Potential of Enzyme-linked Immunosorbent Assay for Detecting Viruses, Fungi, Bacteria, Mycoplasma-like Organisms, Mycotoxins, and Hormones

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Abstract

Enzyme-linked immunosorbent assay (ELISA) is one of the most widely employed serological tests. For example, the double antibody sandwich (DAS-ELISA) technique has been successfully employed for the detection of various rhizobia, to assess the quality of inoculum, and to assess the establishment of the inoculated rhizobial strains in soil. This technique can also distinguish between different strains of Rhizobium. *ELISA has successfully been adapted to direct antigen coating (DAC-ELISA) and protein A coating (PAC) procedures for the detection and assay of several peanut viruses. DAC-ELISA is a simple and useful tool for virus detection in field surveys and in seed samples. Both DAC- and PAC-ELISA are more convenient to use for the detection of large numbers of antigens than DAS-ELISA. More recently, a competitive ELISA procedure has been developed to detect up to 2 ng aflatoxin B_1 in groundnut seed samples.*

Introduction

Nakane and Pierce (1966) coupled low molecular weight antibodies to produce enzymatically active immunological conjugates, and visualized the immune reaction with histochemical-staining techniques. Utilizing this principle, enzyme immunoassays, of the type popularly referred to as enzyme-linked immunosorbent assay (ELISA), were developed by Engvall and Perlmann (1971) and van Weeman and Schuurs (1971). Subsequently, several modifications were introduced to the ELISA test procedures. ELISA is currently by far the most widely used test in serological diagnosis. It permits de tion of antigens, of widely varying size and m. ology, and is adaptable to a wide range of plant extracts and conditions. This paper discusses the uses of various forms of ELISA at ICRISAT for the detection and/or quantification of peanut viruses, mycoplasma-like organisms (MLOs), *Rhizobium* strains, and aflatoxin B_1.

Detection of Peanut Viruses

Most workers use the double antibody sandwich form of ELISA (DAS-ELISA) described by Clark and Adams (1977) for the detection of plant viruses. In this procedure the solid phase (often wells of polystyrene or polyvinylchloride microtitre plates) was coated with immunoglobulins (Ig), which were mainly immuno gamma globulins (IgG). Partially purified Ig were prepared from antisera by precipitation with either ammonium sulfate or sodium sulfate. Test samples were then added and the Ig trapped the virus antigen. Enzyme-labeled antivirus Ig were added, which attached to the trapped virus antigens (Fig. 1). Finally, a suitable substrate was added to produce colored hydrolysates, thus permitting visual scoring and quantitative measurement, by colorimetry, of the amounts of antigen trapped. The DAS-ELISA procedure is known to be highly specific and often detects closely related strains (Koening 1978). The main disadvantage of this test is

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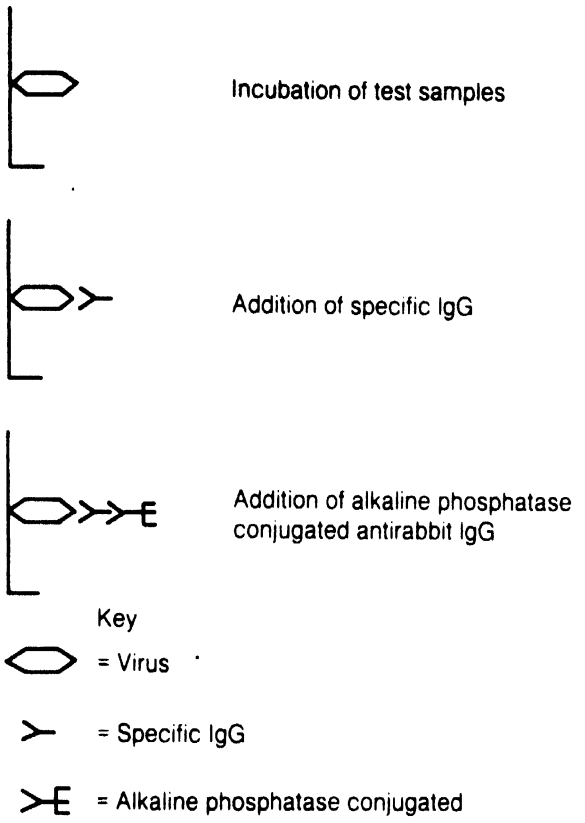

Figure 1. Principle of the double antibody sandwich (DAS) form of enzyme-linked immunosorbent assay (ELISA).

that it requires preparation of different Ig conjugates for each virus to be tested. To overcome this disadvantage we have tested several "indirect ELISA" procedures for the detection of three peanut viruses. Rabbit Fc specific Ig prepared in goats and conjugated with alkaline phosphatase was utilized in these procedures. A single conjugate could be utilized with antisera for different viruses. We describe below the two indirect-ELISA procedures that we have been able to apply successfully for the detection of several peanut viruses. They facilitated both the detection of a broad range of serologically related viruses, and the utilization of high dilutions of crude antisera.

For the direct antigen coating procedure (DAC-ELISA), the method developed by Mowat (1985) was used with minor modifications (Hobbs et al. 1987b). In the first step, plant extracts prepared in a carbonate buffer were applied directly to the wells. In the second step, diluted unfractionated antiserum was added. Ig attached to virus antigens were detected by the addition of enzyme conjugates of rabbit Fc specific Ig prepared in goats (Fig. 2). This method is by far the simplest of all the ELISA test procedures and can be completed within 3 hours. The main disadvantage of DAC-ELISA is that adsorption of the viral antigen to the well surface depends to some extent on the host components present in the plant extracts (Mowat 1985). We found DAC-ELISA to be very useful for virus detection in disease surveys (Reddy 1986, Hobbs et al. 1987b) since plant extracts could be used for coating plates without the necessity of using Ig for trapping viral antigens. The procedure has also been successfully used for detecting peanut mottle virus (PMV) in groundnut seed, thus facilitating screening of groundnut germplasm for seed-transmitted PMV. Small portions of cotyledon from 25 individual seed were mixed, ground in approximately 2.0 mL of carbonate coating buffer, and added to a well of an ELISA plate. As 8 plates, each with 96 wells, could be processed in a single day, we were able to test over 16 000 seeds per day for PMV presence.

Since host components tend to adsorb to the well surface, DAC-ELISA is not suitable for quantitative estimation of viruses present in crude plant extracts. DAC-ELISA is not suitable for investigating serological relationships, unless the virus is present in high concentration in crude extracts, and antisera having few, or no, antibodies against plant host components, are available. As a result we tried various indirect ELISA procedures described by Barbara and Clark (1982), van Regenmortel and Burck-

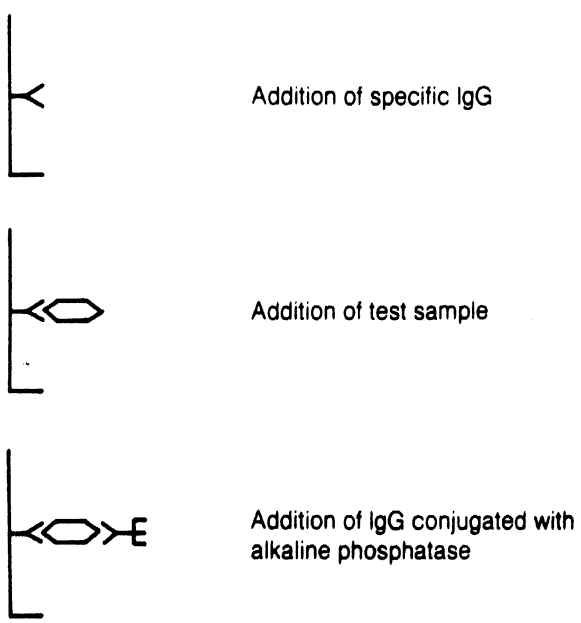

Figure 2. Principle of the direct antigen coating (DAC) form of enzyme-linked immunosorbent assay (ELISA).

ard (1980), and Edwards and Cooper (1985). We found that the protein-A coating ELISA (PAC-ELISA) procedure described by Edwards and Cooper (1985) was simpler to use and permitted cross absorption of antisera with healthy plant extracts in order to minimize nonspecific reaction. In the 4-step PAC-ELISA the plates were first coated with 1 ng mL^{-1} of protein-A. In the second step, high dilutions of unprocessed antisera were added. The Fc portion of IgG present in the antisera was bound to protein-A. In the third step, test samples were added and the $F(ab)_2$ portion of IgG trapped the virus. In the fourth step high dilutions of antisera, usually cross-absorbed with healthy plant components, were added. IgG present in antisera attached to the viral antigens. The Fc portion of this IgG was detected by the addition of conjugated antirabbit Fc specific antibodies produced in goats (Fig. 3).

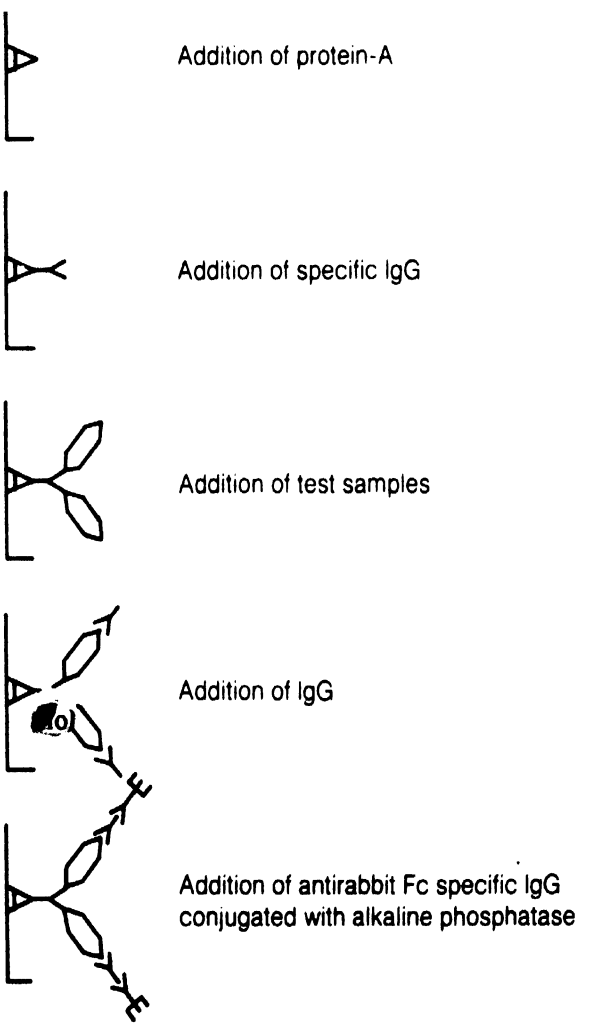

Figure 3. Principle of the protein-A coating (PAC) form of enzyme-linked immunosorbent assay (ELISA).

In Table 1, the results obtained on three peanut viruses (tomato spotted wilt (TSWV), peanut mottle (PMV), and peanut clump (PCV) viruses), using DAC-, PAC-, and DAS-ELISA procedures, are presented. Experimental details are reported by Hobbs et al. (1987b). Sensitivity of the DAC and PAC procedures was comparable to that of the DAS procedure, even under the short incubation period (45 min at 37°C for each step) conditions (Table 1).

Detection of Mycoplasma-like Organisms

We have utilized the PAC-ELISA procedure for detection of mycoplasma-like organisms (MLOs) in crude extracts of leaflets, stems, and pegs of groundnut plants showing witches' broom symptoms. The antisera produced for partially purified MLOs contained antibodies to host plant antigens. The PAC-ELISA procedure permitted cross-absorption of antisera to healthy plant antigens, thus minimizing the nonspecific reaction (Hobbs et al. 1987a). We have used this procedure for the detection of MLOs in several field-collected groundnut samples infected with witches' broom.

Detection of *Rhizobium*

The *Rhizobium* strain NC 92 was used to produce polyclonal antibodies in rabbits. In DAS-ELISA, *Rhizobium* numbers could be estimated utilizing optical density measured at 405 nm in peat-based inoculum (Nambiar and Anjaiah 1985). Estimates by the ELISA procedure were close to the values obtained from the plate count and plant infection techniques; even from samples stored at 37°C for 8 days (Table 2). The DAS-ELISA method is preferred to plant infection and antibiotic sensitivity tests, as large numbers of samples can be processed more economically and in a shorter time. Using an indirect ELISA procedure, utilizing $F(ab)_2$ fragments, *Rhizobium* populations were estimated quantitatively in several soil samples. With neither direct nor indirect ELISA procedure was it possible to estimate *Rhizobium* numbers when the populations were below 10^3 cells g^{-1} soil. Antiserum produced against *Rhizobium* strain NC 92 was also successfully used for the estimation of the percentage of nodules formed by this strain in soils inoculated with the NC 92 strain (Nambiar et al. 1984).

Table 1. Detection of three peanut viruses using the direct antigen coating (DAC), protein-A coating (PCA) and double antibody sandwich (DAS) form of ELISA.

Sample[1]	Absorption value—A410[7]		
	DAC	PAC	DAS
1. Groundnut seed[2]			
Healthy	0.07	0.11	0.08
PMV-infected[3]	1.39	0.97	0.96
2. Groundnut tissue[4]			
Healthy	0.08	0.13	0.08
PMV-infected[3]	1.50	1.25	0.69
3. Groundnut tissue[2]			
Healthy	0.19	0.10	0.28
TSWV-infected[5]	1.24	0.36	1.05
4. Groundnut tissue[2]			
Healthy	0.12	0.14	0.09
PCV-infected[6]	0.38	0.46	0.31

1. All dilutions were based on the original mess of the tissues.
2. 1:100 dilution.
3. Peanut mottle virus (PMV).
4. 1:1000 dilution.
5. Tomato spotted wilt virus (TSWV).
6. Peanut clump virus (PCV).
7. Mean absorbance A of three wells for each dilution in three separate experiments.

Table 2. Enumeration of rhizobia in peat inoculant, stored at 37° C for 8 days[1].

Enumeration method	Number of rhizobia estimated (10-8 cells g^{-1})
Plate count method	1.01
Plant infection method	2.41
Direct ELISA	1.37

1. Source: Nambiar and Anjaiah 1985.

Detection of Aflatoxin B_1

Aflatoxins are extremely potent hepatocarcinogens affecting a wide range of animals including man (Butler 1974). Of the four aflatoxins (B_1, B_2, G_1, and G_2) naturally occurring in crop produce, B_1 is the most potent and common. The method most commonly used for aflatoxin detection and quantification is thin-layer chromatography (TLC). This method is time-consuming, expensive, and permits analysis of only limited numbers of samples. Immunoassays have also been used for aflatoxin determination. They are sensitive, highly specific, and, being cheap, permit analyses of large numbers of samples. Since aflatoxins are low molecular-weight compounds, they do not possess antigenicity. However, if the aflatoxin molecule is conjugated with a protein, such as bovine serum albumin (BSA), it can be used to produce a specific antiserum (Fig.4). The oxime derivative of aflatoxin B_1 (aflatoxin B_1—carboxymethyloxime) conjugated to BSA was found to be suitable for production of aflatoxin B_1 specific antibodies of high titre (Gaur et al. 1980). We describe below a competitive ELISA procedure which we have used successfully for the detection of aflatoxin B_1 in groundnut seed samples. In principle the method adopted is similar to that described by Morgan et al. (1986).

Commercially obtained hapten for aflato[illegible]B_1 (oxime-bovine serum albumin, BSA) was used to prepare a polyclonal antiserum in rabbits. The oxime-BSA was adsorbed to the wells of an ELISA plate (NUNC)®. BSA was then added to saturate the wells. Antiserum produced for oxime-BSA was diluted to 1:40 000 and mixed with various concentrations of pure aflatoxin B_1 standard, or samples of groundnuts with antisera containing various concentrations of aflatoxin, and then preincubated at 37° C for 1 hour. Prior to mixing, the groundnut seed samples were homogenized in 50% aqueous methanol, filtered, and 10-fold dilutions of the extract

in saline were used. Test samples and pure toxin, following incubation with antiserum, were added to ELISA plates, precoated with the oxime-BSA. Ig present in the antiserum, not neutralized by toxin, adsorbed to oxime-BSA. In the final step, Ig attached to oxime-BSA were detected by alkaline phosphatase conjugated rabbit Fc specific Ig. The intensity of color produced by the substrate, P-nitrophenyl phosphate, was inversely proportional to the concentration of toxin present. By employing a standard curve prepared for the pure toxin (Fig. 5) it was possible to estimate the aflatoxin B_1 concentrations in the test samples (Table 3). Results of the ELISA test were compared with those from analyses by TLC and there was general agreement between the tests.

Conclusions

At ICRISAT we have successfully used various form. of ELISA in the study of viruses, MLOs, *Rhizobium*, and aflatoxins. We have used only polyclonal antisera. Specific antisera, especially monoclonal antibodies, are essential for the differentiation of several native strains of rhizobia such as IC 6006 and IC 6009. Polyclonal antibodies for these strains cross-reacted with several native *Rhizobium* populations. Similar problems were experienced

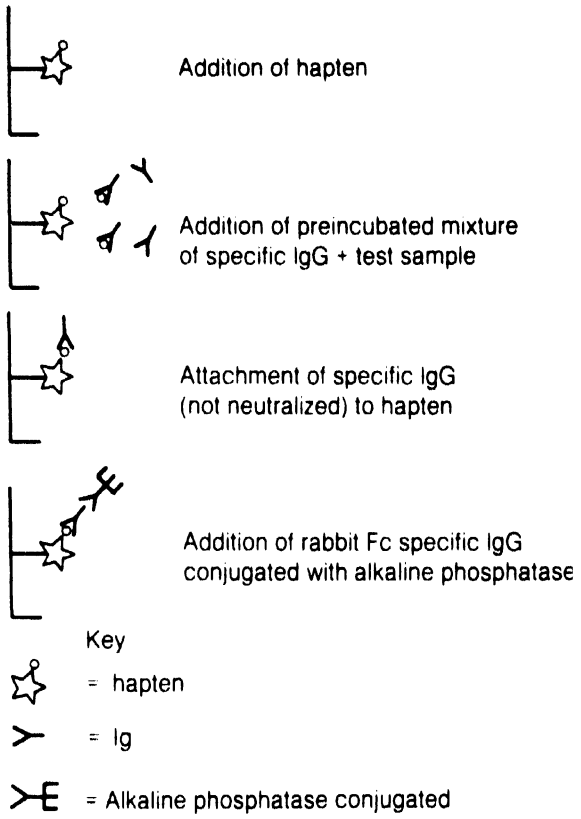

Figure 4. Principle of a competitive enzyme-linkec immunosorbent assay procedure for aflatoxin B estimation.

Table 3. Quantification of aflatoxin in groundnut samples by competitive ELISA.

Sample no. (naturally infected seeds)	Genotype	Toxin conc. ng g^{-1} seed	
		Mean	SE±
1	Ah 7223	1.73	0.461
2	J 11	0.52	0.161
3	U4-47-7	0.51	0.071
4	C55-437	1.19	0.499
5	UF 71513	2.75	0.999
6	PI 337394 F	1.37	0.482
7	JL 24	1.04	0.293
8	JL 24	2.39	0.723
9	TMV 2	2.19	0.602
10	EC 76446 (292)	4.13	0.887
11	EC 76446 (292)	2.43	0.531
12	NC Ac 17090	0.38	0.018
13	NC Ac 17090	2.06	0.525
14	NC Ac 17090	0.57	0.132
15	ICG 3204	1758	588
16	ICG 3206	16115	5522
17	Breeding line	259424	88476
18	Breeding line	259263	88522

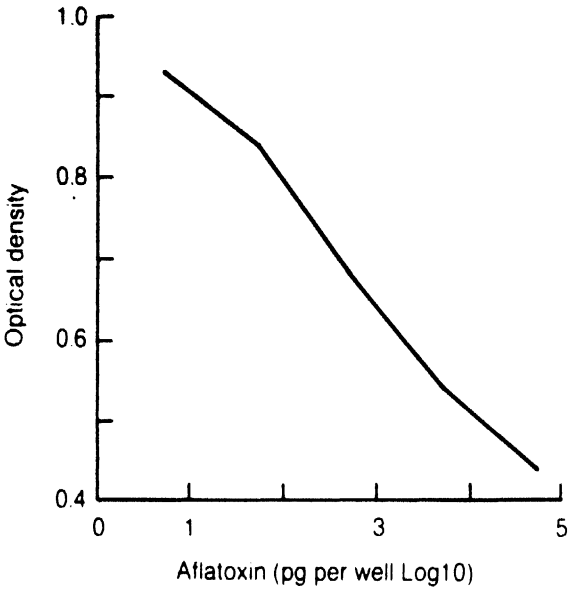

Figure 5. Competitive enzyme-linked immunosorbent assay for aflatoxin B_1. The standard competitive curve was prepared for toxin prepared in methanol-saline, as described in the text. The response was linear between 10 and 100 ng of the toxin. Results represent the average of five determinations.

when polyclonal antisera were used for detecting mycorrhizal fungi in roots. Since ELISA has already been used for distinguishing several species in the Endogonaceae (Aldwell et al. 1985), monoclonal antibodies (MAbs) offer immense potential for the detection of mycorrhizal fungi by ELISA.

We hope to extend our initial research results on aflatoxin B_1 to other aflatoxins and to other mycotoxins. In the competitive ELISA procedure adopted, small variations in absorption values resulted in wide differences in the estimates of toxin concentration. To overcome this setback we are testing other ELISA procedures: these include enzyme-labeled toxins, use of MAbs, and different enzyme and substrate combinations. We are also trying to improve the procedures for extracting the toxin from seed samples. For the detection of individual forms of a toxin, e.g., aflatoxin B_1, G_1 or G_2, MAbs are essential.

We are also interested in using MAbs for the estimation of gibberellins and other plant growth hormones in plant tissue.

Bacterial wilt of groundnut, caused by *Psuedomonas solanacearum* E.F. Smith, is an important disease in parts of southeast Asia. Since the bacterium is known to occur as several different, morphologically indistinguishable strains (Hayward 1986), the ELISA test combined with the utilization of MAbs offers excellent prospects for the detection of these strains.

References

Aldwell, F.E.B., Hall, I.R., and **Smith, J.M.B.** 1985. Enzyme-linked immunosorbent assay as an aid to taxonomy of the Endogonaceae. Transactions of the British Mycological Society 84:399-402.

Barbara, D.J., and **Clark, M.F.** 1982. A simple indirect ELISA using $F(ab)_2$ fragments of immunoglobulins. Journal of General Virology 58:315-322.

Butler, W.H. 1974. Aflatoxins. Pages 1-28 *in* Mycotoxins (Purchase, F.M., ed.). New York, USA: Elsevier.

Clark, M.F., and **Adams, A.N.** 1977. Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. Journal of General Virology 34:475-483.

Edwards, M.L., and **Cooper, J.I.** 1985. Plant virus detection using a new form of indirect ELISA. Journal of Virological Methods 11:309- 319.

Engvall, E., and **Perlmann, P.** 1971. Enzyme-linked immunosorbent assay (ELISA). Quantitation assay of immunoglobulin G. Immunochemistry 8:871-874.

Gaur, P.K., El-Nakib, O., and **Chu, F.S.** 1980. Comparison of antibody production against aflatoxin B_1 in goats and rabbits. Applied and Environmental Microbiology 40:678-680.

Hayward, A.C. 1986. Bacterial wilt caused by *Pseudomonas solanacearum* in Asia and Australia: an overview. Pages 15-24 *in* Bacterial wilt disease in Asia and the South Pacific: proceedings of an International Workshop, Los Banos, Laguna, Philippines (Persley, G.J., ed.). ACIAR Agricultural Proceedings no.13. Canberra, Austrália: Australian Centre for International Agricultural Research.

Hobbs, H.A., Reddy, D.V.R., and **Reddy., A.S.** 1987a. Detection of a mycoplasma-like organism in peanut plants with witches' broom using indirect enzyme-linked immunosorbent assay (ELISA). Plant Pathology 36:164

Hobbs, H.A., Reddy, D.V.R., Rajeshwari, R., and **Reddy, A.S.** 1987b. Use of direct antigen coating and protein A coating ELISA procedures for detection of three peanut viruses. Plant Disease 71:747-749.

Koenig, R. 1978. ELISA in the study of homologous and heterologous reactions of plant viruses. Journal of General Virology 40:309-318.

Morgan, M.R.A., Kang, A.S., and **Chan, H.W.S.** 1986. Aflatoxin determination in peanut butter by enzyme-linked immunosorbent assay. Journal of the Science of Food and Agriculture 37:908-914.

Mowat, W.P. 1985. Tulip chlorotic blotch virus, a second potyvirus causing tulip flower break. Annals of Applied Biology 106:65-73.

Nakane, P.K., and Pierce, G.E., Jr. 1966. Enzyme-labelled antibodies: .preparation and application for the localization of antigens. Journal of Histochemistry and Cytochemistry 14:929-931.

Nambiar, P.T.C., and Anjaiah, V. 1985. Enumeration of rhizobia by enzyme-linked immunosorbent assay (ELISA). Journal of Applied Bacteriology 58:187-193.

Nambiar, P.T.C., Srinivasa Rao, B., and Anjaiah, V. 1984. Studies on competition, persistence, and methods of application of a peanut Rhizobium strain, NC 92. Peanut Science 11:83-87.

Reddy, D.V.R. 1986. Report on a visit to Thailand and Indonesia. Legume Pathology Report. Patancheru, A.P. 502 324, India: International Crops Research Institute for the Semi-Arid Tropics. (Limited distribution.)

van Regenmortel, M.H.V., and Burckard, J. 1980. Detection of a wide spectrum of tobacco mosaic virus strains by indirect enzyme-linked immunosorbent assays (ELISA). Virology 106:327-334.

van Weeman, B.K., and Schuurs, A.H.W.M. 1971. Immunoassay using antigen enzyme conjugates. FEBS (Federation of European Biochemical Societies) Letters 15:232-236.