rhizobium* sp. A-3, *Rhizobium* sp. U-1, *R. leguminosarum* bv. *phaseoli* FB-77 and *R. leguminosarum* bv. *phaseoli* N-3 appeared after 56 and 48 h of incubations, respectively, on yeast extract mannitol agar (YEMA) plates, which not only delays the observation but can also encourage contamination and therefore may cause difficulty in interpreting the results. *In situ* replica immunoblot assay (RIBA), in contrast, provides a rapid, strain specific and highly reliable alternative for quantitative estimation of root nodule bacteria using specific and homologous antiserum. Using replica immunoblot assay (RIBA), all rhizobial species were detected in nodule suspensions after 24 h of growth whereas the *Bradyrhizobium* species (*vigna*) was specifically detected after 48 h of growth. The colonies of fast growing species of *Rhizobium* which were even not visible on YEMA plates after 24 h of incubations were specifically detected using their homologous antisera and produced a purple spot on the membranes. Maximum RIBA counts (84×10^4 cells/ml of nodule suspension) were observed in the nodular suspension of frenchbean inoculated with *R. leguminosarum* bv. *phaseoli* FB-77, after 56 h of growth on YEMA plates whereas the nodule suspension prepared from mungbean nodules showed lowest RIBA counts (0.6×10^4 cells/ml) after 48 h of growth on YEMA plates. The nodular antigen suspension of greengram when reacted with its homologous antiserum showed 100% positive immunoreaction (1.5×10^4cells/ml), indicating a greater rhizobial specificity in each nodule as compared to other nodular suspensions. In general, the plate counts and RIBA counts of *Bradyrhizobium* sp. (*vigna*) and rhizobial species varied at different incubation periods. The rhizobial population counts obtained by plate counts and direct RIBA method at different time intervals were fairly close to each other and the immunoblot estimates were highly correlated with the plate counts ($r = 0.95$). Testing of rhizobial colonies using conventional agglutination (AG), immunodiffusion (DID), and immunofluorescent technique (IF) would require handling of individual colonies (Khan, 1996). Direct replica immunoblot assay (RIBA), however, is an easy and rapid method that

Table 1. *Rhizobium* and *Bradyrhizobium* strain specificity in nodules as determined by replica immunoblot assay (RIBA)[a] at different incubation periods

Nodules formed by	Time (h)	Nodule suspensions ($\times 10^4$/ml)	
		Plate count	RIBA count
Bradyrhizobium sp. (*vigna*)	24	–	–
	48	–	0.6
	56	1.5	1.5
Rhizobium cicerii	24	–	20
	48	65	63
	56	83	81
Rhizobium sp. A-3	24	–	29
	48	55	53
	56	62	59
Rhizobium sp. U-1	24	–	28
	48	67	64
	56	81	71
R. leguminosarum bv. *phaseoli* FB-77	24	–	20
	48	60	56
	56	87	84
R. leguminosarum bv. *phaseoli* N-3	24	–	25
	48	42	35
	56	68	65

Each value is a mean of three replicate plates and each individual experiment was repeated three times. [a]Correlation between plate counts and RIBA counts, r = 0.95.

revealed the strain specificity of each colony easily. It requires only 6 h for specific identification and enumeration of fast and slow growing species of rhizobia as compared to 5–7 days required for plate count or more than 20 days for development of nodules on legume hosts. Micro-colonies (<0.6 mm) which were even difficult to visualize were picked up on the nitrocellulose membranes and immunostained using specific antiserum raised against *Bradyrhizobium* sp. (*vigna*), *R. cicerii*, *Rhizobium* sp. A-3, *Rhizobium* sp. U-1, *R. leguminosarum* bv. *phaseoli* FB-77 and *R. leguminosarum* bv. *phaseoli* N-3 and alkaline phosphatase conjugated second antibody to yield a positive purple spot on the nitrocellulose membrane. The purple spot produced as a result of interaction between epitopes and specifically raised antibody is the mirror image of live rhizobial cells. Interestingly, nodules produced on each leguminous host showed 2–16% serologically unrelated spots on the nitrocellulose membranes (white) after 56h growth, which suggested the appearance of indigenous rhizobial

population within a single nodule. Our finding is in close agreement to the findings of Galiana et al. (1994) who also found rhizobial diversity in the nodules of *Acacia mangium* inoculated with *Bradyrhizobium* strains. Further, using direct RIBA, specific rhizobial populations were enumerated somewhat earlier than normal plate counting method.

The traditional analysis of rhizobia has been performed by means of plate count or a Most Probable Number (MPN) plant grow technique in which a serially diluted culture is applied to aseptic host plants and the viable *Rhizobium* number is counted from the point of nodule extinction after several weeks of growth. A major problem associated with the plate count method is that it does not determine strain specificity whereas the problem with plant grow out technique is the length of time required for completion of nodule development on host plants. Also, the grow-out procedure is incapable of distinguishing between strains within a *Rhizobium* species. In contrast, the direct RIBA developed and discussed is rapid, specific and reliable for quantification and determination of specifically inoculated rhizobial strains in nodules. In addition, in situ RIBA, not only checks strain specificity of all colonies on a single plate but also helps to determine the rhizobial diversity within a single nodule of each legume crop individually. Use of nitrocellulose membranes in the RIBA test means that there is no involvement of equipment and the volume of antigen, antibody and other reagents used is low and therefore the test is relatively inexpensive. Further, a large number of nodules can be included in a single test. The advent of selected individual rhizobial strains in the commercial market has created a need for rapid analysis at the strain level. The method (RIBA), therefore, can also be used to enumerate the viable cells of *Rhizobium* strains in carrier-based biofertilizers and can help to check the quality of rhizobial inoculants.

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