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## Enumeration of rhizobia by enzyme-linked immunosorbent assay (ELISA)

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The use of the enzyme-linked immunosorbent assay (ELISA) to enumerate rhizobia in peat carrier and in soil has been investigated. The ELISA technique takes less time than the conventional plant infection technique often used to enumerate rhizobia present in the presence of other micro-organisms. A minimum of  $10^2$ - $10^3$  cells are required for a detectable ELISA reaction, limiting the use of this technique when the number of rhizobia is low.

It is necessary to count rhizobia in samples containing other micro-organisms in the quality assessment of *Rhizobium* inoculants and in the study of *Rhizobium* ecology and competition in the field. Bacteriologically controlled plant infection tests are generally used to enumerate *Rhizobium* populations in soil and inoculum carriers. In this method test-plants grown aseptically are inoculated with aliquots from a dilution series of the sample being examined. The number of rhizobia in the soil/carrier can be calculated from the proportion of test plants that form nodules at each dilution (Tuzimura & Atanabe 1961; Date & Vincent 1962; Brockwell 1963). This method, which is time consuming and laborious has been discussed in detail recently by Brockwell (1980); In this paper we discuss the use of the enzyme linked immunosorbent assay (ELISA) for enumerating rhizobia.

The direct or double antibody sandwich form of ELISA has been widely used for the detection of viruses in plant tissues (Clark & Adams 1977; Clark 1981; Bar-Joseph & Garnsey 1981) and *Rhizobium* in nodules (Kishinevsky & Bar-Joseph 1978; Morley & Jones 1980). The principle of direct ELISA is as follows. Specific antibodies are adsorbed to a solid surface in wells in a polystyrene Microtitre plate (Dynatech Laboratories). The test sample containing rhizobia is incubated in the coated wells

and any rhizobia recognized by the antibody are bound. The subsequent reaction of the bound rhizobia with enzyme-labelled specific antibody results in the formation of a complex. The antigen (rhizobia) is sandwiched between antibody molecules. This complex is detected by the addition of an enzyme-specific substrate with which the enzyme reacts to form a coloured product.

Direct ELISA is specific for a particular antigen and can be used to detect the antigen whose anti-serum is used in the experiment, or to detect cross reacting antigens. Barbara & Clark (1982) suggested a modified method, called indirect ELISA, which has a broad based reaction and can be used for detecting a wider range of related strains. The procedures in the indirect ELISA test are similar to those of direct ELISA except for the following steps. (1) The Microtitre plates are coated with  $F(ab')_2$  fragment instead of IgG, (2) The bound  $F(ab')_2$  fragment-antigen complex is allowed to react with the antiserum (IgG) and (3) IgG is detected by goat anti-rabbit Fc enzyme conjugate.

When dilutions of other reagents are constant in the above two tests, the intensity of the reaction is proportional to the concentration of antigen. We have attempted to quantify *Rhizobium* populations (a) in a peat based carrier, by direct ELISA where only a single *Rhizobium*

strain is usually present, (b) in soil, by indirect ELISA where quantification of all *Rhizobium* strains nodulating a given host plant is desired.

## Materials and Methods

### CHEMICALS AND REAGENTS

Unless otherwise mentioned all chemicals and reagents used were obtained from Sigma Chemicals, St Louis, USA.

### ANTIGEN SOURCE

#### *Rhizobium* strain and preparation of peat inoculants

The *Rhizobium* strains used in these experiments were NC 92 (obtained from Professor G.H. Elkan, North Carolina State University, Raleigh, USA), 5a/70 (obtained from Dr Rina Lobel, Volcani Center, Israel) and IC 6006 (isolated from groundnut nodule collected from fields at International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), near Hyderabad, India). Peat inoculants of these strains were prepared as described by Thompson (1980). Mixtures of inoculants were prepared by mixing peat inoculants in the desired proportions just before the assay.

#### Soil

For enumeration of rhizobia in fields, soil samples were collected using soil sampling tubes, 19 mm internal diam. (Soil Test Inc., Evanston, Illinois, USA) at 0–15 cm depth from alfisols and vertisols at ICRISAT center.

### PREPARATION AND DILUTION OF ANTIGEN

Serial dilutions of peat inoculum or soil were prepared as follows: peat inoculum or soil (1 g) was mixed with 9 ml of antigen extraction buffer (phosphate-buffered saline, PBS: 0.02 mol/l phosphate, 0.15 mol/l NaCl, 0.003 mol/l KCl, pH 7.4, plus 0.05% Tween 20 and 2% polyvinylpyrrolidone, PVP-40T). Serial dilutions at 1:50, 1:100, 1:500, 1:10<sup>3</sup>, 1:5 × 10<sup>3</sup>, 1:1 × 10<sup>4</sup>, 1:5 × 10<sup>4</sup> and 1:1 × 10<sup>5</sup> were made in antigen extraction buffer.

### ANTISERUM

Antiserum against *Rhizobium* strain NC 92 was prepared as follows. The *Rhizobium* strain was grown on a defined solid medium, as described by Bergersen (1961) for 6 d at 30°C. The cells were harvested in 5–10 ml of physiological saline and washed 3–4 times in the same solution. One ml of culture suspension containing approximately 10<sup>9</sup> cells/ml, emulsified with an equal volume of Freund's incomplete adjuvant was injected intramuscularly at weekly intervals into New Zealand White rabbits. Fifteen days after the third injection, the rabbits were bled by puncturing the marginal ear vein, and the titre of the antiserum determined. It was usually about 1:1600.

### DIRECT ELISA

#### *Purification of antibody from antiserum*

Gamma globulins (antibody) were collected by sodium sulphate precipitation (Van Weemen & Schuurs 1971). The serum was diluted in an equal volume of distilled water and an equal volume of 36% aqueous sodium sulphate added. The precipitated protein was collected by centrifuging for 15 min at 6000 g, washed twice with 18% aqueous sodium sulphate, resuspended in PBS and dialysed at 4°C against three changes of PBS for 3 h each. The antibody was diluted with PBS to give a final protein concentration of 1 mg/ml as estimated spectrophotometrically.

#### *Antibody-enzyme conjugation*

The purified antibody was conjugated with the enzyme, alkaline phosphatase, as described by Kishinevsky & Bar-Joseph (1978).

#### *Detection and estimation of antigen*

The procedure for direct ELISA was followed as described by Kishinevsky & Bar-Joseph (1978).

### INDIRECT ELISA

The method followed was as described by Barbara & Clark (1982) for plant viruses, modified for *Rhizobium*, as follows.

*Extraction of  $\gamma$ -globulin and F(ab')<sub>2</sub> fragment preparation*

Gamma globulins were extracted from antisera raised against *Rhizobium* strain NC 92 as described above. The  $\gamma$ -globulins were digested with pepsin to yield F(ab')<sub>2</sub> fragments as follows. The reconstituted  $\gamma$ -globulins were resuspended in 0.07 mol/l sodium acetate, (pH 4.0) containing 0.05 mol/l NaCl. The  $\gamma$ -globulin concentration was adjusted to 3 mg protein/ml, by diluting with sodium acetate buffer. Pepsin diluted in distilled water (1:10000) was added to a final concentration of 45  $\mu$ g pepsin/mg  $\gamma$ -globulin. The mixture was incubated at 37°C overnight and then dialysed against three changes of PBS for 3 h each at 4°C.

*Anti Fc fragment-enzyme conjugation*

Goat anti-rabbit IgG (Fc fragment, gamma chain specific; commercially available, Cappel Laboratories, Cochranville, USA), was purified as described for  $\gamma$ -globulins as in step one of direct ELISA. The anti Fc fragment was conjugated with alkaline phosphatase as described in step two of direct ELISA.

*Detection and estimation of antigen*

The procedure for indirect ELISA was as follows: (1) polystyrene Microtitre plates were coated with 200  $\mu$ l of *Rhizobium* (strain NC 92) specific F(ab')<sub>2</sub> fraction (in 0.05 mol/l sodium carbonate buffer containing 0.02% NaN<sub>3</sub>, pH 9.6) and incubated at 30°C for 4 h. (2) Soil samples collected from the fields, were weighed and serially diluted in antigen extraction buffer. Aliquots (200  $\mu$ l) were added to the coated plates and allowed to react with immobilized F(ab')<sub>2</sub> fragment (overnight at 4°C). (3) Antiserum (strain NC 92 specific, conc. 1 mg/ml) diluted in (1:10000) conjugate buffer (PBS plus 0.05% Tween 20 plus 2% PVP and 0.2% ovalbumin), were added to react with the trapped antigen (3 h at 30°C). (4) Anti Fc fragment-enzyme conjugate (200  $\mu$ l, reactive only to the Fc portion of IgG) diluted in conjugate buffer (1:10000) was added and incubated for 3 h at 30°C. (5) Immobilized enzyme was visualized by adding enzyme substrate and incubating at 30°C for 20 min. (6) Reactions were stopped with 3 mol/l NaOH and the O.D. at

405 nm was recorded. The plates were washed three times between each stage with PBS Tween (PBS plus 0.05% Tween).

## PLATE COUNTS

The spread plate method was employed (Vincent 1970). Samples were serially diluted in sterile tap water and 0.1 ml of the dilution was spread in triplicate on congo red agar media in Petri dishes. The plates were incubated for 6–8 days at 27  $\pm$  2°C and the number of colonies in plates containing 30–300 colonies were recorded.

## ENUMERATION OF RHIZOBIA BY THE PLANT INFECTION TECHNIQUE

Rhizobia in peat and soil were enumerated by the plant infection method as described by Brockwell (1980). The experiments were done to enumerate 'cowpea cross inoculation group' rhizobia and so siratro (*Macroptilium atropurpureum*) was used as the test plant. The plants were grown in nitrogen-free mineral agar medium (Date & Vincent 1962). Ten grams of soil sample or peat were diluted in 90 ml sterile water, mixed well and serially diluted in ten fold dilutions. From each sample dilution 1 ml was inoculated on 5-day-old siratro seedlings in three replicates. The inoculated plants were grown in a light chamber at 23–25°C for 30 days. The most probable number (MPN) of 'cowpea cross inoculation group' rhizobia in the sample was calculated from the distribution of nodulated test plants as described by Brockwell (1980).

## Results and discussion

Figure 1 shows the relation between the *Rhizobium* number in a peat inoculum and the corresponding optical density (O.D.) values obtained from the direct ELISA procedure. All values are means of four replications. The optical density measured did not show a linear relationship across the range of *Rhizobium* numbers (10<sup>3</sup>–10<sup>8</sup> cells) tested. This is probably due to the fact that the intensity of the colour developed is dependent on the percentage of reagents (antiserum, antibody, antiserum-enzyme conjugate) bound and equilibrium reached during each incubation step, rather than directly proportional to the

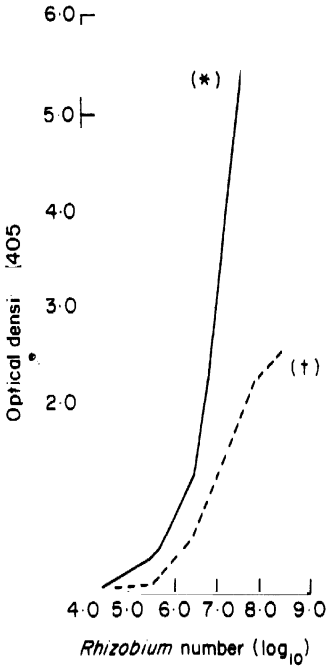


Fig. 1. Relationship between *Rhizobium* number ( $\log_{10}$ ) and intensity of alkaline phosphatase reaction estimated as O.D. using direct ELISA.  $\gamma$ -globulin enzyme conjugate dilutions used were 1 : 1000 (\*) and 1 : 2000 (+).

concentration of individual reagents added. Linear relationship can be obtained, however, for a narrow range of *Rhizobium* numbers at a

given enzyme-conjugate dilution. Figures 2(a) and (b) show separate regression analyses, one for the population range of  $10^3$ - $10^5$  cells and the other for  $10^5$ - $10^8$  cells. At the lower population level higher concentration of  $\gamma$ -globulin-enzyme conjugate was required. A similar situation was observed in the case of indirect ELISA (Fig. 3). Hence different regression equations were used to calculate the unknown population depending on the O.D. measurement. In general, the standard specifications by the inoculum quality laboratories are above  $10^6$  cells/g carrier, and the sensitivity of the regression equation is well within these limits.

The numbers of NC 92 rhizobia in a mixed peat based inoculum and in a mixture of other micro-organisms, are estimated by plate count, plant infection technique, and direct ELISA, are shown in Tables 1 and 2, respectively. These results indicate that estimations by the ELISA procedure are close to the values obtained from the plate count and plant infection techniques.

Quite often the quality of peat inoculant is poor because of the reduction in rhizobia numbers during storage. Interference due to antigens from the dead cells was examined in two experiments. The *Rhizobium* population in peat containing  $10^9$ - $10^{10}$  cells/g was enumerated after incubating at 37°C for 8 d or 40°C for 15 d. The results presented in Table 3 indicate that loss of viability in the peat carrier, does not

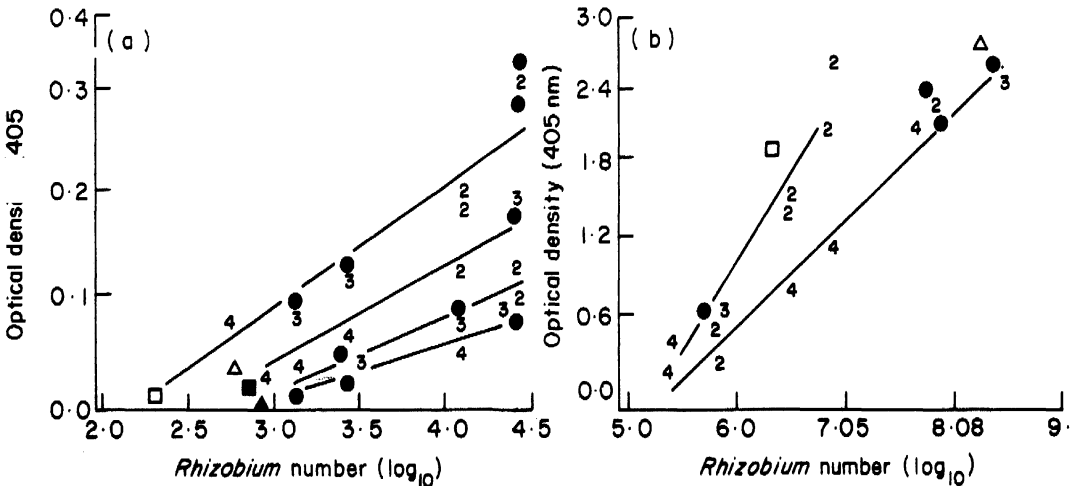


Fig. 2. Linear regression analyses of *Rhizobium* numbers and intensity of alkaline phosphatase reaction estimated as O.D. by direct ELISA. The assays were done at 4  $\gamma$ -globulin enzyme conjugate dilutions for lower *Rhizobium* numbers ( $10^3$ - $10^5$ , a) and at two dilutions at higher *Rhizobium* numbers ( $10^5$ - $10^8$  b). (a)  $\square$ -1 : 250,  $y = -0.254 + 0.144 \times (r^2 = 0.85)$ ;  $\Delta$ -1 : 500,  $y = -0.222 + 0.086 \times (r^2 = 0.84)$ ;  $\blacksquare$ -1 : 750,  $y = -0.217 + 0.073 \times (r^2 = 0.95)$ ;  $\blacktriangle$ -1 : 1000,  $y = -0.174 + 0.058 \times (r^2 = 0.98)$ . (b)  $\square$ -1 : 1000,  $y = -4.94 + 0.9 \times (r^2 = 0.97)$ ;  $\Delta$ -1 : 2000,  $y = -7.84 + 1.48 \times (r^2 = 0.92)$ . Other details are described in materials and methods.

**Table 1.** Comparison of different *Rhizobium* enumeration methods for detecting a strain in mixed inoculum†

Enumeration method	Estimated <i>Rhizobium</i> cells/g peat carrier
Plate count	
Total	$3.27 \times 10^{10}$
NC 92	$1.35 \times 10^{10}$
Plant Infection Technique	$4.20 \times 10^{10}$
Direct ELISA (NC 92)*	$1.29 \times 10^{10}$

Confidence limit (95%) for plant infection technique is  $1.0 \times 10^{10}$ – $17.2 \times 10^{10}$ .

\* Direct ELISA using NC 92 antiserum.

† Mixed peat inoculum containing  $1.35 \times 10^{10}$  of strain NC 92,  $7.5 \times 10^9$  of strain 5a/70 and  $9.9 \times 10^9$  of strain IC 6006 cells/g.

**Table 2.** Effect of contaminants in the carrier on *Rhizobium* (strain NC 92) enumeration by direct ELISA.

Enumeration method	Estimated <i>Rhizobium</i> cells/g
Plate count	$4.10 \times 10^9$
Plant infection technique	$2.40 \times 10^9$
Direct ELISA	$1.82 \times 10^9$

Confidence limit (95%) for plant infection technique is  $0.67 \times 10^9$ – $8.57 \times 10^9$ .

The plate had a contaminant load (mainly fungal) of  $4 \times 10^8$  cells/g.

**Table 3.** Use of ELISA for the detection of loss in viability of *Rhizobium* population in peat carrier

Enumeration method	Estimated <i>Rhizobium</i> cells/g	
	40°C for 15 days*	37°C for 8 days†
Plate count	$9.70 \times 10^8$	$1.01 \times 10^8$
Plant infection technique	$2.30 \times 10^8$	$2.41 \times 10^8$
Direct ELISA	$6.04 \times 10^8$	$1.37 \times 10^8$

Confidence limit (95%) for plant infection technique, \*  $0.66 \times 10^8$ – $8.04 \times 10^8$ ; †  $0.67 \times 10^8$ – $8.57 \times 10^8$ .

\* Peat inoculum with  $1.4 \times 10^{10}$  NC 92 *Rhizobium* cells/g was incubated at 40°C for 15 d.

† Peat inoculum containing  $1 \times 10^9$  NC 92 *Rhizobium* cells/g was incubated for 8 d at 37°C.

interfere in enumeration of live rhizobia by ELISA.

Time and manual labour required for the plate count is less than that required for ELISA, but the plate count method is not satisfactory when other micro-organisms are present in the carrier. The plant infection technique, which was developed to enumerate rhizobia in the pre-

sence of contaminants is time consuming and laborious. The test plant has to be grown for 25–30 d before it can be scored for nodulation. A minimum of 30 test plants growing in test tubes are required for each sample. A single ELISA Microtitre plate can be used for testing eight samples and the results can be known within two days. One person can process 20 Microtitre plates in a day.

The plant infection technique needs elaborate and expensive light chambers. Most of the equipments needed for the ELISA technique are available in a biochemical laboratory and is required mainly for the  $\gamma$ -globulin conjugation step.

Enumeration of a particular strain is desirable where a single strain is recommended for inoculation. We have selected strain NC 92 in our experiments because it is a potential inoculant strain for groundnut (Nambiar *et al.* 1984). The results given in Tables 1 and 2 show that ELISA can be used to enumerate a specific strain even in the presence of other non-cross reactive *Rhizobium* strains, or other micro-organisms. This is not possible with the plate count.

#### ENUMERATION OF SOIL RHIZOBIUM POPULATION BY INDIRECT ELISA

In ecological studies one requires to quantify different *Rhizobium* strains in soil that nodulate a given host (e.g., for groundnut 'cowpea cross inoculation group'). No positive reaction was observed with any soil dilution tested when direct ELISA was employed, using purified  $\gamma$ -globulin raised against strain NC 92 for coating the plates. This indicates that even if purified  $\gamma$ -globulin raised against a native isolate is used it may not quantify other non-cross reacting strains. Hence we used indirect ELISA which has a broad based specificity to enumerate *Rhizobium* populations in soil. Regression analyses of the data obtained from these experiments are shown in Fig. 3. Using these regression equations we have enumerated the *Rhizobium* population in soils collected from seven different fields. Table 4 shows that indirect ELISA can be used to quantify *Rhizobium* (Cowpea group) populations in soil. However at low numbers (below  $10^3$  cells/g soil) these enumerations are not possible, at the generally recommended enzyme conjugate dilution (1:250–1:1000), which limits the use of this test in ecological

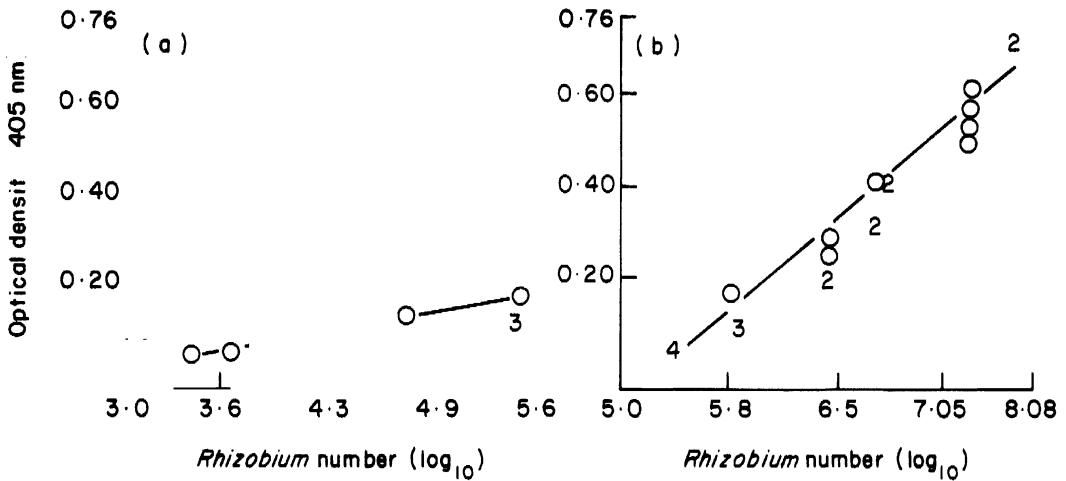


Fig. 3. Linear regression analyses of *Rhizobium* numbers and intensity of alkaline phosphatase reaction as measured by indirect ELISA. The analyses were carried out at lower *Rhizobium* population ( $10^3$ - $10^5$ , a) and at higher population ( $10^5$ - $10^8$ , b). The conjugate dilution used was 1:10 000. In (a)  $y = -0.156 + 0.054 \times x$  ( $r^2 = 0.97$ ). In (b)  $y = -1.354 + 0.258 \times x$  ( $r^2 = 0.95$ ).

Table 4. Comparison of indirect ELISA and plant infection methods for enumerating rhizobia in soil samples

Soil samples	Estimated numbers of rhizobia $\times 10^4$ /g dry soil by	
	Indirect ELISA	Plant infection technique
Vertisol	362	92 (23-367)*
Alfisol	324	92 (23-367)
Alfisol	229	15 (4-52)
Alfisol	11	5 (1-17)
Vertisol	10	2 (1-8)
Alfisol	30	14 (4-52)
Alfisol	33	43 (10-175)

\* confidence limit (95%) for estimations by plant infection technique are given in parentheses.

studies. Under such conditions one could employ indirect ELISA for an initial enumeration, and if the populations are low, i.e., below  $10^3$  cells/g then the plant infection technique can be employed. This will reduce the number of the plant tubes and dilutions that have to be tested.

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