APPLICATION OF THE ENZYME-LINKED IMMUNOSORBENT ASSAY IN THE QUALITY CONTROL OF LEGUME INOCULANTS

by

HESTER HELENA LOCHNER

Submitted to the Faculty of Science
(Department of Microbiology and Plant Pathology)

UNIVERSITY OF PRETORIA

in partial fulfilment of the requirements
for the degree of

M.Sc.

PRETORIA

MAY, 1986

LEADER: PROF. P.L. STEYN
CO-LEADER: PROF. B.W. STRIJDOM

ACKNOWLEDGEMENT

Sincerest gratitude is extended to the following persons:

- Dr. B.W. Strijdom for his interest and guidance during the course of the investigation.

- Prof. P.L. Steyn for his interest, suggestions and assistance in the preparation of the manuscript.

- Dr. B.D. Kishinevsky for his interest, guidance in the ELISA techniques employed and suggestions during this project.

- My colleagues for their support and assistance.

- My parents for their encouragement.

as well as to:

The Department of Agriculture and the Plant Protection Research Institute for financial support and use of facilities.

INDEX

LIST OF ABBREVIATIONS

A_{405}	-	absorbance at 405 nm
B.S.	-	British standards
BSS	-	buffered salt solution
cfu	-	colony forming units
CR-YM agar	-	Congo red-yeast extract-mannitol agar
DAS ELISA	-	double-antibody-sandwich ELISA
d	-	day
ELISA	-	enzyme-linked immunosorbent assay
F	-	value calculated in analysis of variance to test goodness-to-fit of regression lines. F = ratio of the mean squares If F is larger than the tabulated value, we may claim that b is significantly different from 0.
IgG	-	immunoglobulin G
IgM	-	immunoglobulin M
kGy	-	kilo Gray
PBS	-	phosphate-buffered saline
PBS-Tween	-	phosphate-buffered saline with Tween 20
pers comm	-	personal communication
P.P.R.I.	-	Plant Protection Research Institute
PVP	-	polyvinyl pyrrolidone
r	-	correlation coefficient, the value of r will give a valid measure of the size of linear relation between two parameters
S.A.R.C.	-	South African Rhizobium Collection
(strain) antigen	-	rhizobial strain was the source of the antigen
(strain) IgG	-	solution of IgG specific to the rhizobial strain antigen
(strain) coating IgG	-	solution of IgG specific to the rhizobial strain antigen was used to coat the microtitre plates
U-DALS	-	University-Department of Agriculture Laboratory Service
YM agar	-	yeast extract-mannitol agar
YM broth	-	yeast extract-mannitol broth

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1

INTRODUCTION

The legume-*Rhizobium* symbiosis is the most important biological nitrogen-fixing system and its benefit to agriculture is well known (e.g. Fred, Baldwin & McCoy, 1932). For example, grain legumes, lucerne and other legume-based pastures are currently estimated to contribute 95 000 ton (t) of nitrogen per year to South African agriculture (Strijdom & Wasserman, 1984). This is nearly one fifth of the nitrogen administered as fertilizer (Strijdom & Wasserman, 1984). There is also much potential for the further development of biological nitrogen fixation in South Africa. Strijdom & Wasserman (1984) concluded that a total area of approximately 17 million hectare could be brought under legume-based pastures in this country. If this were done, the annual incorporation of nitrogen into the soil would be more than 400 000 t (Strijdom & Wasserman, 1984).

To obtain the maximum benefit from symbiotic nitrogen fixation, legume seed should be inoculated prior to sowing when the appropriate rhizobia are absent from the soil (Strijdom, 1977). A sufficient number of specifically invasive and effective rhizobia must be applied to the seed surface to ensure nodulation of the legume under cultivation (Vincent, 1970). The aim is to establish a vigorous

population of the inoculated strain in the rhizosphere of the seedling to achieve early and effective nodulation of the legume. Successful nodulation by an effective strain of Rhizobium or Bradyrhizobium may result in a substantial increase in the yield of the legume crop (Nutman, 1976).

The production of legume inoculants for agricultural purposes in South Africa dates back to the fifties. The early inoculants were frequently of inferior quality and often resulted in crop failures (Strijdom, 1977). An effective inoculant industry was only established following legislation in 1966 enforcing registration of legume inoculants by manufacturers (Strijdom, 1977).The subsequent improvement in the quality of legume inoculants can inter alia be attributed to the implementation of an independent quality control system, currently in operation, whereby strain selection and quality tests are carried out by microbiologists at the Plant Protection Research Institute (P.P.R.I.) (Strijdom, 1977). This ensures that inferior legume inoculants are not marketed. Legume inoculants available on the South African market are at present of excellent quality and compare favourably with the highest quality inoculants produced in countries such as Australia and the USA (Strijdom, 1977; Strijdom & Jansen van Rensburg, 1981).

A high quality inoculant is defined as one which contains a sufficient and specified number of cells of a suitable Rhizobium strain, from the date of manufacture until the

expiry date (Jansen van Rensburg & Strijdom, 1969). Quality control tests presently used supply information on the number of viable cells and contaminats in the peat carrier by means of plate counts while strain identity is confirmed by serological methods. Further improvement of the quality control system is desirable, as the plate count technique is time-consuming, laborious and therefore expensive. The preparation of inoculants is also a lengthy process, especially when slow-growing rhizobia are used, thus any reduction in the checking and approval of the inoculant before marketing will be of economical benefit to inoculant manufacturers (P.L. Steyn, pers comm) as well as the P.P.R.I.

The enzyme-linked immunosorbent assay (ELISA) is a serological procedure in which a solid phase carrier is used to separate free antigen and antibody from antigen-antibody complexes. Enzyme-linked antibodies are then used for detecting these antigen-antibody complexes. This technique offers an attractive alternative to the plate count technique for enumeration of rhizobia in peat, as it is used to identify strains of rhizobia (e.g. Kishinevsky & Bar-Joseph, 1978), can be completed within two days and has quantitative potential (Clark & Adams, 1977). Because of technical difficulties in the practical application of ELISA, Tchan (1982) suggested that ELISA would only be useful for small scale experimentation rather than for large-scale ecological studies. In contrast, other workers successfully used ELISA to enumerate rhizobia in quality

control tests and ecological studies (Nambiar & Anjaiah, 1985).

The present investigation was undertaken with the object of reducing labour, time and costs attached to quality control tests. The indirect and double-antibody-sandwich (DAS) ELISA techniques were evaluated for their use in the detection and counting of viable rhizobia in peat, in order to determine whether they could safely replace, alone or with a complementary test, the slower and more costly plate count and serological strain identification tests in the quality control system in South Africa.

CHAPTER 2

LITERATURE REVIEW

"Many investigations are lost for years, if not forever, in the jungle of journals and the tangle of tongues."

W.J. Humphrey

The Leguminosae is one of the largest families of flowering plants (Allen & Allen, 1961; Allen & Allen, 1981). For this worldwidely distributed family 16 000 to 19 000 species in about 750 genera are estimated (Allen & Allen, 1981). The nodulating habit among this family, that may lead to an effective legume-Rhizobium symbiosis, is very widespread (Allen & Allen, 1981). Exceptions occur, as the ability to nodulate seems consistently absent within certain sections of the family, e.g. members of the Caesalpinioidae (Allen & Allen, 1981). Trinick (1973) and Akkermans, Abdulkadir & Trinick (1978) reported the ability of Rhizobium sp. to form effective nodules on the non-legume Parasponia rugosa. Aspects of the role leguminous plants have in agricultural programmes throughout the world, are treated inter alia by Ayanaba & Dart (1977), Vincent, Whitney & Bose (1977) and Graham & Harris (1982).

On the South African scene, the Leguminosae is the third largest family of seed plants and consists of about 1 400 species and 101 genera (Philips, 1951; Strijdom & Wasserman,1984). With the exception of members of the

Caesalpinioideae, most of the leguminous species are able to fix nitrogen (Strijdom & Wasserman, 1984). The utilization of biological nitrogen fixation in agriculture of this country is reviewed by Grobbelaar & Strijdom (1971), Strijdom (1977) and Strijdom & Wasserman (1984).

2.1 Necessity of inoculation

Rhizobia are widely distributed in the soil as a result of the natural distribution of the Leguminosae and through the cultivation of leguminous crops (Allen & Allen, 1961; Brockwell, 1980). Despite of this, many soils are devoid of rhizobia able to effectively nodulate legumes introduced to new regions or virgin soils (Brockwell, 1977). These soils may contain no rhizobia or high proportions of ineffective nodulating rhizobia (Parker, Trinick & Chatel, 1977; Peterson & Loynachan, 1981). Specificities in nodule formation and nitrogen fixation exist due to genotype interactions between the host legume and the invading Rhizobium or Bradyrhizobium (Date, 1976). Limitations imposed by this specificity, frequently prevent a legume from achieving a productive symbiosis (Vincent, 1977). An encounter between legume and Rhizobium or Bradyrhizobium, that will result in an effective symbiosis, can therefore not be left to chance (Date & Roughley, 1977; Brockwell, 1977).

Many early records of the need for seed inoculation

have been based on poor nodulation of a legume leading to crop failure (Fred et al., 1932). More efficient means in establishing the need for inoculation are investigations of occurrence, frequency and effectiveness of rhizobia and knowledge of origin, distribution and growth requirements of the legume (Brockwell, 1977).

In many areas in South Africa, the inoculation of agriculturally important legumes such as Vigna anguiculata (van der Merwe, Strijdom & Uys, 1974), Arachis hypogaea (van der Merwe et al., 1974) and Aspalathus linearis (Deschodt & Strijdom, 1976) has doubtful value, whereas the inoculation of Glycine max (Jansen van Rensburg, Strijdom & Kriel, 1976), Lupinus spp. (Nel, 1962), Pisum spp. (Strijdom, 1977), foreign Trifolium spp. (Jones, Strijdom & Theron, 1974) and Medicago spp. (Bartholomew, 1971) is recommended (Strijdom, 1977). Results with the inoculation of beans (Phaseolus vulgaris and Phaseolus multiflorus) indicated inconsistent reaction (Strijdom, 1977). It should be kept in mind that no general recommendations can be made. Each legume should be judged with regard to its specific region of cultivation (Strijdom, 1977).

Allen & Allen (1958) have listed four conditions that warrant the treatment of legume seed with inoculant preparations, i.e. the absence of the same or a symbiotically related legume in the immediate past history of the land, poor nodulation when the same crop was grown on the land previously, when a legume follows a non-leguminous

plant in a rotation and in land reclamation undertakings.

Forecasting the need to introduce rhizobia into a soil is thus one of the most important considerations faced by the agricultural adviser.

2.2 High quality inoculants

The quality of legume inoculants depends on both the number of rhizobia they contain and their effectiveness in fixing nitrogen with the intended host (Roughley, 1976; Vincent, 1977). According to Thompson (1980) and Strijdom & Jansen van Rensburg (1981), high quality inoculants would contain ca. 10^9 colony forming units (cfu).$gram^{-1}$ peat. The inoculant strain should be able to form N_2-fixing nodules on legumes for which it is recommended, under a range of field conditions and over a range of root temperatures (Burton, 1979). Other attributes needed in an inoculant strain are competitiveness in nodule formation, survival and multiplication in soil, peat and on the seed and good growth in culture (Brockwell, Dudman, Gibson, Hely & Robinson, 1968; Date, 1976; Burton, 1976).

Peat has been the most commonly used carrier base for commercial inoculants, and is generally considered the most dependable (Fred et al., 1932; Vincent, 1974;

Strijdom & Deschodt, 1976; Burton, 1979). Not all peats satisfy the requirement for a good Rhizobium or Bradyrhizobium carrier, as they are diverse in nature and may vary widely in ability to support growth and survival of rhizobia (Roughley & Vincent, 1967; Steinborn & Roughley, 1975; Roughley, 1982). Peats of similar chemical composition may differ in suitability as a Rhizobium or Bradyrhizobium carrier (Roughley & Vincent, 1967). In other cases, peats with differences in chemical composition have been used with success in inoculants (Burton, 1965; Roughley & Vincent, 1967; Roughley, 1970; Strijdom & Deschodt, 1976).

Sterilization is one of the most rewarding of various carrier treatments considered beneficial for the survival of rhizobia in inoculants (Strijdom & Deschodt, 1976). Data obtained by independent tests on each of 483 batches of legume inoculants for Glycine max, Medicago sativa and Arachis hypogaea, indicated that gamma-irradiation at a dose of 50 kGy and steam sterilization for 3,5 h at 124 °C were equally effective for the production of high quality inoculants with South African peat (Strijdom & Jansen van Rensburg, 1981). In most cases, gamma-irradiated peat is used by South African manufacturers to produce inoculants (C.J. Otto, pers comm). Problems such as toxicity in overheated peat, and the influence of storage temperature, moisture and access of air have been well documented in a series of papers (Roughley & Vincent, 1967; Steinborn & Roughley, 1975; Roughley, 1982).

The initial cell density of the inoculum used to produce inoculant seems to play a role in inoculant quality (Meade, Higgins & O'Gara, 1985). These authors reported that peat supported the growth and survival of a R. leguminosarum biovar viceae strain when an inoculum with low initial cell density were used. In contrast, when an inoculum with high initial cell density were used, cell numbers declined rapidly when stored at room temperature and 4 °C. Meade et al. (1985) suggested that cells are not necessarily protected by the peat when very high cell densities were used, and that the protective action of peat is greater when cells are grown in it.

In general, the quality of South African inoculants, produced commercially in sterilized peat, is high and contaminated batches are rarely encountered (Jansen van Rensburg & Strijdom, 1974). Given the factors critical to survival of Rhizobium and Bradyrhizobium in peat carrier, and the natural variation to which strains of rhizobia are prone (Herridge & Roughley, 1975) it is essential that the quality of inoculants be monitored.

2.3 Quality control of legume inoculants

Excellent quality of legume inoculants is maintained by manufacturers in Australia (Vincent, 1977), South Africa (Jansen van Rensburg & Strijdom, 1974), New Zealand (Burton, 1982) and Canada (B.W. Strijdom, pers

comm) whose products are subjected to independent quality control.

In South Africa only Rhizobium and Bradyrhizobium strains provided by the Rhizobium unit of the P.P.R.I. may be used for inoculant production (Strijdom, 1977). Not only is the Rhizobium unit responsible for testing, selecting and maintenance of effective Rhizobium and Bradyrhizobium strains, but also to ensure that inoculants contain sufficient numbers of the right kind of rhizobia to cause effective nodulation of legumes for which they are recommended (Jansen van Rensburg & Strijdom, 1974). Inoculants are rejected for marketing if Rhizobium or Bradyrhizobium strain identity is doubtful, if the number of viable rhizobia.g^{-1} moist peat is less than 5×10^{8} cfu.g^{-1} peat, if the pH of the inoculant is below 6,5 or above 7,5, if contaminants are present on plates streaked with 10^{-5} dilutions and if the mass of an inoculant packet is less than 254 g (Strijdom & Jansen van Rensburg, 1981). Manufacturers are allowed an expiry date which is in most cases six months after the date of commencement of the tests (Strijdom, 1977).

In South Africa, sterilized peat is the only form used to produce inoculant (Strijdom & Jansen van Rensburg, 1981). Tests on the broth used to inoculate the carrier are not conducted, since the final number of rhizobia in the peat is independent of the number added in the broth (Roughley, 1968). The important checkpoint in determining the quality

of legume inoculants is therefore tests with manufactured inoculant (Vincent, 1977).

The plate count (viable count) is used to enumerate rhizobia in inoculant produced from sterile peat (Vincent, 1970; Vincent, 1974). This technique requires the preparation of a set of serial dilutions to provide 30 to 300 colonies at some step in the series. 0,1 cm^3 of the diluted suspension is then spread over the surface of agar and incubated. The number of rhizobia in the carrier is calculated by recording the number of colonies on the plates. Counts are multiplied by the dilution factor and for statistical purposes, are best converted to logarithms (Vincent, 1970). When considering the incubation period of 4 to 5 d for the faster-growing rhizobia, and up to 10 d for the slower-growing rhizobia, this method is time-consuming. The plate count also provides information on the presence of contaminants (Vincent, 1970). Autoclaved carriers are usually free of contaminants, whereas gamma-irradiation may not provide full sterilization (Strijdom & Deschodt, 1976). Any significant contamination will justify culture rejection (Vincent, 1977).

When testing inoculant produced from sterile peat carrier, a serological strain check becomes the only proof of strain identity (Thompson, 1980). Strain identity is confirmed by means of spot tests on single colonies, using the agglutination method (Strijdom & Jansen van Rensburg, 1981). Cell clumps and instability of some antigens in

saline can complicate assessment of results (Vincent, 1970).

Bacteriologically controlled plant infection tests are generally used to count Rhizobium and Bradyrhizobium cells in legume inoculants produced from non-sterile peat (Vincent, 1977; Thompson, 1980). In this method, test plants grown aseptically, are inoculated with aliquots from a dilution series of the suspension being examined. The number of rhizobia in the peat can be calculated from the proportion of test plants that forms nodules at each dilution (Brockwell, 1963). As discussed by Brockwell (1980) this method is laborious and time-consuming. Olsen, Rice, Stemke & Page (1983) found the plant infection technique, when used to evaluate the quality of alfalfa inoculant, only specific at the Rhizobium species level.

The conventional quality control tests have proved to be successful for evaluation of inoculant quality (Vincent, 1977; B.W. Strijdom, pers comm). However, one will be able to economize materials, time and labour if rhizobia can be identified as well as counted in peat, by a single test.

2.4 Methods of identification of strains of rhizobia

Techniques available for recognition of Rhizobium and

Bradyrhizobium strains are the use of genetic markers such as antibiotic resistance (Schwinghamer & Dudman, 1973) and auxotrophy (Johnston & Beringer, 1975); culture characteristics (Norris, 1958); symbiotic rating (Brockwell, 1971); sensitivity to phage and bacteriocin (Schwinghamer & Reinhardt, 1963); nodule characters (Cloonan, 1963) and antigenic properties (Fred et al., 1932). Natural markers tend to be less reliable or more difficult to apply than serological differences or induced mutant markers (Schwinghamer & Dudman, 1980). Genetic markers pose possibilities of unseen modifications to carefully selected strains (Jones & Bromfield, 1978; Josey, Beynon, Johnston & Beringer, 1979). Serological techniques are frequently favoured for identification of Rhizobium and Bradyrhizobium strains because of their specificity and reproducibility (Knootz & Faber, 1961; Vincent, 1970; Dudman, 1977).

2.5 Serology of rhizobia

2.5.1 Antigenic characteristics of rhizobia

Early workers recognized several serotypes within species of Rhizobium and Bradyrhizobium (Stevens, 1923; 1925; Wright, 1925; Wright, Sarles & Holst, 1930). Stevens (1923) demonstrated the antigenic heterogeneity of rhizobia able to nodulate legumes within a single cross-inoculation group and on one

plant of a species. Rhizobia from closely adjoining areas and from different nodules on the same plant could fall into distinct serogroups (Hughes & Vincent, 1942). No common group somatic antigens were found for Rhizobium strains of pea and clover (Kleczkowska & Thornton, 1944), nor for B. japonicum strains (Knootz & Faber, 1961; Skrdleta, 1969a). Purchase, Vincent & Ward (1951) postulated 16 O antigens in only 15 strains of R. meliloti. All Rhizobium and Bradyrhizobium species studied contain antigenically distinct strains that may or may not share common antigens, i.e. no species is antigenically homogenous (Dudman, 1977). Bushnell & Sarles (1939) and Kleczkowska & Thornton (1944) found that cross agglutination could take place between rhizobia of different species. According to Graham (1976) serological methods have been little used in the study of rhizobia. Strain identification within a species is at present the prime use of serology in the study of rhizobia.

The usefulness of any marker technique depends on the stability of the markers with time (Diatloff, 1977). It is generally held that the antigenic characteristics of Rhizobium and Bradyrhizobium strains are stable and do not change with cultural conditions, plant passage or length of storage (Stevens, 1923; Wright, 1925; Fred et al., 1932; Purchase et al.,1951; Vincent, 1954; Diatloff, 1977). Although marked serological specificity has been demonstrated for strains of Rhizobium sp. (e.g. Purchase et al., 1951; Graham, 1963; Holland, 1966) and Bradyrhizobium sp. (e.g. Means, Johnson & Date, 1964; Date & Decker, 1965;

Skrdleta, 1965) few long term field experiments to test stability have been reported (Diatloff, 1977). Strains of Bradyrhizobium sp. (Lotononis) have been exposed to the rigours of the field environment, including inundation and the periodic absence of the legume host for a period of 12 years, yet no change was detected in the antigenic properties of the strains (Diatloff, 1977). It should be noted that Bradyrhizobium sp. (Lotononis) is taxonomically atypical of the rhizobia (Norris, 1958; Vincent, 1982). According to Brockwell, Schwinghamer & Gault (1977), strains of R. leguminosarum biovar trifolii have maintained their recognizable surface antigens for several to many years. Jansen van Rensburg & Strijdom (1985) employed the gel immunodiffusion technique for the detection of inoculant strains introduced into field soils four to eight years before their study. Their results indicated antigenic stability of inoculant strains of R. leguminosarum biovar trifolii, R. meliloti, B. japonicum and Bradyrhizobium sp. (Lotus). Wilson, Humphrey & Vincent (1975) reported decrease in antigenic specificity of R. meliloti strains after prolonged culture in the laboratory, whereas freshly isolated, completely unrelated cultures of R. meliloti showed a degree of specificity in their agglutination reactions similar to that recorded in 1941 (Vincent, 1982).

A number of workers noted effects of the nature of the growth medium on the serological behaviour of rhizobia. Vintikova, Srogl & Skrdleta (1961) stated that the nature of the growth medium can affect the antigenic properties of

R. meliloti. Vincent & Humphrey (1968) found a shaken liquid culture inferior to one grown on a moist agar surface for the demonstration of flagellar agglutination. Media that favour extracellular polysaccharide production, can give a suspension of high viscosity, that may interfere with agglutination (Humphrey & Vincent, 1963). The calcium status of the medium can influence the antigenic structure of cells of R. leguminosarum biovar trifolii by altering the strength of cell surfaces (Vincent & Humphrey, 1968). Calcium deficiency also permits outward diffusion of internal components. According to Dudman (1977), different immunodiffusion patterns can be obtained by growing the same strain in the same medium in broth or agar form. He found antigens of R. meliloti to be more susceptible to the nature of the growth medium than antigens of R. leguminosarum biovar trifolii. It seems necessary to use standardized media when growing strains for the purpose of serological comparison.

Mutations, which affect many features of rhizobia, generally have little effect on their antigenic characteristics (Vincent, 1982). According to Kleczkowska & Thornton (1944), ability to cross-inoculate, susceptibility to bacteriophage and effectiveness in nitrogen fixation in the host plant were shared by antigenically different strains. Kleczkowska & Thornton (1944), as well as Vincent (1944), found antigenically identical strains to differ in nitrogen-fixing ability. Little effect on antigenic characteristics of strains who lost their symbiotic capacity

have been reported (Almon & Baldwin, 1933; Vincent, 1944; 1954; Kleczkowska, 1950). Parasitic and chlorotic strains studied by Knootz & Faber (1961), were antigenically similar to the wild-type strains. Although Wright (1925) reported effectivity differences between two serologically distinct groups of R. meliloti, results in general indicated that no relationship between physiological and antigenic characteristics of rhizobia exists.

Genetic changes in strains associated with colony form and symbiotic efficiency have been responsible for changes in antigenicity (Vincent, 1982). Lorkiewicz & Dusinski (1963) reported antigenic differences between 'smooth' and 'rough' colonies of R. leguminosarum biovar trifolii. The most consistent change detected in preparations of 'rough' mutants was the loss of rhamnose (Lorkiewicz & Russa, 1971). Colony variants of some strains of R. leguminosarum biovar trifolii may lack an antigen possessed by the parent strain (Vincent, 1982), or may be antigenically identical to the parent strain (Dudman, 1968). By comparing surface and internal antigens of a R. leguminosarum biovar trifolii and R. leguminosarum biovar viciae wild-type strain respectively before and after genetic modification, Kaushik, Dadarwal & Venkatraman (1973) indicated that the detailed nature of rhizobial antigens can be subjected to serious genotype modifications. It should be noted that these results include the study of only a few strains of two Rhizobium species.

When taking reassuring evidence (Diatloff, 1977; Brockwell *et al.*, 1977; Jansen van Rensburg & Strijdom, 1985) into account, antigenic properties as a marker for *Rhizobium* and *Bradyrhizobium* strain identification seem reliable.

2.5.2 Serological techniques

Various serological techniques have been used in the study of rhizobia (Dudman, 1977; Vincent, 1982). Techniques like complement fixation, immunoelectrophoresis and quantitative precipitation have not proved convenient for identifying *Rhizobium* and *Bradyrhizobium* strains (Vincent, 1970; Dudman, 1977).

Agglutination was the first serological technique used for strain identification of e.g. *R. meliloti* (Dunham & Baldwin, 1931; Hughes & Vincent, 1942), *R. leguminosarum* biovar *trifolii* (Dunham & Baldwin, 1931; Read, 1953; Vincent & Waters, 1953; 1954; Scheffler & Louw, 1967), *B. japonicum* (Dunham & Baldwin, 1931; Johnson & Means, 1963; 1964; Means *et al.*, 1964; Johnson, Means & Weber, 1965; Skrdleta, 1965; Damirgi, Frederick & Anderson, 1967; Caldwell & Vest, 1968; Caldwell & Weber, 1970; Gibson, Dudman, Weaver, Horton & Anderson, 1971), *R. leguminosarum* biovar *viciae* (Dunham & Baldwin, 1931) and chick pea strains (Okon, Eshel & Henis, 1972).

Surface-located antigens are detected by the agglutination technique (Dudman, 1977). By distinguishing between flagellar and somatic antigens, strain recognition is enhanced as somatic antigens are more strain-specific than flagellar antigens (Vincent, 1941; Purchase et al., 1951; Graham 1963; Loos & Louw, 1964). Antibody adsorptions have improved strain recognition by the agglutination technique (Vincent, 1941; 1942; Kleczkowska & Thornton, 1944). Other problems encountered using this technique are instability of cultures in saline and non-agglutinatability of sub-cultures of some strains (Knootz & Faber, 1961; Vincent, 1982).

Dudman (1964) applied the Ouchterlony gel immunodiffusion technique to detect soluble antigens of rhizobia. Although gel immunodiffusion is a much faster method for identification of isolates with homologous antiserum than agglutination, immunodiffusion results did not always correlate with flagellar agglutination results (Scheffler & Louw, 1967). Whereas isolates exhibiting strong somatic cross-agglutination shared diffusible antigens, this was not the case for isolates exhibiting weak somatic cross-agglutination (Scheffler & Louw, 1967). They concluded that immunodiffusion was not an absolute means of strain identification. As somatic antigens are highly strain specific, Vincent (1941; 1942), Holland (1966), Skrdleta (1969b) and Parker & Grove (1970) stated that greater confidence can be placed in identification of strains made by immunodiffusion than by agglutination. The immunodiffusion technique assisted inter alia in strain

identification of R. meliloti (Dudman, 1964; Sinha & Peterson, 1980; Jansen van Rensburg & Strijdom, 1985), R. leguminosarum biovar trifolii (Holland, 1966; Scheffler & Louw, 1967; Dudman & Brockwell, 1968; Jansen van Rensburg & Strijdom, 1985) B. japonicum (Jansen van Rensburg & Strijdom, 1969; 1985; Skrdleta, 1969a; 1969b; 1973; Dudman, 1971) and Bradyrhizobium sp. (Lotus) (Jansen van Rensburg & Strijdom, 1985). By using immunodiffusion, group antigens have been detected among fast-growing (Vincent & Humphrey, 1970) and slow-growing rhizobia (Vincent, Humphrey & Skrdleta, 1973). Kremer & Wagner (1978) reported the use of gel immunodiffusion for detection of rhizobial antigen in soil. However, cell breakage (Vincent & Humphrey, 1970) or leakage of internal antigens from calcium deficient cells (Humphrey & Vincent, 1965) can complicate recognition of serotypes within a species.

Cloonan & Humphrey (1976) introduced the indirect haemagglutination test for the detection of strains of R. leguminosarum biovar trifolii in nodules of subterranean clover plants. This technique depends on lipopolysaccharide on the cell surfaces adsorbing the specific antibody and so blocking the combination with, and agglutination of specific lipopolysaccharide-conjugated red blood cells (Cloonan & Humphrey, 1976). According to these authors, the sensitivity of the indirect haemagglutination test for detection of rhizobial lipopolysaccharide is greater than the sensitivity of the agglutination or immunodiffusion techniques.

The fluorescent antibody technique is a convenient and reliable method of strain recognition based on surface antigens, and has been used for direct typing of small and large nodules (Schmidt, Bankhole & Bohlool, 1968; Trinick, 1969), for strain identification (Jones & Russell, 1972), to recognize bacteroids (Staphorst & Strijdom, 1972), to detect a non-invasive cohabitant in nodules (van der Merwe, Strijdom & Jansen van Rensburg, 1972) and to study doubly infected nodules (Lindemann, Schmidt & Ham, 1974). According to van der Merwe & Strijdom (1973), the fluorescent antibody technique was more sensitive than gel immunodiffusion to detect cross-reactions among isolates from nodules of Arachis hypogaea. This technique also permits qualitative examination of the behaviour of rhizobia as free-living organisms in soil (Schmidt et al., 1968; Bohlool & Schmidt, 1970) and the approximate quantitative assessment of specific rhizobia in soil (Schmidt, 1974). This method should not be attempted with inferior reactants or optical equipment, and requires technical sophistication (Vincent,1982).

Kishinevsky & Bar-Joseph (1978) were the first to apply the enzyme-linked immunosorbent assay (Engvall & Perlmann, 1971; Van Weemen & Schuurs, 1971) to the identification of strains of Bradyrhizobium sp. (Arachis). Since then, the ELISA technique has been used to identify strains of Bradyrhizobium sp. (Lupinus) (Kishinevsky & Gurfel, 1980), R. leguminosarum biovar trifolii (Kishinevsky & Gurfel, 1980; Morley & Jones, 1980; Hodgson & Waid, 1981;

Kishinevsky, Maoz, Gurfel & Nemas, 1984), R. meliloti (Kishinevsky & Gurfel, 1980; Kishinevsky & Maoz, 1983; Kishinevsky et al., 1984; Martensson, Gustafsson & Ljunggren, 1984), R. leguminosarum biovar viciae (Berger, May, Berger & Bohlool, 1979; Kishinevsky & Gurfel, 1980; Kishinevsky & Maoz, 1983), Bradyrhizobium spp. of the cowpea miscellany (Kishinevsky & Gurfel, 1980; Kishinevsky, Gurfel, Lobel & Nemas, 1982; Kishinevsky & Maoz, 1983; Kishinevsky et al., 1984) and B. japonicum (Fuhrmann & Wollum II, 1985) in culture and nodules; to examine serological diversity of slow-growing rhizobia (Ahmad, Eaglesham & Hassouna, 1981); to identify Canadian selected R. meliloti strains in commercial inoculants (Olsen, Rice, Stemke & Page, 1981; 1983); to determine competitive abilities of two R. meliloti strains (Rice, Olsen & Page, 1984); for minimal antigenic characterization of eight R. meliloti strains (Olsen & Rice, 1984); in ecological (Jones & Morley, 1981; Renwick & Jones, 1985) and competition studies (Martensson & Gustafsson, 1985) of R. leguminosarum biovar trifolii; and for the enumeration of rhizobia in peat and soil (Nambiar & Anjaiah, 1985; Renwick & Jones, 1985). According to Engvall & Perlmann (1972) and Maolini & Masseyeff (1975), the ELISA technique is as sensitive as radioimmunoassays. Kishinevsky & Gurfel (1980) reported the ELISA technique to be more sensitive than agglutination and immunodiffusion tests. This technique involves the consecutive immobilization of antigens and antibodies on an insoluble carrier surface. Non-relevant substances are removed from the reaction site, and the 'captured' antigens or antibodies in the test sample

are detected by means of enzyme-linked antibodies. The amount of enzyme-linked antibodies attached to the antigen-antibody complexes on the carrier surface is measured by the amount of substrate that the enzyme degrades (Voller, Bidwell & Bartlett, 1976a).

Advantages of the ELISA technique over other serological techniques are the use of very small amounts of immunoreactants per test (Renwick & Jones, 1985), the elimination of health risks due to radiation (O'Sullivan, Bridges & Marks, 1979), no dependence upon the formation of immunoprecipitates (Clark & Adams, 1977), ability to store the conjugates for long periods without loss of activity (Voller et al., 1976b; O'Sullivan et al., 1979), stability of enzyme labels and the relative low cost of enzyme labels when compared to radio-active and fluorescent labels (Voller et al., 1976a). According to Clark & Adams (1977) the ELISA technique has quantitative potential.

When considering the serological methods used for identification of strains of Rhizobium and Bradyrhizobium spp., the ELISA technique, combining simplicity of operation with specificity and rapidity of performance (B.D. Kishinevsky, pers comm), is the method of choice for routine use.

2.5.3 Enzyme-linked immunosorbent assay (ELISA)

Horseradish peroxidase, β-galactosidase and alkaline phosphatase are the most widely used enzymes in heterologous ELISA systems (O'Sullivan et al., 1979). Alkaline phosphatase as marker enzyme is generally used for strain identification of rhizobia (Kishinevsky & Bar-Joseph, 1978; Berger et al., 1979; Kishinevsky & Gurfel, 1980; Ahmad et al., 1981; Hodgson & Waid, 1981; Olsen et al., 1981; 1983; Kishinevsky et al., 1982; Kishinevsky & Maoz, 1983; Kishinevsky et al., 1984; Olsen & Rice, 1984; Rice et al., 1984; Fuhrmann & Wollum II, 1985; Nambiar & Anjaiah, 1985). Morley & Jones (1980) reported the use of fluorescent substrate (3-O-methylfluorescein phosphate) instead of the generally used p-nitrophenyl phosphate to improve the sensitivity of ELISA. The enzyme β-galactosidase, linked to purified gamma-globulin by a hetero-bifunctional reagent, has been used as marker by Martensson et al. (1984) and Martensson & Gustafsson (1985).

Antibodies, linked to alkaline-phosphatase, are preferably used in ELISA, because the conjugate can be stored in the liquid form and it is therefore easier to remove small aliquots without wastage (Voller, Bidwell & Bartlett, 1977). p-Nitrophenyl phosphate as substrate is favoured because there are no recognized health hazards associated with it (Voller et al., 1977).

The success of the ELISA technique depends upon the skill of the operator and the use of high quality reactants (B.D. Kishinevsky, pers comm). If adequate sensitivity is to be

obtained, a number of variable factors require consideration.

Disposable microtitre plates are convenient for large scale use, since only small volumes of reagents are required (Voller et al., 1976a). According to Clark & Adams (1977) and Fuhrmann & Wollum II (1985), considerable variation in reaction strength and reproducibility of ELISA absorbance values occurred due to the use of microtitre plates from different sources. Clark & Adams (1977) encountered irregular, non-specific reactions in some batches of microtitre plates, notably in the outer row of wells. Harding (1982) reported that variability in ELISA reactions occurred when microtitre plates, even from the same batch, were used. It seems that different grades of materials used to manufacture plates can severely affect results (Voller et al., 1977). Procedures such as avoiding the use of certain wells on a plate, or using only plates from a particular manufacturer, have been proposed to avoid this lack of uniformity (Clark & Adams, 1977). Renwick & Jones (1985) suggested replication and randomization of both test and control wells to allow statistical analysis.

The ELISA technique consists of a series of incubations of different reagents separated by washing steps (Clark & Adams, 1977). According to them, washing steps must be sufficient to remove traces of reactants that could cause non-specific reactions. Since it is important to ensure that all wells are treated in exactly the same way, washing procedures are critical (Voller et al., 1976a). Microtitre

plates are washed by emptying the plate, refilling wells with phosphate buffered saline containing a wetting agent (Tween 20) and allowing this to stand for 2 to 3 min. Washing is repeated three times, after which the plate is shaken dry and the next reagent is added at once (B.D. Kishinevsky, pers comm). The Tween 20 is included to prevent post-coating adsorption of protein to the well surface (Clark & Adams, 1977).

The production of specific antisera with high titres is important to ELISA (Voller et al., 1976a;B.D. Kishinevsky, pers comm). Of the antibody molecules that arise upon injection of an animal, five classes are recognized (Rowe, 1970). Immunoglobulin M (IgM) and G (IgG) are important in in vitro serological reactions (Dudman, 1977). Antibodies of classes IgG and IgM differ not only in molecular weight, i.e. 150 000 and 900 000 respectively, but also in order of appearance after injection and in reactive properties (Dudman, 1977). A low level of antibody, belonging mainly to the IgM class, appears first. Upon subsequent injections of the same antigen, a quicker build-up of antibody, mainly of the IgG class, to high final levels occurs (Pike, 1967). IgM antibodies are more efficient in agglutination reactions with particulate antigens and less effective in precipitation reactions with soluble antigens than IgG antibodies (Pike, 1967).

According to Barret (1970), antiserum produced from lipopolysaccharides of cell walls of Enterobacteriaceae,

contains mainly IgM. Engvall & Perlmann (1972) reported that the indirect ELISA measures mainly antibodies of the IgG class. This may be the reason why Martensson et al. (1984) found that antibodies produced against purified Rhizobium cell wall lipopolysaccharides, are of low specificity and sensitivity in the indirect ELISA. Since purified cell wall lipopolysaccharides are poor antigens in rabbits (Williams & Chase, 1967), antisera against strains of Rhizobium and Bradyrhizobium are produced by using whole cell antigens (B.D. Kishinevsky, pers comm).

Two or more animals are immunized at the same time, since the same antigen suspension can evoke a different immunological response from rabbit to rabbit (Chase, 1967). These differences can be reflected in titre and in the ratio between IgM and IgG (Humphrey & Vincent, 1973). The change-over from IgM to IgG production seems to vary from antigenic determinant to antigenic determinant (Humphrey & Vincent, 1973). They suggested that the ratio of IgM to IgG for any antigenic determinant, depends on the point of termination of antibody production. Overdosing an animal with antigen may lead to immunological unresponsiveness (Halliday, 1971). A number of factors, such as the method used to prepare antigen, immunization schedule followed and the time between primary immunization and bleeding can be manipulated to enhance antiserum quality (Vincent, 1982).

Since antisera produced from whole cell antigens often include contaminants which can react non-specifically in

ELISA, an immunologically pure IgG is isolated from the antiserum for use in ELISA (B.D. Kishinevsky, pers comm).

All the macromolecular constituents of microbial cells are potential antigens (Dudman, 1977). As antibodies are not specific for entire large antigens, but for small portions of the antigen molecule about 30 to 50 Å long, antibodies are formed against immuno-dominant groups of the antigen (Dudman, 1977). Two antigens may be different in total composition, but if they share antigenic determinants, they may give rise to antibodies with similar reactive sites that may cross-react with other antigens (Dudman, 1977). Like other serological techniques, the ELISA technique suffers from an incapacity to distinguish among strains sharing strong or many antigenic determinants (Fuhrmann & Wollum II, 1985). Olsen et al. (1983) as well as Kishinevsky et al. (1984) were able to remove cross-reaction of Rhizobium and Bradyrhizobium strains by thorough, repetitive adsorption of antisera with appropriate antigen. Adsorbed IgG can be used directly in indirect ELISA tests, but this IgG should be purified before use in DAS ELISA tests (B.D. Kishinevsky, pers comm). Homologous antigen as positive control, heterologous antigen to test for cross-reactions and wells without antigen as blanks, should be included in ELISA tests.

Heated whole cell and nodule suspensions give rise to higher final absorbance values than non-heated suspensions in homologous reactions in ELISA (Kishinevsky &

Bar-Joseph, 1978; Berger et al., 1979; Kishinevsky & Gurfel, 1980; Fuhrmann & Wollum II, 1985). This is probably due to the release of thermostable antigens from killed cells (Kishinevsky & Bar-Joseph, 1978) or the exposure of additional antigenic sites (Berger et al., 1979) provoked by heating. It is known that heat treatment (30 min at boiling point) also destroys flagellar antigen (Vincent, 1970). Kishinevsky & Gurfel (1980) found mechanical disruption of cell suspensions (20 min at 0 °C) as effective as heat treatment (30 min at boiling point) and ultrasonic treatment (14 min at 0 °C) to increase final ELISA absorbance values. Rhizobial antigen suspensions are thus treated, in most cases by heating, to increase final ELISA absorbance values.

Antigens or antibodies can be conjugated to enzymes without destroying the reactivity of either (Voller et al., 1976a). According to these authors, alkaline phosphatase is a stable, highly reactive, readily available and widely used enzyme. The dialdehyde, glutaraldehyde, can link proteins (e.g. IgG and alkaline phosphatase) through their amino-residues to form a Schiff's base (Avrameas, 1969). Followed by further reactions, the IgG and alkaline phosphatase polymerize as extended chains (O'Sullivan et al., 1979). This one-step glutaraldehyde method, like most conjugation methods, produce enzyme-enzyme and protein-second protein conjugates (O'Sullivan et al., 1979). Boorsma & Kalsbeek, (1975) reported that extensive self-linkage and loss of IgG activity occur with this

method. IgG-alkaline phosphatase conjugates have been characterised as electrophoretically heterogeneous (Avrameas, 1969). The extent of cross-linking is thus difficult to control and the reactivity of conjugates may vary from batch to batch (B.D. Kishinevsky, pers comm.). However, the one-step glutaraldehyde method is easy to perform and conjugates yield reproducible ELISA results and can retain activity for more than one year when stored concentrated with a preservative at 4 °C (Voller et al., 1976b). By using a hetero-bifunctional reagent to link β-galactosidase to antibodies, Martensson & Gustafsson (1985) were able to avoid enzyme-enzyme and/or antibody-antibody linkages. These authors used polystyrene test tubes to perform ELISA tests. Due to the centrifugation step, microtitre plates cannot be used in their ELISA procedure.

The conjugate incubation step is followed by washing, the addition of substrate and a timed addition of reagent to terminate the enzyme reaction (O'Sullivan et al., 1979). The reactivity of alkaline phosphatase with the highly soluble p-nitrophenyl phosphate is terminated with a concentrated alkaline solution (Voller et al., 1977). The yellow product remains stable for some time and can be read spectrophotometrically (Voller et al., 1977). The amount of yellow product is proportional to the amount of enzyme present, which is directly related to the quantity of antigen or antibody in the microtitre plate well (Voller et al., 1977).

Enzyme-linked anti-species IgG can be replaced by enzyme-linked protein A in the indirect ELISA test (Kishinevsky & Maoz, 1983). This is possible, as Staphylococcus aureus protein A reacts with the Fc part of IgG of most mammalian species, including rabbits (Kronvall, Seal, Finstad & Williams, 1970). Linkage of protein A to IgG by the two-step glutaraldehyde method resulted in conjugates that performed better in the indirect ELISA method than enzyme-linked anti-species IgG conjugates (Engvall, 1978; Barabara & Clark, 1982; Kishinevsky & Maoz, 1983). When purified antigens are not available, the use of enzyme-linked protein A rather than enzyme-linked anti-species IgG may lead to an improved assay (Barbara & Clark, 1982).

ELISA reactivity may vary for different antigen-antibody combinations (Kishinevsky & Gurfel, 1980). According to Ahmad et al. (1981), ELISA reactivity correlates with colony morphology. In general, strains that produce moderate to high amounts of extracellular polysaccharides had low reactivity in ELISA (Ahmad et al., 1981). These authors considered mucilage a possible physical barrier to antigen-antiserum reactions, but washing of the cells did not increase reactivity.

Erratic ELISA results were obtained with DAS ELISA when attempting to identify some R. meliloti and R. leguminosarum biovar viciae strains (Kishinevsky & Gurfel, 1980; Olsen et al., 1983). Since IgG fractions of the antiserum were

active in the indirect assay (Kishinevsky & Gurfel, 1980), and the antiserum active and specific in agglutination tests (Kishinevsky & Gurfel, 1980; Olsen et al., 1983), these authors postulated that loss of ELISA reactivity occurs during preparation of enzyme-linked antibodies specific to the strains tested. The binding ability of these anti-strain antibodies might have been reduced or destroyed by conjugation. This phenomenon might have been due to antigen which is inadequately linked to coating or enzyme-linked, strain-specific antibodies (Kishinevsky & Gurfel, 1980), or because of differences in rabbit antisera suitability for ELISA (Koenig, 1978). However, the reason for this phenomenon is not clear (Kishinevsky et al., 1984).

As small changes in procedure and experimental conditions may lead to large differences in final results (Voller et al., 1976a), standardization of each step is the key to precision in ELISA.

At present, two ELISA procedures are of importance in the identification of strains of rhizobia, i.e. the indirect ELISA procedure (Berger et al., 1979; Ahmed et al., 1981; Hodgson & Waid, 1981; Kishinevsky & Maoz, 1983; Olsen & Rice, 1984; Martensson et al., 1984; Fuhrmann & Wollum II, 1985; Martensson & Gustafsson, 1985) and the

direct or DAS ELISA procedure (Kishinevsky & Bar-Joseph, 1978; Kishinevsky & Gurfel, 1980; Morley & Jones, 1980; Jones & Morley, 1981; Kishinevsky et al., 1982; Olsen et al., 1981; 1983; Rice et al., 1984; Nambiar & Anjaiah, 1985).

2.5.3.1 Indirect ELISA

This procedure involves the non-specific adsorption of antigen onto the solid phase carrier, incubating with strain-specific IgG, then incubating with enzyme-linked anti-species IgG or enzyme-linked protein A and finally adding specific substrate. Washing between each stage is necessary to remove excess reagents. The amount of substrate converted to end product is proportional to the amount of antibody and antigen present (Voller, Bidwell & Bartlett, 1973).

In contrast to the DAS ELISA method, the indirect ELISA method enables identification of different strains of rhizobia with a single conjugate (Kishinevsky et al., 1984). Technical difficulties in the practical use of indirect ELISA have been encountered (Tchan, 1982; Olsen & Rice, 1984). Engvall & Perlmann (1972) reported that the amount of antigen that can adsorb to polystyrene surfaces is limited. During incubations, some of the adsorbed antigen is

released from the tubes (Engvall, Jonsson & Perlmann, 1971) and the amount released is dependent on the total amount adsorbed (Engvall & Perlmann, 1972). Since reliable passive adsorption of antigen to microtitre plate wells depends on the nature of the antigen and adsorbing surface (Salonen & Vaheri, 1979; Lehtonen & Viljanen, 1980), differences in binding strength and stability were encountered when using different antigen preparations and batches of microtitre plates (Tchan, 1982; Olsen & Rice, 1984).

Olsen & Rice (1984) recommended evaporation of antigen in wells at elevated temperatures to adsorb rhizobial antigen firmly to well surfaces, and to reduce differential antigen detachment during incubation and washing of the plates. Ahmad et al. (1980) and Kishinevsky & Maoz (1983) did not encounter any problems to passively adsorb rhizobial antigens to well surfaces. The way in which rhizobial antigen can be attached to solid surfaces seems to differ for different antigen-antibody combinations.

Nambiar & Anjaiah (1985) attempted to quantify rhizobia of the 'cowpea' cross inoculation group in soil by using a modified indirect ELISA method (Barbara & Clark, 1982). They used the $F(ab')_2$ fragment of IgG, prepared by pepsin digestion of IgG, to coat the microtitre plate. After incubation with test sample, the bound $F(ab')_2$ fragment-antigen complex was incubated with IgG, then with enzyme-linked protein A or goat anti-rabbit Fc enzyme conjugate. Substrate was finally added, and end-product

assessed spectrophotometrically. The usual washing steps were conducted (Barbara & Clark, 1982). Barbara & Clark (1982) considered the $F(ab')_2$ assay useful in small scale tests for detecting antigens which do not warrant production of individual conjugates.

Martensson & Gustafsson (1985) investigated competition between two R. leguminosarum biovar trifolii strains in soil used for commercial inoculant production. According to these authors, their ELISA method is an effective quantitative assay. They demonstrated that strain 7612 dominated over strain 285 in the soil, even when strain 285 constituted 90% of the initial inoculum.

2.5.3.2 Double-antibody-sandwich ELISA (DAS ELISA)

The DAS ELISA method involves adsorption of specific antibody onto the solid phase carrier (Kishinevsky & Bar-Joseph, 1978). The sample being assayed for antigen is then incubated with the solid phase, washed and antigen-specific enzyme-conjugated antibody is added. The enzyme-linked antibody is of the same specificity as the sensitizing antibody. Substrate is added and the amount of reaction product formed is proportional to the quantity of antigen contained

in the sample (Kishinevsky & Bar-Joseph, 1978). For most strains of rhizobia, 10^{4} to 10^{5} cfu.cm^{-3} can easily be detected with confidence (Kishinevsky & Bar-Joseph, 1978).

The DAS ELISA method has been used successfully for the identification of R. meliloti in commercial alfalfa inoculants (Olsen et al., 1983). Inoculants were prepared by using non-sterile peat. These authors performed plate counts and used the DAS ELISA test to screen colonies picked from plates for strain identity. By determining the ratio of identified strain colonies to total colonies, they were able to calculate the number of rhizobia of specific strain.g^{-1} inoculant. In contrast to the conventionally used laborious, time-consuming plant infection technique, the results were available within 5 d, thus permitting a quick evaluation of inoculant quality (Olsen et al., 1983).

Kishinevsky et al., (1982) used the DAS ELISA method to estimate the population density of rhizobia directly in peat without a time-consuming plate count or plant infection count. They could detect a minimum of $3,0 \times 10^{6}$ rhizobial cfu.g^{-1} peat. Uninoculated peat did not interfere with ELISA results and no colour reactions were observed with peat cultures of serologically unrelated strains (Kishinevsky et al., 1982).

Renwick & Jones (1985) reported that the fluorescent ELISA method, when used in its range of sensitivity, and

antibiotic resistance plating agreed well when used to determine the number of cfu of a R. leguminosarum biovar trifolii strain in peat and brown earth soil. Because of the absence of cross-reactive antigens in the peat soil, these authors could detect 2,0 X 10^{5} cfu.g^{-1} peat soil. However, brown earth soil particles incited fluorescence (Renwick & Jones, 1985). This resulted in a higher background, and can be avoided by using a non-fluorescent substrate (Renwick & Jones, 1985).

Nambiar & Anjaiah (1985) investigated if DAS ELISA could be used to enumerate rhizobia of the 'cowpea' cross inoculation group in peat and soil. According to these authors, a linear relationship was not obtained between absorbance values and the number of Bradyrhizobium sp. (Arachis) cfu over the whole range of numbers (10^{3} to 10^{8} cfu.g^{-1} peat) tested, but a linear relationship was obtained for a narrow range of Bradyrhizobium numbers at a given enzyme conjugate dilution. They concluded that estimates by DAS ELISA are close to values obtained from plate counts and the plant infection technique; that loss in viability of cells in the peat carrier does not interfere in enumeration of viable rhizobia by DAS ELISA; and that a minimum of 10^{2} to 10^{3} cfu.g^{-1} peat are required

for a detectable DAS ELISA reaction.

2.5.3.3 Comparative evaluation of the two ELISA methods

Kishinevsky et al. (1984) compared the two ELISA methods with regard to specificity and sensitivity. When using the same antigens and antisera, the two ELISA methods agreed well in ability to detect strains (Kishinevsky et al. 1984). Serologically related strains could be detected by indirect ELISA, whereas DAS ELISA was more strain - specific (Kishinevsky et al., 1984). The sensitivity of the two methods to detect different Rhizobium and Bradyrhizobium strains varied (Kishinevsky et al., 1984). Greater sensitivity was obtained with the indirect than the DAS ELISA method to detect strains of Bradyrhizobium sp. (Arachis) in culture. In the case of a R. leguminosarum biovar trifolii strain, sensitivity of the two methods was comparable. For detection of nodule antigen, the indirect ELISA was ca. ten times more sensitive than DAS ELISA (Kishinevsky et al., 1984).

2.6 Coda

The work reviewed indicates the importance of the independent quality control system practised in

South Africa, i.e. to ensure that high quality inoculants reach the farmer.

Formerly, the bacteriologically controlled plant infection test was performed to assess the quality of legume inoculants. Although reliable estimates of the number of Rhizobium and Bradyrhizobium cells in inoculants were obtained with this technique (Jansen van Rensburg & Strijdom, 1974), it is laborious and time-consuming.

Quality control tests became more accurate, quicker and easier to apply when inoculant manufacturers started to use sterilized peat for production of inoculants. Today inoculant manufacturers produce inoculants only with sterile peat as carrier. These inoculants must contain at least $5,0 \times 10^{8}$ cfu.g^{-1} peat of the desired strain, supplied by the P.P.R.I., over a period of six months. Before marketing, samples of each batch of inoculant produced are tested with regard to Rhizobium or Bradyrhizobium numbers and strain identity. Results of the serological strain check are available on the same day, but 10 to 14 d are required before viable counts of slow-growers are available. Quality control is therefore time-consuming and laborious due to the use of the plate count technique to enumerate rhizobia in the peat.

Economical losses sustained by inoculant manufacturers due to time-consuming quality control tests, can be reduced if a quicker technique is substituted for the plate count

technique. A further improvement would be if this technique can simultaneously identify the strain present in the inoculant. The most promising dual purpose technique tested so far for Rhizobium and Bradyrhizobium strain identification and cell enumeration, is ELISA. This technique involves the use of a solid phase carrier to facilitate the rapid separation of free antigen, antibody and non-relevant substances e.g. peat particles from antigen-antibody complexes. An enzyme marker is used to detect antigen-antibody complexes. ELISA offers sensitivity, specificity, speed, ease of use and is of relatively low cost.

Two ELISA methods are generally used in the study of rhizobia, i.e. the indirect ELISA and DAS ELISA. They differ from each other mainly in the sequence whereby immunoreactants are added to the microtitre plate wells and by the specificity of the antibodies linked to the enzyme. An universal conjugate can be used in indirect ELISA, but a specific conjugate is needed for each strain tested in DAS ELISA. The indirect ELISA is therefore the method of choice for routine use. DAS ELISA, if used to determine the quality of only the most important inoculants, may prove worthwhile to economize quality tests. In South Africa DAS ELISA could be used, as only ca. four slow-growing and three fast-growing strains of the 14 inoculant strains are important in 90% of the inoculants produced.

Although a number of recent publications indicated that a single ELISA test can be used for counting rhizobia in peat and soil, there are obvious problems when using ELISA as quality control test, e.g. its inability to distinguish between viable and non-viable cells. It was thus hypothesized that a single indirect or DAS ELISA test cannot be used for reliable enumeration of viable rhizobia in peat. The present investigation was undertaken to support or disprove the hypothesis.

CHAPTER 3

MATERIALS AND METHODS

3.1 Bacterial strains

The strains of rhizobia used in these studies were obtained from the South African Rhizobium Collection (S.A.R.C.) at the Plant Protection Research Institute. The strains used and their history are listed in Table 1.

The mother cultures were streaked on Congo red-yeast extract-mannitol agar (CR-YM agar) to check for contaminants and to ensure that the colonial morphology of each strain was uniform. Congo red is weakly absorbed by rhizobia, whereas most other species of bacteria absorb it strongly to form pink colonies on the CR-YM agar (Vincent, 1970). The mother cultures were also streaked on nutrient agar with peptone, and the plates were incubated for 2 d at 28 °C. Most rhizobia grow poorly on this medium (Vincent, 1970). Abundant growth on this medium thus indicates the presence of contaminants. However, R. meliloti grows readily on this medium.

3.2 Media

The following media were prepared:

TABLE 1 Strains of rhizobia, their origin and history

Species	Strain	Synonym(s)	Host	Country of origin	Yr obtained
Bradyrhizobium japonicum	WB1[a]	-	*Glycine max*	Roodeplaat, RSA	1966
Bradyrhizobium sp. (*Lotus*)	XHT1[a]	CC8145	*Lotus pedunculatus*	Australia	1976
Bradyrhizobium sp. (*Desmodium*)	XBL6	471	*Desmodium intortum*	Zimbabwe	1968
Bradyrhizobium sp. (*Lotononis*)	XCT9	CB376	*Lotononis bainesii*	Unknown[b]	1968
Bradyrhizobium sp. (*Lupinus*)	VK10[a]	W11425	*Lupinus* and *Ornithopus* species	Unknown[b]	1977
Rhizobium loti	XCV14[a]	NZP2238, LC265DA	*Lotus corniculatus*	Ireland	1976
Rhizobium leguminosarum biovar *trifolii*	SR4[a]	TA1	*Trifolium repens*	Unknown[b]	1968
Rhizobium meliloti	RF14[a]	U45	*Medicago* sp.	Uruguay	1968
Rhizobium meliloti	RF6	-	*Medicago* sp.	RSA	1959

[a]Strains used for commercial inoculant production in South Africa.
[b]Obtained from U-DALS, Australia by the P.P.R.I.

3.2.1 Yeast extract-mannitol broth (YM broth)(Allen, 1959)

Mannitol	10,0 g
Yeast extract (a)	100,0 cm^3
K_2HPO_4	0,5 g
$MgSO_4.7H_2O$	0,2 g
NaCl	0,1 g

Distilled water was added to a final volume of 1 dm^3. The pH of the medium was adjusted to 6,8 to 7,0 using 0,1 M NaOH or 0,1 M HCl. An E520 Metrohm Herisau pH meter, equipped with a glass electrode, was used to determine the pH. The medium was then autoclaved at 121 °C for 20 min.

(a) Yeast extract

To one dm^3 of distilled water, 100 g baker's yeast blocks (Gold Star) was added. After autoclaving at 108 °C for 3 h, sediment was allowed to settle overnight. The supernatant was carefully decanted, distributed in approximately 200 cm^3 quantities in medicinal flasks, and autoclaved at 121 °C for 40 min. The yeast extract was stored at 4 °C.

3.2.2 Yeast extract-mannitol agar (YM agar)

Yeast extract-mannitol broth was prepared and 12 g agar (May and Baker Ltd.).dm^{-3} of medium added before autoclaving. YM agar slants, used for the maintenance of cultures of rhizobia, contained

10,0 $g.dm^{-3}$ $CaCO_3$ to neutralize acidity (Vincent, 1970).

3.2.3 Congo red-yeast extract-mannitol agar (CR-YM agar) (Hahn, 1966)

YM broth was prepared, and 15 g agar.dm^{-3} and 10 cm^3 of a 1/400 aqueous solution of Congo red were added before the solution was autoclaved for 5 min at 121 °C. The agar medium was distributed in approximately 200 cm^3 quantities in flasks, in order to avoid repeated heating when the medium is melted, autoclaved for 20 min at 121 °C and stored at 4 °C after cooling.

3.2.4 Nutrient agar

'Lab-Lemco' Powder (b)	10,0 g
Peptone	10,0 g
NaCl	5,0 g
Agar	15 g

Distilled water was added to a final volume of 1 dm^3. The pH of the medium was 7,5. The medium was distributed in approximately 200 cm^3 quantities in medicinal flasks, autoclaved at 121 °C for 20 min and stored at 4 °C after cooling.

(b) Chemical analysis of 'Lab-Lemco' powder

Moisture	5,3% m/m
Ash	16,5% m/m
NaCl	5,3% m/m
Phosphorus as P_2O_5	12,5% m/m
Total nitrogen	11,6% m/m
Amino nitrogen	1,2% m/m
Calcium	0,16% m/m
Magnesium	0,04% m/m
Copper	1 ppm
Iron	112 ppm
pH 7,0	

3.3 Buffers

3.3.1 Phosphate buffered saline (PBS), 0,15 M

NaCl	8,0 g
KCl	0,2 g
KH_2PO_4	0,2 g
$Na_2HPO_4.12H_2O$	2,9 g
NaN_3	0,2 g

Deionized water was added to a final volume of 1 dm^3. The pH of the buffer was adjusted to 7,4 with 3 M NaOH. A stock solution (10 times concentrated) without NaN_3 was prepared for storage and diluted as required.

3.3.2 Phosphate buffered saline with Tween 20 (PBS-Tween)

0,5 cm^3 Tween 20 was added to 1 dm^3 PBS. The PBS-Tween used for washing the plates contained no NaN_3.

3.3.3 Carbonate buffer (coating buffer), 0,05 M; pH 9,6

Na_2CO_3	1,59 g
$NaHCO_3$	2,93 g
NaN_3	0,2 g

Deionized water was added to a final volume of 1 dm^3.

3.3.4 Substrate buffer

Diethanolamine	97 cm^3
NaN_3	0,2 g

The diethanolamine was dissolved in 800 cm^3 deionized water and the pH adjusted to 9,8 with 36% HCl. The solution was made up to a final volume of 1 dm^3 with deionized water.

3.3.5 Conjugate dilution buffer

The enzyme-linked IgG was diluted in PBS- Tween containing 2% polyvinyl pyrrolidone (PVP) and 0,2% bovine serum albumin.

3.3.6 Buffered salt solution (BSS) (B.D. Kishinevsky, pers comm)

Solution A

$CaCl_2$	0,14 g
NaCl	8,00 g
KCl	0,40 g
$MgSO_4.7H_2O$	0,20 g
$MgCl_2.6H_2O$	0,20 g

Distilled water was added to a final volume of 1 dm^3.

Solution B

A 6% KH_2PO_4 solution was prepared, using distilled water.

Solution C

A 19% Na_2HPO_4 solution was prepared, using distilled water.

The three solutions were sterilized separately for 20 min at 121 °C. 100 cm^3 of solution A, 0,1 cm^3 of solution B and 0,1 cm^3 of solution C were mixed aseptically. The pH of the resulting solution was 7.

3.4 Methods

3.4.1 Antiserum preparation

Antisera against strains WB1, VK10, XHT1, RF6 and SR4 were kindly supplied by C.J. Otto (P.P.R.I.).

Antisera against strains XBL6, XCT9 and RF14 were raised, each in two rabbits, using whole cell antigens (See Section 2.5.3).

The strains of rhizobia were grown on YM agar slants. Strains XBL6 and XCT9 were incubated for 10 d at 28 °C, whereas the fast-growing strain RF14 was cultivated for 5 d before being harvested aseptically and suspended in sterile saline. The cells were pelleted in sterile centrifuge tubes by centrifugation at 12 100 x g for 15 min. Centrifugation was performed in a SS-34 rotor in a Sorvall SS-4 superspeed centrifuge at 4 °C. The pellet was resuspended in sterile saline and the centrifugation step repeated twice. The pellet was finally resuspended to provide a turbid suspension of approximately 1 X 10^{9} cells.cm^{-3}.

The following immunization schedule was followed:

A pre-immune blood sample, collected from the large marginal ear vein of each rabbit, was retained as a check on absence of antibodies prior to injection. The turbid cell suspension (the inoculum) was emulsified with an equal volume of Freund's complete adjuvant (Difco Labs., Detroit 1, Michigan, U.S.A.) by progressive incorporation of the suspension in the adjuvant. For the primary injection, 1 cm^{3} of the emulsion was injected intramuscularly. Two and three weeks later, an intravenous injection with 1 cm^{3} of the turbid cell suspension was given to each rabbit. The rabbits were bled on the fifth day after the final

injection, having recieved no food within 24 h prior to bleeding in order to reduce the fat content of the blood. Blood was collected dropwise in clean glass centrifuge tubes (each containing a wooden stick to facilitate removal of the clot) from a small incision made in the marginal ear vein. The blood was incubated for 1 h at 37 °C to facilitate clotting. (The wooden stick with the clot was stirred slightly after 30 min to help extrusion of the serum). The clot was removed and the serum incubated overnight at 4 °C. Any erythrocytes remaining in the serum were removed by centrifugation at 3 020 x g at 4 °C for 10 min.

The antiserum obtained was subdivided into 2 cm^3 quantities in small screw-cap bottles. Merthiolate (BDH Chemicals, Poole, England) was added as preservative (1:10 000). The antiserum was stored at -18 °C.

3.4.2 Determination of the titres of the antisera (Kishinevsky & Bar-Joseph, 1978)

Tube agglutination tests were carried out to determine the titres and specificity of the antisera. Doubling dilutions of each antiserum were made with saline. 0,5 cm^3 of each dilution was pipetted into tubes. Each tube then received 0,5 cm^3 of the cell suspension containing approximate-

ly 1 X 10^{9} $cells.cm^{-3}$ saline. Flagellar antigens were denaturated by heating the cell suspension for 30 min in McCartney bottles immersed in boiling water. Control tubes containing 0,5 cm^{3} cell suspension and 0,5 cm^{3} saline were included. Both homologous and heterologous combinations of antigens and antisera were used. The tubes were incubated at 37 °C overnight. The titre of each antiserum was recorded as the highest dilution in which an agglutination reaction was visible. Only antisera with high homologous agglutination titres (at least 1280) were used for the ELISA test.

3.4.3 Purification of immunoglobulins (IgG)

Whole antisera often contain contaminants which produce 'background' reactions that are undesirable and reduce the efficiency of the ELISA test (B.D.Kishinevsky, pers comm). To overcame this problem, it was necessary to isolate an immunologically pure IgG from the antiserum which was then conjugated to the enzyme.

Immunoglobulins were purified from antiserum by ammoniumsulphate ($(NH_4)_2SO_4$) precipitation and DE 23 cellulose filtration (Clark & Adams, 1977; Kishinevsky & Bar-Joseph, 1978). One cm^{3} of

antiserum was diluted with 9 cm^3 deionized water. Proteins were precipitated from the suspension by dropwise addition of saturated $(NH_4)_2SO_4$ solution to give a final concentration of 35%. After a standing period of 60 min at room temperature, the solution was centrifuged at 27 000 x g for 20 min at 4 °C to collect the precipitate. The pellet was resuspended in 2,5 cm^3 half-strength PBS, and desalted using a 1,4 X 5 cm Sephadex G 25M column (PD-10 column, Pharmacia, Sweden). The PD-10 column was equilibrated in approximately 50 cm^3 half- strength PBS. The 2,5 cm^3 sample was applied and the effluent discarded. The IgG (a high molecular mass component) was eluted with 3,5 cm^3 half-strength PBS.

The IgG were then further purified by passage through a 0,8 X 10 cm column containing 5 cm^3 DE 23 cellulose (Whatman Ltd., Maidstone Kent), pre-equilibrated in half-strength PBS. The effluent was collected in approximately 2 cm^3 fractions. The effluent was monitored at 280 nm by using a Varian Superscan 1 ultra-violet visible spectrophotometer and all fractions containing the first protein peak combined. The IgG fraction was then adjusted to concentrations of 1 $mg.cm^{-3}$ ($\varepsilon^{0,1\%}_{278\,nm,1\,cm}$ = 1,4) with half-strength PBS and 0,02% (m/v) sodium azide (NaN_3), and stored in 2 cm^3 amounts in silicone-treated glass tubes at -18 °C.

3.4.4 Conjugation of alkaline phosphatase with protein A

The 'two-step' glutaraldehyde method (Engvall, 1978; Kishinevsky & Maoz, 1983) was used. The enzyme alkaline phosphatase E.C.3.1.3.1. (Boehringer Mannheim, EIA grade) was used. 0,25 cm^3 of the enzyme (2,5 mg) solution was transferred to a dialysis tube (Visking, size 1-8/32", Medicell International Ltd, London) and immersed in a 28 cm^3 glass McCartney bottle with 25 cm^3 0,5% glutaraldehyde (Sigma, 25%, electron-microscope grade) in PBS. After stirring overnight at room temperature, the enzyme solution was dialyzed three times against 500 cm^3 PBS to remove the excess glutaraldehyde. The conjugate was prepared by mixing the glutaraldehyde-treated enzyme with 1 mg protein A (Sigma P8143) in 0,1 cm^3 PBS and was left overnight at room temperature. The conjugate was stored at 4 ^{o}C after adding 1% bovine serum albumin (Sigma A7030).

3.4.5 Conjugation of alkaline phosphatase with the purified immunoglobulins

The enzyme alkaline phosphatase E.C.3.1.3.1. (Boehringer Mannheim, EIA grade) was linked to the IgG by the 'one-step' glutaraldehyde method (Avrameas, 1969; Kishinevsky & Bar-Joseph, 1978). 1,4 cm^3 IgG solution (1 $mg.cm^{-3}$ in half-strength

PBS with 0,02% NaN_3) was added to 0,3 cm^3 (3 mg) alkaline phosphatase (2 500 U/mg). Fresh glutaraldehyde solution (Sigma, 25%, electron microscope grade) was added to a final concentration of 0,06%. The reaction mixture was incubated at room temperature for 4 h (a very pale yellow-brown colour may develop) and dialyzed in dialysis tubing (Viking size 1-8/32") against three changes of cold PBS (500 cm^3) to remove the excess glutaraldehyde. The conjugates were stored at 4 °C after adding 0,5% (m/v) bovine serum albumin (Sigma A7030).

Because of volume changes and possible IgG losses during the conjugation procedure, all references to the conjugate were given in terms of dilutions of the conjugate rather than absolute concentrations.

3.4.6 Preparation of antigen

3.4.6.1 Cells of rhizobia in culture

(B.D.Kishinevsky,pers comm)

The Rhizobium and Bradyrhizobium strains were grown on YM agar slants at 28 °C for 5 and 10 d respectively, after which they were harvested from the surface with PBS and centrifuged at 12 100 x g at 4 °C. The pellets were suspended in PBS with

0,05% Tween 20, pH 7,4 (the solution used for the extraction of rhizobial antigen in the DAS ELISA procedure). The suspension was centrifuged at 12 100 x g for 20 min at 4 °C, the supernatant decanted and the pellet resuspended in PBS-Tween. This washing step was repeated twice. The pellet was finally suspended in PBS-Tween. The absorbance (A) of the suspension was determined at 420 nm with a Spectronic 20 photometer (Bausch & Lomb), and standardized against a previously compiled standard curve to give a cell density of approximately 10^9 cells. cm^{-3}. The cell suspension was heated for 30 min in 28 cm^3 glass McCartney bottles immersed in boiling water, since ELISA values of heated cell suspensions were significantly higher than those obtained for the unheated suspensions. The suspension was stored at 4 °C.

Cell suspensions prepared for use in the indirect ELISA method were suspended in PBS without Tween-20.

3.4.6.2 Cells of rhizobia in the peat carrier

See Section 3.4.13.4

3.4.7 ELISA techniques

3.4.7.1 Indirect ELISA

The procedure described by Ahmad et al. (1981) with some modifications (Kishinevsky & Maoz, 1983) was used.

200 µl of inoculant or cell suspension was placed in the wells of a flat bottom polystyrene microtitre plate (Linbro/Titertek, Flow Labs., Inc., Conn. USA) and incubated overnight at 4 °C. The plate was covered with plastic film to prevent evaporation. Unadsorbed antigen was removed by flooding the wells three times for 3 min each with PBS-Tween (The washing step). The Tween 20 was included to prevent post-coating adsorption of protein to the surfaces of the wells.

Following washing, the plate was shaken dry. 200 µl of IgG diluted in PBS-Tween was placed in the wells, the plate covered with plastic film and incubated at room temperature for 2 h. The washing step was repeated and the plate shaken dry.

200 µl enzyme-linked protein A, appropriately diluted in the dilution buffer (Section 3.3.5), was placed in the wells, covered and incubated at room temperature for 2 h.

Following washing, residual liquid was shaken out. 200 µl of freshly prepared 0,6 mg.cm^{-3} p-nitrophenyl phosphate in substrate buffer was added to each well. After incubation for 30 min at room temperature, the reaction was terminated by adding 50 µl 3 M NaOH to each well.

The results were assessed by visual observation and by measuring the absorbance at 405 nm (A_{405}) in a Titertek Multiskan Photometer (Flow Labs, Inc., Conn. USA).

3.4.7.2 DAS ELISA

The DAS ELISA procedure, as applied by Kishinevsky & Bar-Joseph (1978) to identify strains of Bradyrhizobium sp. (Arachis) in pure culture and single nodules, was used.

200 µl purified strain-specific IgG, appropriately diluted in coating buffer, was added to each well of the microtitre plate. The plate was covered with plastic film to prevent evaporation, and incubated at 37 °C for 3 h. The plate was emptied, washed by flooding the wells with PBS-Tween and left for 3 min. The washing process was repeated twice, the plate emptied and shaken dry.

200 µl of test sample, diluted in PBS-Tween, was

added to appropriate wells. The plate was covered and incubated at 4 °C overnight. After the plate had been washed, residual liquid was shaken out.

200 µl of alkaline phosphatase-linked IgG conjugate, appropriately diluted in conjugate dilution buffer, was added to each well. The plate was covered as before. After incubation at 37 °C for 4 h, the plate was washed and shaken dry.

200 µl of freshly prepared enzyme substrate solution (0,6 $mg.cm^{-3}$ p-nitrophenyl phosphate in 10% diethanolamine buffer) was added to each well and incubated at room temperature. The enzyme-substrate reaction was terminated after 30 min with the addition of 50 µl 3 M NaOH. The hydrolyzed substrate concentration was determined spectrophotometrically at 405 nm.

3.4.8 Determination of the optimal concentration of reagents

For each new batch of purified IgG and conjugate prepared, optimal concentrations were determined using a chequerboard titration protocol (Voller *et al.*, 1976a).

In the indirect ELISA procedure, IgG at concentra-

tions of 4 $\mu g.cm^{-3}$; 8 $\mu g.cm^{-3}$ and 12 $\mu g.cm^{-3}$ and enzyme-protein A conjugate at dilutions of 1:2 000, 1:4 000 and 1:8 000 were tested. In the DAS ELISA procedure, coating IgG at concentrations of 2 $\mu g.cm^{-3}$, 4 $\mu g.cm^{-3}$, 6 $\mu g.cm^{-3}$ and 8 $\mu g.cm^{-3}$ and enzyme-linked antibody at dilutions of 1:100; 1:200; 1:400 and 1:800 were tested. Successive 10-fold dilutions of cell suspension were used to estimate the level of dilution of IgG and conjugate solutions giving the desired colour gradient, or the best separation of positive from negative test samples. Both homologous and heterologous reactions were tested. In order to standardize experimental conditions for subsequent tests, optimal concentrations of immunoreactants were also determined when using inoculant suspensions.

3.4.9 Preparation of inoculant

3.4.9.1 Preparation of peat

250 g portions of finely ground (150 to 200 mesh, B.S.) Putfontein peat, sealed in polythene bags, were kindly supplied by Stimuplant (Pty) Ltd. The pH of the peat had previously been adjusted to between 6,5 and 7,5 with $CaCO_3$ and the moisture content was ca. 45%. The peat was either steam sterilized or sterilized by gamma-irradiation.

Peat sterilized by gamma-irradiation was sealed off in low-density polythene bags (Fig. 1 A). A cardboard container with sealed bags of peat, not exceeding a total mass of 22 kg, was exposed to gamma-irradiation on two sides from a ^{60}Co source. The irradiation dose was determined by red poly (methyl methacrylate) dosimetry and verified by serum sulphate chemical dosimetry (Strijdom & Jansen van Rensburg, 1981). The peat received a 75 kGy dose. When steam sterilization was used, 250 g quantities in high-density polythene bags were autoclaved for 1 h at 124 °C. The open ends were not sealed prior to sterilization, but were folded back.The peat was allowed to cool in the autoclave before the bags were sealed in a laminar flow unit (Fig. 1 B).

3.4.9.2 Preparation of the inoculum

Two loopsful of growth from the surface of a YM agar slant were suspended in 100 cm^3 YM broth. The fast-growing strains were cultivated at 28 °C for 5 d on a rotary shaker. The slow-growing strains were cultivated for ca. 12 d. At this stage the total number of cells of rhizobia in the suspension was determined, using a Wild M20 phase contrast microscope and a bacterial counting chamber (Petroff-Hausser) having a depth of 0,002 cm.

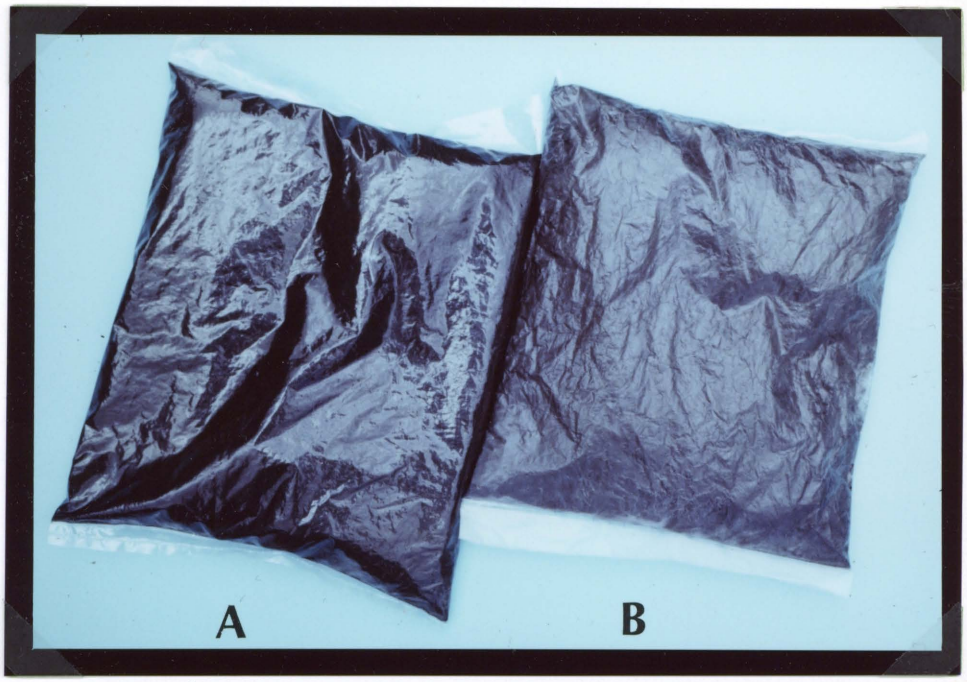


Fig. 1 Packets of sterilized Putfontein peat

A - Gamma-irradiated peat, sealed in a low-density polythene bag

B - Steam sterilized peat, sealed in a high-density polythene bag

One cm^3 broth culture was diluted 100 times. A small drop of this suspension was delivered with a Pasteur pipette to the space at the edge between the slide and the coverslip. The count was done as soon as the flowing movement had ceased. Phase illumination and 400 times magnification were used. Cells touching the top and left side boundaries were included in the count. Those touching the bottom and right side boundaries were excluded. The cells in 160 small squares (ca. 10 to 16 cells per square) were counted. The following equation was used to calculate the number of cells.cm^{-3} :

$$\text{Number of cells.cm}^{-3} = \frac{\text{Number of cells counted}}{\text{Number of squares}} \text{ X dilution X f}$$

f - factor for size of square = $2 \text{ X } 10^7$ for the small squares (Vincent, 1970).

Most of the broth cultures had reached a density of ca. 10^9 cells.cm^{-3} at the end of the incubation period.

3.4.9.3 Inoculant production

Inoculants were produced using Bradyrhizobium sp. (Arachis) strain XBL6, Bradyrhizobium japonicum

strain WB1, Bradyrhizobium sp. (Lotus) strain XHT1 and Rhizobium leguminosarum biovar trifolii strain SR4.

Each packet of sterilized peat was inoculated aseptically by puncturing the surface with a sterilized plastic syringe. A 20 cm^3 portion of broth culture was injected into each packet and the hole sealed. The contents of each packet were mixed by hand for 5 min. Packets of inoculant were incubated at 28 °C for approximately 14 d. After 5 d of incubation, the contents of each packet were again mixed by hand for 5 min in order to distribute the cells evenly throughout the peat. The method of inoculant production by the manufacturers was essentially the same as described here, the main difference being the cultivation of the strains in well-aerated broth in small fermentors of 20 or 40 dm^3 capacity. The carbon source used in the broth may differ (e.g. sucrose instead of mannitol). A sterilized automatic syringe, connected to a fermentor containing the broth culture, was used to inoculate the packets of sterilized peat aseptically. An example of legume inoculant at the retail outlet is given in Fig. 2.

Fig. 2 Legume inoculants at the retail outlet

3.4.10 Quality tests

After two randomly selected packets of inoculant of each batch produced by two manufacturers were received, the following information was recorded:

1. Name of manufacturer
2. Inoculant produced
3. Batch number
4. Date of receipt of inoculant
5. Testing date
6. Method used by the manufacturer to sterilize the packets of peat before inoculating the broth culture

3.4.10.1 The plate counting procedure

Viable counts of rhizobia in peat were made by the spread plate method (Postgate, 1969) with slight modifications (S. Dutrieux, pers comm), after the mass of each packet of inoculant had been determined.

The contents of each packet were suspended aseptically in 800 cm^3 of sterile water (initial dilution). The suspension was left on a rotary shaker (Labotec) for 30 min before a dilution series was prepared. One cm^3 of the suspension was transferred into 99 cm^3 of distilled water and shaken for 5 sec, then 1 cm^3 transferred to

a second bottle and subsequent 10-fold dilutions were made. The last three dilutions (subsequent dilutions), i.e. 10^{-5}, 10^{-6} and 10^{-7}, were used to spread over the surfaces of agar plates. Four CR-YM agar plates and one nutrient agar plate, dried overnight at room temperature, were used for each dilution. Each plate received 0,1 cm^3 of the dilution which was spread over its agar surface until dry by means of a sterile L-shaped glass rod. The spread plates were inverted and incubated at 28 °C. Fast-growing strains were incubated for 5 to 7 d and slow-growing strains ca. 12 d. Counts were made on plates containing between 30 and 300 colonies. The number of cfu.g^{-1} peat was calculated by using the following equation:

$$\text{Number of cfu.g}^{-1}\text{ peat} = \frac{A \times B_1 \times B_2}{C}$$

$$= \frac{A}{0,4\ cm^3} \times 4 \times B_2$$

A - Total number of colonies on 4 plates

B - Initial dilution factor

B - Subsequent dilution factor

C - Total amount of cell suspension spread on 4 plates

3.4.11 Preliminary experiments to investigate the use of the indirect ELISA method to estimate the number of rhizobia in peat

For each of strains XBL6 and WB1, two packets of inoculant were produced as described in Section 3.4.9.3. Viable counts and the indirect ELISA method were conducted as follows:

3.4.11.1 Viable counts

Each packet of inoculant was mixed for 2 min by hand in order to distribute cells evenly in the peat. 10 g of inoculant was aseptically suspended in 40 cm^3 of sterile PBS in a 250 cm^3 Duran bottle. Subsequent steps were as described in Section 3.4.10.1.

3.4.11.2 Indirect ELISA

After 1 cm^3 of inoculant suspension was removed and dilution series prepared for plate counts, the remaining inoculant suspension was heated in boiling water for 30 min. A five-fold dilution series was prepared by mixing each inoculant suspension for 2 min on a Gallenkamp flask shaker. One cm^3 of inoculant suspension was immediately transferred into 4 cm^3 of PBS with a 1 cm^3 pipette. The indirect ELISA method was

conducted as described in Section 3.4.7.1, with the XBL6 IgG used at a concentration of 4 $\mu g.cm^{-3}$ and the WB1 IgG at a concentration of 5 $\mu g.cm^{-3}$. In both cases the alkaline phosphatase-linked protein A was used at a dilution of 1:4 000. Heated peat and cell suspensions (Section 3.4.6.1) were included as controls.

3.4.11.3 Effect of centrifugation of the inoculant suspension on indirect ELISA A_{405} values

Soybean inoculant, prepared from strain WB1 (Section 3.4.9.3), was used. After mixing a packet of inoculant for 2 min by hand, 20 g of inoculant was aseptically suspended in 80 cm^3 of sterile PBS. Subsequent steps for viable counts were as described in Section 3.4.10.1.

The remainder of the inoculant suspension was heated for 30 min in boiling water. After the inoculant suspension had been shaken for 2 min on a Gallenkamp flask shaker (speed 8), four 20 cm^3 portions of the suspension were centrifuged for 10 min at 121 x g, 3 020 x g, 12 100 x g and 27 000 x g respectively. A dilution series was prepared for each of the four supernatants obtained. The successive dilution steps used were five times, two times and two times. The indirect ELISA test was conducted as described in Section 3.4.7.1 with

the WB1 IgG used at a concentration of 5 µg.cm^{-3} and the alkaline phosphatase-linked protein A at a dilution of 1:4 000. Three replicates of each dilution were tested. A dilution series of a heated peat suspension and a suspension of WB1 cells were used as controls. Enzyme reactions were terminated after an incubation period of 60 min at room temperature.

3.4.12 Preliminary experiments to investigate the use of DAS ELISA to estimate the number of rhizobia in peat

For each of strains XBL6, XHT1 and WB1, four packets of inoculant were produced (See Section 3.4.9.3). Plate counts and the DAS ELISA test were performed as follows:

3.4.12.1 Viable counts

Each packet of inoculant was mixed for 2 min by hand in order to distribute the cells evenly in the peat. 20 g of inoculant was aseptically suspended in 80 cm^3 of sterile PBS in a 250 cm^3 Duran bottle. Subsequent steps were as described in Section 3.4.10.1. Plate counts of the 12 inoculant suspensions were done on day one. Two CR-YM agar plates instead of four were used for each dilution in order to complete

the viable counts in one day.

3.4.12.2 DAS ELISA

After the dilution series were prepared for the plate counts, the remainder of the inoculant suspensions were heated in boiling water for 30 min. One drop of a 0,02% NaN_3 solution was added to each inoculant suspension and stored at 4 °C. On day two, the DAS ELISA test was conducted on the four XBL6 inoculant suspensions. Sedimentation of the large peat particles took place during storage (Fig. 3). The supernatant of each suspension was sucked off with a 10 cm^3 pipette with a rubber bulb. A five-fold dilution series of each supernatant was prepared using PBS-Tween. Each remaining supernatant was then well mixed with its corresponding sediment, and a five-fold dilution series of each suspension was prepared. The DAS ELISA test was conducted as described in Section 3.4.7.2, with the XBL6 IgG used at a concentration of 4 $\mu g.cm^{-3}$ and the XBL6 strain-specific conjugate diluted 200 times. Three replicates of each dilution were tested. Heated peat and cell suspensions (Section 3.4.6.1) were used as controls.

On day three and four, the DAS ELISA test was conducted as before on the XHT1 and WB1 inoculant

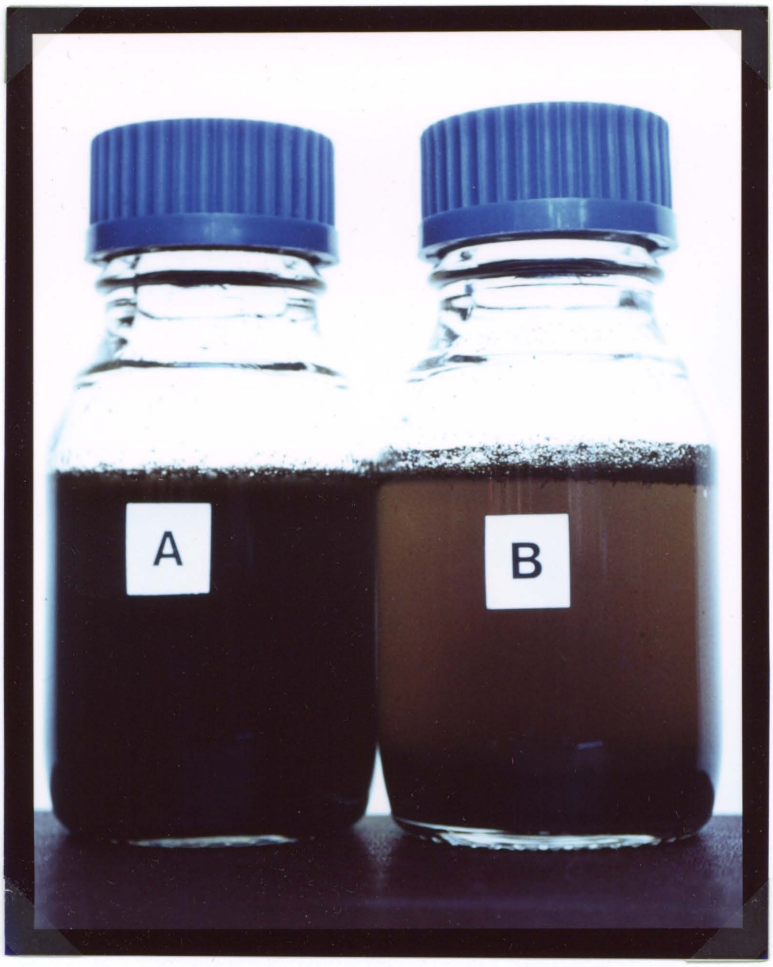


Fig. 3 Soybean inoculant suspensions used in preliminary experiments to investigate the use of DAS ELISA to estimate the number of viable B. japonicum strain WB1 cells in peat.

A - Suspension immediately after mixing

B - Suspension after peat particles were allowed to settle for 12 h

suspensions respectively. The XHT1 IgG was used at a concentration of 2 $\mu g.cm^{-3}$ and the XHT1 strain-specific conjugate diluted 400 times. The optimal concentration used for strain WB1 IgG was 2 $\mu g.cm^{-3}$ at a WB1 strain-specific conjugate dilution of 1:500.

3.4.13 Reduction of inhibition

Inoculants produced from strains WB1 and XBL6 were used to determine if the inhibition of the DAS ELISA reaction at low dilutions of inoculant suspensions could be removed or reduced by certain treatments.

3.4.13.1 Screening for a suitable treatment to reduce inhibition

A packet of inoculant was mixed by hand for 2 min. 10 g of inoculant was aseptically suspended in 40 cm^3 of sterile PBS. Plate counts were conducted as described in Section 3.4.10.1. The remainder of the inoculant suspension was heated in boiling water for 30 min, mixed on a Vortex mixer (speed 8) for 2 min and divided into four portions of 8 cm^3 each in 28 cm^3 glass McCartney bottles. After portion one was mixed for 60 sec on a Vortex mixer (speed 8), a five-fold dilution series was prepared using

PBS-Tween. Portion two was left for 24 h at 4 °C. The tip of an Eppendorf micropipette (1000 µl) was immersed 1 cm into the suspension. In total ca. 4 cm^3 supernatant was removed, mixed and a five-fold dilution series prepared. Portion three was mixed well and divided into two centrifuge tubes, after which they were centrifuged for 10 min at 121 x g and 3 020 x g respectively. Five-fold dilution series were prepared as before. Portion four was filtered using filter paper with large pores for rapid filtration (Ederol nr. 11, øm 12,5). Two portions of 4 cm^3 PBS-Tween were forced through the filter by means of a 10 cm^3 plastic syringe, to wash remaining antigen through the filter. The filtrate was used for preparing the dilution series and the extra dilution factor was taken into account in calculations. DAS ELISA was conducted as described in Sections 3.4.7.2 and 3.4.12.2. The first six dilutions of each treatment were tested.

3.4.13.2 Reduction of inhibition by centrifugation

Soybean inoculant prepared from strain WB1 was used in this experiment (Section 3.4.9.3). A packet of inoculant was mixed for 2 min by hand in order to distribute the cells evenly in the peat. 20 g of inoculant was aseptically suspended in

80 cm^3 of sterile PBS. Subsequent steps were the same as in Section 3.4.11.3, the only difference being the ELISA method followed. Plate counts were conducted as described in Section 3.4.12.1.

DAS ELISA was conducted as described in Section 3.4.7.2 with the WB1 IgG used at a concentration of 4 $\mu g.cm^{-3}$ and a WB1 strain specific conjugate diluted 1:600. Three replicates of each dilution were tested. Dilutions of heated peat and WB1 cell suspensions were used as controls.

3.4.13.3 Reduction of inhibition by sedimentation

The same procedure was followed as described in Section 3.4.13.2, the only difference being the treatment of the inoculant suspension before preparing the dilutions. Five 10 cm^3 portions of the well-mixed inoculant in 28 cm^3 glass McCartney bottles were left at 4 °C for 3 h, 6 h, 9 h, 12 h and 24 h respectively. The sixth 10 cm^3 was mixed for 60 sec on a Vortex mixer (speed 8) before the tip of an Eppendorf micro-pipet (1 000 µl) was immersed 2 cm into the suspension. A total of ca. 8 cm^3 suspension was transferred to a second McCartney bottle and was used to prepare the dilution series. Following standing, the five portions were treated the same as the sixth 10 cm^3 portion. The WB1 IgG in

DAS ELISA was used at a concentration of $2\ \mu g.cm^{-3}$ at the conjugate dilution of 1:500.

3.4.13.4 Comparison of the most successful treatments

Preparation of the soybean inoculant suspension by including a centrifugation step (121 x g for 10 min) was compared with soybean inoculant suspension prepared by using the sedimentation step of 3 h at $4^{o}C$. The experimental procedures are described in Sections 3.4.13.2 and 3.4.13.3. DAS ELISA was conducted at a WB1 IgG concentration of $2\ \mu g.cm^{-3}$ and a WB1 strain specific conjugate dilution of 1:500. With freshly prepared antibody and conjugate, the WB1 IgG concentration used was $4\ \mu g.cm^{-3}$ at a WB1 strain specific conjugate dilution of 1:600.

3.4.13.5 Standardized procedure for preparation of soybean inoculant suspensions

The method used in successive tests to prepare soybean inoculant suspension was as follows:
X g of well-mixed inoculant was placed in Y cm^3 PBS$^{(e)}$. The suspension was left on a shaker (Labotec) for 30 min before it was heated in a

Note: (e) Sterilised PBS was used when viable counts were also determined

boiling water bath for 30 min. 20 cm^3 portions of well-mixed, heated suspension were transferred to 28 cm^3 McCartney bottles. After a standing period of 3 h, the tip of an Eppendorf micropipette (1 000 µl) was immersed 2 cm into the supernatant and a total of 8 cm^3 supernatant was transferred to a second McCartney bottle and used to make appropriate dilutions using PBS-Tween.

3.4.14 Standard curve for estimating the number of strain WB1 cfu in peat by DAS ELISA

3.4.14.1 Use of laboratory produced inoculants

Soybean inoculants produced in the laboratory was used to compile a standard curve. 100 cm^3 YM broth was inoculated with two loopsful of growth from a CR-YM agar slant. After incubation of ca. 10 d, 5 cm^3 portions of inoculum were used to inoculate aseptically each of 12 Erlenmeyer flasks with 500 cm^3 YM broth. Following incubation of 12 d, the cells were harvested aseptically by centrifugation at 12 100 x g for 20 min. Each cell pellet thus obtained was aseptically suspended in approximately 20 cm^3 sterile BSS, and mixed aseptically with the other suspended pellets in a 500 cm^3 sterile centrifuge tube. BSS was added to a final volume of 300 cm^3. A total cell count was conducted on the well-mixed cell

suspension (Section 3.4.9.2). The cells were concentrated by centrifugation at 12 100 x g for 20 min and suspended in BSS to give a final concentration of ca. $1,2 \times 10^{11}$ cells.cm^{-3}. A 50 cm^3 portion of the well mixed suspension was transferred to a second Duran bottle that contained 50 cm^3 sterile BSS. Subsequent dilution steps used were 5 times and 2 times. 20 cm^3 portions of each dilution were injected into gamma-irradiated and steam-sterilized packets of peat. Two packets were prepared for each dilution. Each packet was mixed by hand for 2 min and incubated for 14 h at 28 °C.

Packets of the laboratory produced inoculant that contained ca. 1×10^{10}, 5×10^{9} and 1×10^{9} cfu.g^{-1} peat were used to compile the standard curve. Of the six packets used, three were prepared using steam-sterilized peat and three by using gamma-irradiated peat. 10 g of well-mixed inoculant was suspended in 90 cm^3 PBS. This suspension was treated as in Section 3.4.13.4. Plate counts and the DAS ELISA test were conducted as described in Sections 3.4.10.1 and 3.4.7.2. The WB1 IgG was used at a concentration of 4 µg.cm^{-3} and a conjugate dilution of 1:700.

3.4.14.2 Use of commercially produced inoculant

A standard curve was obtained by monitoring the numbers of Bradyrhizobium japonicum WB1 cfu in 18 packets of inoculant by plate counts and DAS ELISA. The packets were sampled randomly from 9 batches, two from each batch, produced by two manufacturers. The two inoculant manufacturers used peat from the same source. Soybean inoculant suspensions were prepared as described in Section 3.4.13.4. Plate counts were conducted as described in Section 3.4.10.1 and the DAS ELISA test as described in Section 3.4.7.2.

3.4.15 Comparison of DAS ELISA and plate counts

The quality of 43 packets of soybean inoculant, produced by two manufacturers, were tested as described in Sections 3.4.10, 3.4.13.4 and 3.4.7.2. Packets of inoculant were incubated at 28 °C for at least 10 d before two randomly selected packets of each batch were collected by the P.P.R.I. Each inoculant suspension was diluted 120 times and tested at a WB1 IgG concentration of 4 $\mu g.cm^{-3}$ and a WB1 strain-specific conjugate dilution of 1:700.

3.4.16 Screening methods to determine if B. japonicum strain WB1 cells in peat are viable

3.4.16.1 Inoculant particles

Packets of gamma-irradiated and steam-sterilized peat, inoculated with 20 cm^3 portions of a strain WB1 broth culture (ca. 1 X 10^9 cells. cm^{-3}) were prepared. After an incubation period of ca. 12 d, each packet of inoculant was mixed by hand for 2 min. Inoculant particles were thinly distributed onto agar surfaces by means of sterile aluminium cups with perforated bottoms. Three CR-YM agar plates and one nutrient agar plate were used for each packet of inoculant. The plates were incubated at 28 °C for 8 d.

3.4.16.2 Drops of inoculant suspension

A 10-fold dilution series was prepared for each inoculant suspension, using sterile PBS. Four drops of each of the 10^{-1}, 10^{-3}, 10^{-5} and 10^{-6} dilutions were transferred to a CR-YM agar and nutrient agar plate. The plates were inverted and incubated for 8 d at 28 °C.

3.4.16.3 The Miles and Misra drop plate technique (Vincent, 1970)

For each inoculant suspension, a suitable dilution series was prepared (Dilutions likely to contain up to 20 viable rhizobia per 0,02 cm^3),

using sterile PBS. Each plate was divided into 4 sectors and numbered. One CR-YM agar plate and one nutrient agar plate were used for each dilution of inoculant suspension tested. Four 20 µl portions of a well-mixed dilution were transferred to a plate (one 20 µl portion per sector) by using an Eppendorf micropipette (10 to 100 µl). Each 20 µl drop was spread over an area of approximately 2 cm^2. The plates were inverted and incubated at 28 °C for 4 to 5 d for the faster-, and up to 10 d for the slower-growing rhizobia. Counts were made when the colonies were still distinct (non-confluent). To calculate the number of cfu.cm^{-3} of inoculant suspension, the mean of the four replicate counts was multiplied by 50 times the dilution factor. To calculate the number of cfu.g^{-1} peat, the dilution factor that arose when X g of the inoculant had been suspended in Y cm^{-3} sterile PBS, was taken into account.

3.4.16.4 Comparison of plate counts and the drop plate technique

Plate counts were carried out as described in Section 3.4.10.1 on 32 packets of soybean inoculant (the packets of inoculant were supplied by Stimuplant). Viable counts by means of the Miles and Misra drop plate technique (Section

3.4.16.3) were also determined for each inoculant suspension tested by the plate counting procedure.

3.4.17 The effect of non-viable Bradyrhizobium cells on DAS ELISA

3.4.17.1 Effect of steam sterilization on inoculant antigen

Three packets of soybean inoculant were prepared from strain WB1 (Section 3.4.9.3). Each packet of inoculant was divided aseptically into three 80 g portions in sterile 250 cm^3 Duran bottles. 20 g of each portion was aseptically suspended in 80 cm^3 of sterile PBS in a 250 cm^3 Duran bottle. Plate counts (Section 3.4.12.1) and the DAS ELISA test (Section 3.4.12.2) were conducted on each suspension. The remaining portions were steam sterilized for 3 h at 121 ^{o}C. After cooling, plate counts and the DAS ELISA test were again conducted on each portion in order to determine if complete sterilization of the peat inoculant was obtained, and if any killed cells could be detected by DAS ELISA. The experiment was repeated three times.

3.4.17.2 Effect of gamma-irradiation on inoculant antigen

The same procedure was followed as described in Section 3.4.17.1, the only difference being the

method of sterilization used. The 80 g portions of inoculant were sealed in low density polythene bags, packed in a cardboard container and exposed to gamma-irradiation. The portions of inoculant received a 50 $kGy.kg^{-1}$ dose.

3.4.18 Statistical procedures

Statistical techniques and tables used may be found in Steel & Torrie (1960) and Snedecor & Cochran (1967).

CHAPTER 4

RESULTS

4.1 Quality of antisera

In order to determine the quality of antisera raised against five Bradyrhizobium and four Rhizobium strains, somatic agglutination titres were measured by tube agglutination tests. Titres of antisera, tested in homologous as well as heterologous reactions, are shown in Table 2. High somatic agglutination titres, ranging from 1 600 to 12 800, were obtained in homologous reactions of all antisera. The exception was antiserum raised against strain RF14 with a titre of 400. Attempts to increase the somatic agglutination titre of strain RF14 antiserum with weekly intravenous booster injections, administered to the rabbits for one month, and by repeating the immunization schedule with two other rabbits, were unsuccessful. Antiserum raised against strain RF14 furthermore cross-reacted with strain RF6 antigen. Consequently, it was decided not to use strain RF14 antiserum in subsequent experiments, as antisera with homologous agglutination titres of at least 1 280 are required for use in the ELISA technique (B.D. Kishinevsky, pers comm). No other heterologous antigen-antibody reactions could be detected

TABLE 2 Cross-agglutination reactions of heat-treated cells of slow- and fast-growing rhizobia with homologous and heterologous antisera

Source of antigen	Source of antiserum and agglutination titre[a]								
Strains	WB1	XHT1	XBL6	XCT9	VK10	XCV14	SR4	RF14	RF6
WB1	1 600	-	-	n	-	-	-	-	-
XHT1	-[b]	3 200	-	n	-	-	-	-	-
XBL6	-	-	3 200	n	-	-	-	-	-
XCT9	-	-	n[d]	12 800	n	-	-	-	-
VK10	-	-	-	n	1 600	-	-	-	-
XCV14	a[c]	a	a	n	a	a	a	a	a
SR4	-	-	-	n	-	-	3 200	-	-
RF14	-	-	-	n	-	-	-	400	-
RF6	-	-	-	n	-	-	-	200	12 800

[a] All values represent reciprocals of the highest serum dilution in which agglutination occurred. There were no replicates.

[b] No antigen-antiserum reaction was detected at a 1:100 dilution of antiserum.

[c] Autoagglutination occurred

[d] Not determined

at 1:100 dilutions of antisera. Homologous and heterologous somatic agglutination titres were not determined for strain XCV14 antiserum, as auto-agglutination of this strain occurred in saline.

4.2 Quality of enzyme-linked antibodies

The alkaline phosphatase-linked protein A was of high quality as low non-specific reactions and high A_{405} values were obtained in indirect ELISA. Using XBL6 IgG at a concentration of 4 $\mu g.cm^{-3}$ and WB1 IgG at a concentration of 5 $\mu g.cm^{-3}$, a moderate conjugate dilution of 1:4 000 was used in subsequent tests.

In contrast, not all the alkaline phosphatase-linked anti-strain specific IgG prepared for use in DAS ELISA were of desired quality. Fig.4 shows the performance of enzyme-linked anti-strain specific IgG of slow-growing strains of rhizobia in DAS ELISA, when compared at conjugate dilutions of 1:100 and 1:400. High DAS ELISA values (A_{405}) were obtained in homologous reactions of all three slow-growing strains, when tested at a conjugate dilution of 1:100 (Fig. 4 A). The A_{405} value of 0,93, obtained when XHT1 IgG was tested against strain VK10 cell suspension, indicated that strains XHT1 and VK10 shared common antigenic determinants. All other heterologous reactions were negative. Non-specific reactions in these wells

Fig. 4 The effect of two dilutions of three strain-specific conjugates on A_{405} values obtained in the serological identification of five different strains of rhizobia by DAS ELISA

A - 1:100 dilution of conjugates

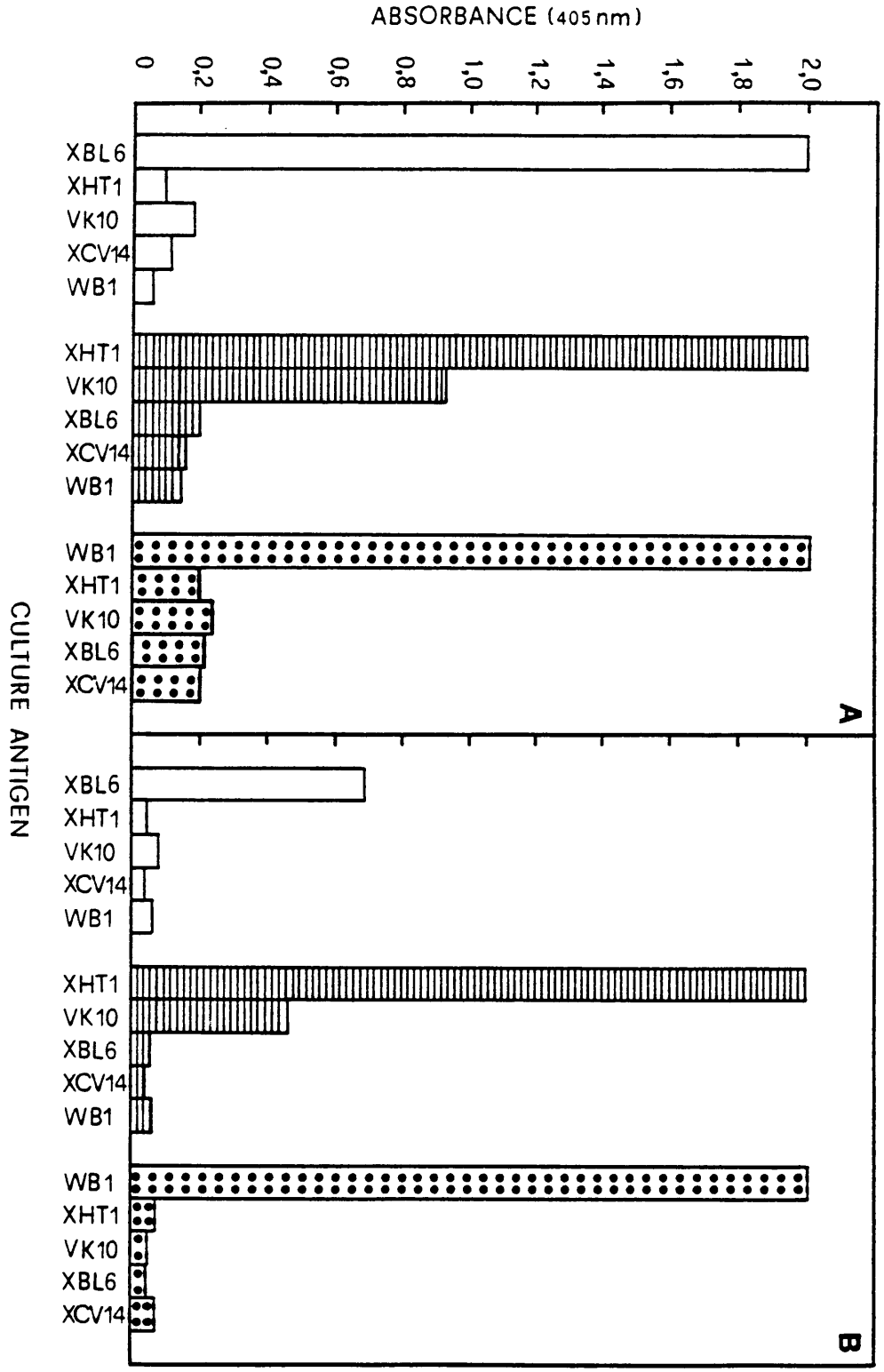
B - 1:400 dilution of conjugates

 - XBL6-specific conjugate

 - XHT1-specific conjugate

 - WB1-specific conjugate

Homologous and heterologous antigen-antibody combinations were tested. Each XBL6, XHT1 and WB1 IgG concentration was 6 $\mu g.cm^{-3}$. Cell suspensions contained <u>ca</u>. 10^9 $cells.cm^{-3}$. A_{405} values are the means of four replicates.



were low ($A_{405} \leq 0,22$). Similar high A_{405} values were obtained in homologous reactions of strains WB1 and XHT1 at a 1:400 conjugate dilution, whereas the A_{405} value in the homologous reaction of strain XBL6 decreased markedly (Fig. 4 B). The quality of the WB1-specific and XHT1-specific conjugates were superior to the quality of the XBL6-specific conjugate. A_{405} values of non-specific background reactions were lower at the conjugate dilution of 1:400 ($A \leq 0,08$) than at the 1:100 dilution.

The performance of fast-growing strains of rhizobia in DAS ELISA at conjugate dilutions of 1:100 and 1:400 is shown in Fig. 5. Moderate high DAS ELISA values were obtained in homologous reactions of the fast-growing strains XCV14 and RF6 at the 1:100 conjugate dilutions (Fig. 5 A). An A_{405} value of 0,92 indicated that strains RF6 and RF14 shared common antigenic determinants. Non-specific reactions in heterologous wells were high ($A \leq 0,58$). When the XCV14-specific and RF6-specific conjugates were diluted 1:400 in an attempt to reduce non-specific background reactions, homologous A_{405} values decreased markedly (Fig. 5 B).

It appeared that conjugates specific to the fast-growing strains were weaker and less specific than the conjugates specific to the slow-growing strains.

Fig. 5 The effect of two dilutions of two strain-specific conjugates on A_{405} values obtained in the serological identification of different strains of rhizobia by DAS ELISA

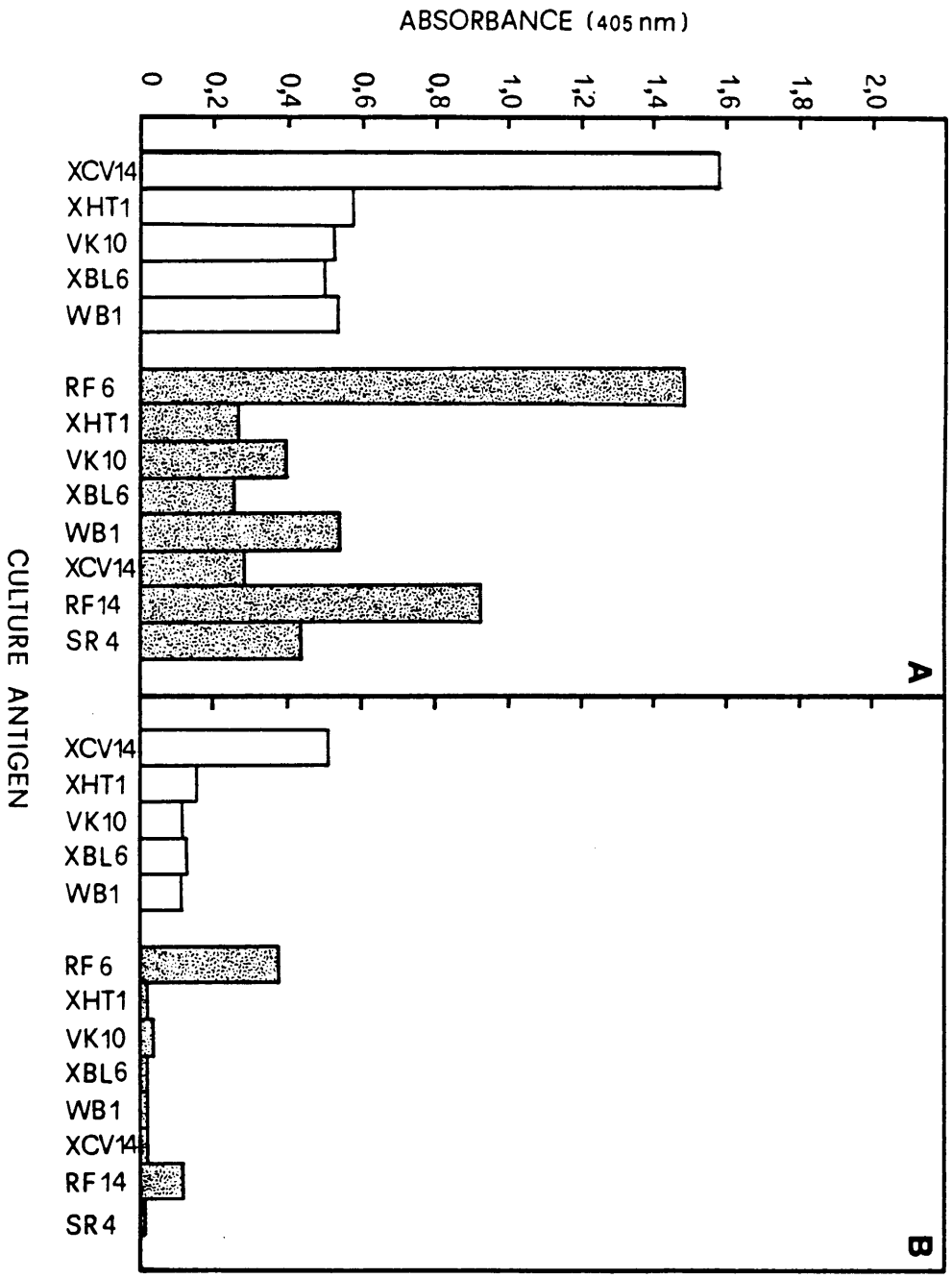
A - 1:100 dilution of conjugates

B - 1:400 dilution of conjugates

 - XCV14-specific conjugate

 - RF6-specific conjugate

Homologous and heterologous antigen-antibody combinations were tested. Each XCV14 and RF6 IgG concentration was 6 $\mu g.cm^{-3}$. Cell suspensions contained *ca*. 10^9 cells.cm^{-3}. A_{405} values are the means of four replicates.



4.3 Optimal concentrations of immunoreactants

As ELISA reactivity varied for different antigen-antibody combinations, optimal concentrations of immunoreactants were determined for each combination by a chequerboard titration protocol. An example of a preliminary titration of strain RF6 cell suspension, at three concentrations of coating RF6 IgG and three dilutions of RF6-specific conjugate, is given in Fig. 6. While concentrations of coating IgG had little effect on final A_{405} values, the dilution of conjugate used was critical. In this case, an RF6 IgG concentration of 2 $\mu g.cm^{-3}$ and a RF6-specific conjugate dilution of 1:100 were adopted for subsequent tests. The lowest limit of positive detection of antigen by ELISA was taken as A_{405} values twice the average A_{405} value of the negative control.

4.4 Preliminary investigation into the use of indirect ELISA for enumeration of rhizobia in peat

Attempts to use the indirect ELISA technique for detection of antigen of strain XBL6 in the peat carrier were unsuccessful. Even when the enzyme reaction was not terminated after 30 min as is common practice, positive detection of antigen in the well-mixed inoculant suspension was not possible. Similar negative results were obtained

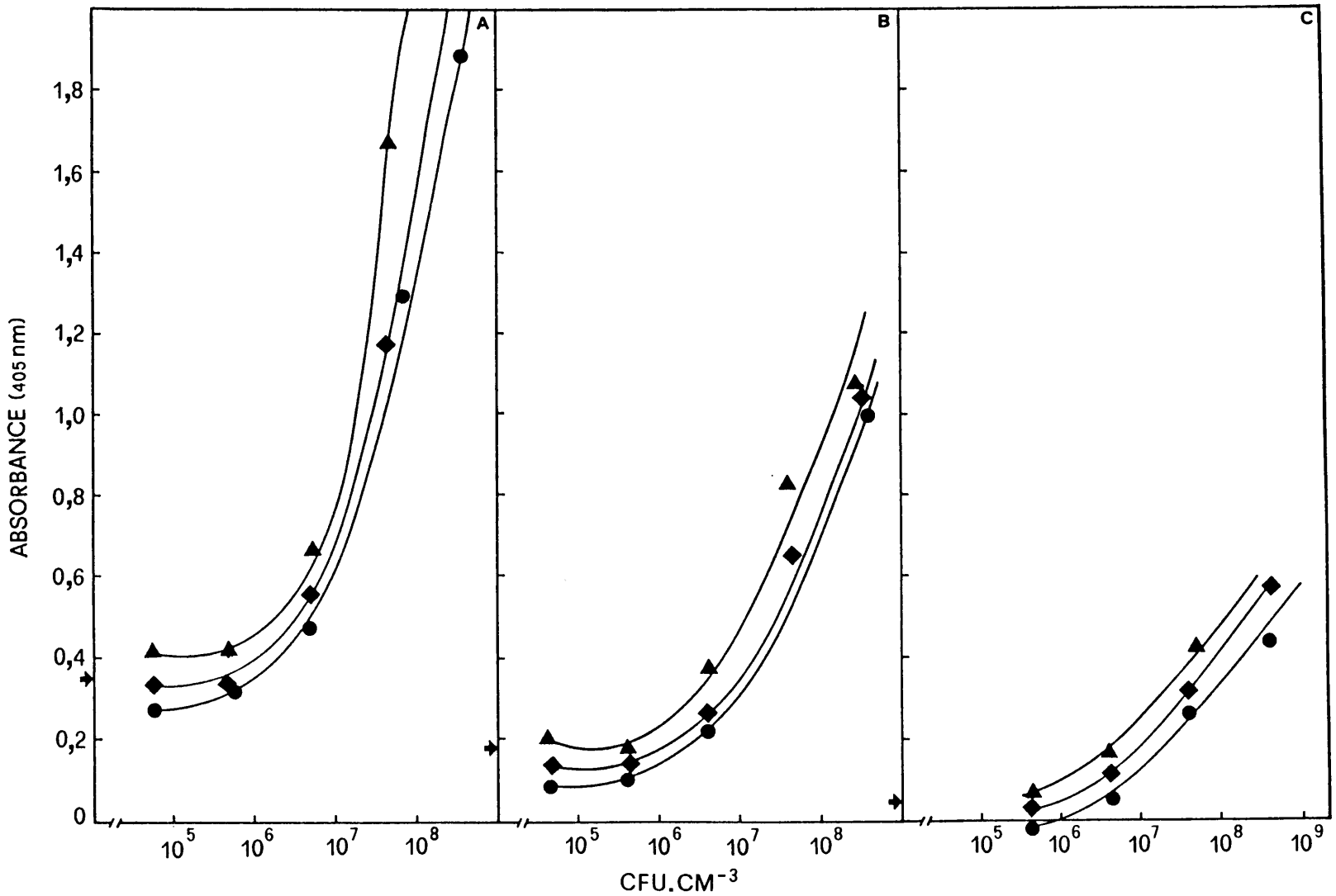
Fig. 6 Optimal concentrations of immunoreactants for detection of strain RF6 antigen in PBS-Tween by DAS ELISA

A - 1:100 dilution of RF6-specific conjugate

B - 1:200 dilution of RF6-specific conjugate

C - 1:400 dilution of RF6-specific conjugate

RF6 coating IgG was tested at concentrations of 2 $\mu g.cm^{-3}$ (●—●), 4 $\mu g.cm^{-3}$ (◆—◆) and 8 $\mu g.cm^{-3}$ (▲—▲). A 10-fold dilution series of strain RF6 cell suspension was tested. The arrows represent highest A_{405} values obtained with controls.



with soybean inoculant, as no yellow colouring occurred in the wells containing soybean inoculant suspension after 60 min. When four portions of a soybean inoculant suspension, subjected to centrifugation of 10 min at 121 x g, 3 020 x g, 12 100 x g and 27 000 x g respectively, were tested in indirect ELISA, weak yellow colouration could be observed in some wells after 30 min. Termination of the enzyme reaction after an incubation period of 60 min resulted in indirect ELISA values shown in Fig 7. ELISA colour reactions became progressively weaker with higher speeds of centrifugation of inoculant suspension. Centrifugation of inoculant suspension at 121 x g seemed the most promising pre-treatment for detection of antigen of strain WB1 in peat, as highest A_{405} values were obtained in this case. The sudden decrease in A_{405} values that occurred at concentrations $\geq 6{,}0 \times 10^{7}$ cfu.g^{-1} peat, made it impossible to distinguish for example between $1{,}0 \times 10^{6}$ and $2{,}0 \times 10^{7}$ cfu.g^{-1} peat at $A_{405} = 0{,}5$. Preliminary results indicated that interference with the indirect ELISA reaction occurred, possibly by the peat particles during the antigen binding step. The indirect ELISA method used here was considered incapable of enumerating rhizobia in the peat carrier at this stage.

4.5 Preliminary investigation into the use of DAS ELISA for enumeration of rhizobia in peat

Fig. 7 Effect of centrifugation of soybean inoculant suspension on indirect ELISA A_{405} values.

△-△ 121 x g for 10 min

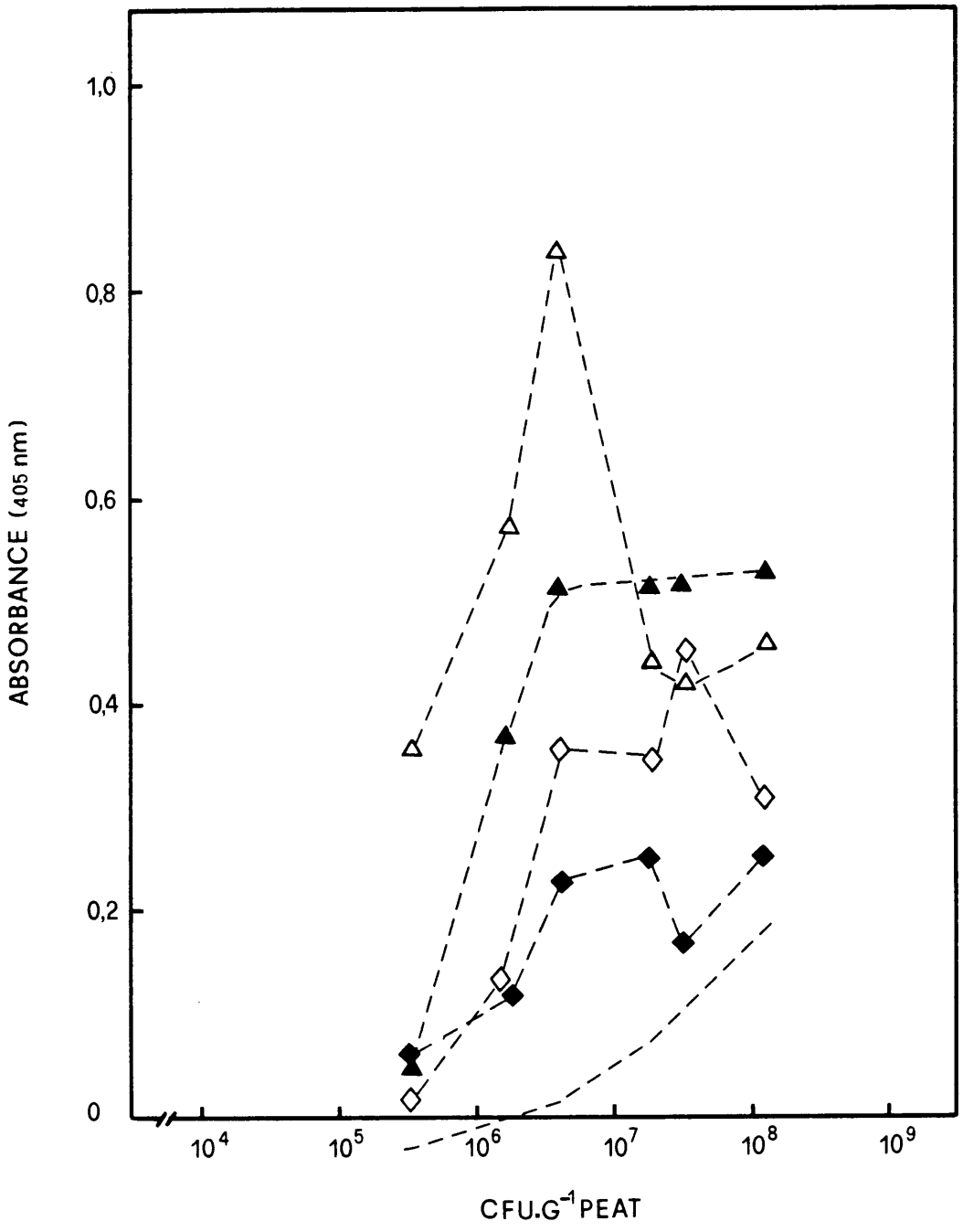
▲-▲ 3 020 x g for 10 min

◇--◇ 12 100 x g for 10 min

◆-◆ 27 000 x g for 10 min

--- uninoculated peat suspension after centrifugation at 121 x g for 10 min

A_{405} values and viable cell counts are the means of three replicates. Concentration of WB1 coating IgG was 5 $\mu g.cm^{-3}$. The protein A conjugate was diluted 1:4 000. Serial five-fold dilutions of inoculant suspension were tested.



Results of preliminary experiments to investigate if DAS ELISA could be used to estimate the number of cfu of strain WB1 in peat inoculant are presented in Fig. 8, 9, and 10. Higher A_{405} values were obtained for all the inoculant suspensions where the supernatant (B), rather than the well-mixed inoculant suspension (A), was used to prepare the five-fold dilution series. The relationship between A_{405} values and the number of Bradyrhizobium cfu.g^{-1} moist peat was not linear over the whole range of numbers of cfu tested for any of the inoculant suspensions (Fig. 8 B, 9 B and 10 B). When estimating a DAS ELISA reading range for the strain WB1 antigen-antibody combination (Fig. 8 B), A_{405} values would range from ca. 0,35 to 0,85, as a linear relationship could be observed over the narrow range of ca. $5,0 \times 10^{6}$ to $5,0 \times 10^{7}$ cfu.g^{-1} peat. For the XHT1 and XBL6 antigen-antibody combinations, a linear relationship was also observed over a narrow range of Bradyrhizobium numbers. In all cases (Fig. 8, 9, and 10) very low non-specific DAS ELISA reactions occurred with uninoculated peat. Due to this low background A_{405} values obtained, ca. $4,0 \times 10^{6}$ strain WB1 cfu (Fig. 8 B), $6,0 \times 10^{4}$ strain XHT1 cfu (Fig. 9 B) and $4,0 \times 10^{5}$ strain XBL6 cfu (Fig. 10 B). g^{-1} moist peat could be observed.

Inhibition of DAS ELISA colour reactions occurred at low dilutions of the majority of inoculant suspensions tested (Fig. 8, 9 and 10). This sudden decrease in A_{405} values made it impossible to distinguish for example

Fig. 8 DAS ELISA A_{405} values and viable cell counts obtained from serial dilutions of each of four soybean inoculants immediately after suspension and after peat particles had settled

A - Five-fold dilutions were made immediately after each inoculant had been suspended in PBS

B - Five-fold dilutions of the supernatant were made after peat particles had been allowed to settle for 12 h.

Concentration of WB1 coating IgG was 2 $\mu g.cm^{-3}$. The WB1-specific conjugate was diluted 1:500. A_{405} values and viable cell counts are the means of three replicates. Bars represent the standard deviation. An arrow represents the highest average A_{405} value obtained with the uninoculated peat control.

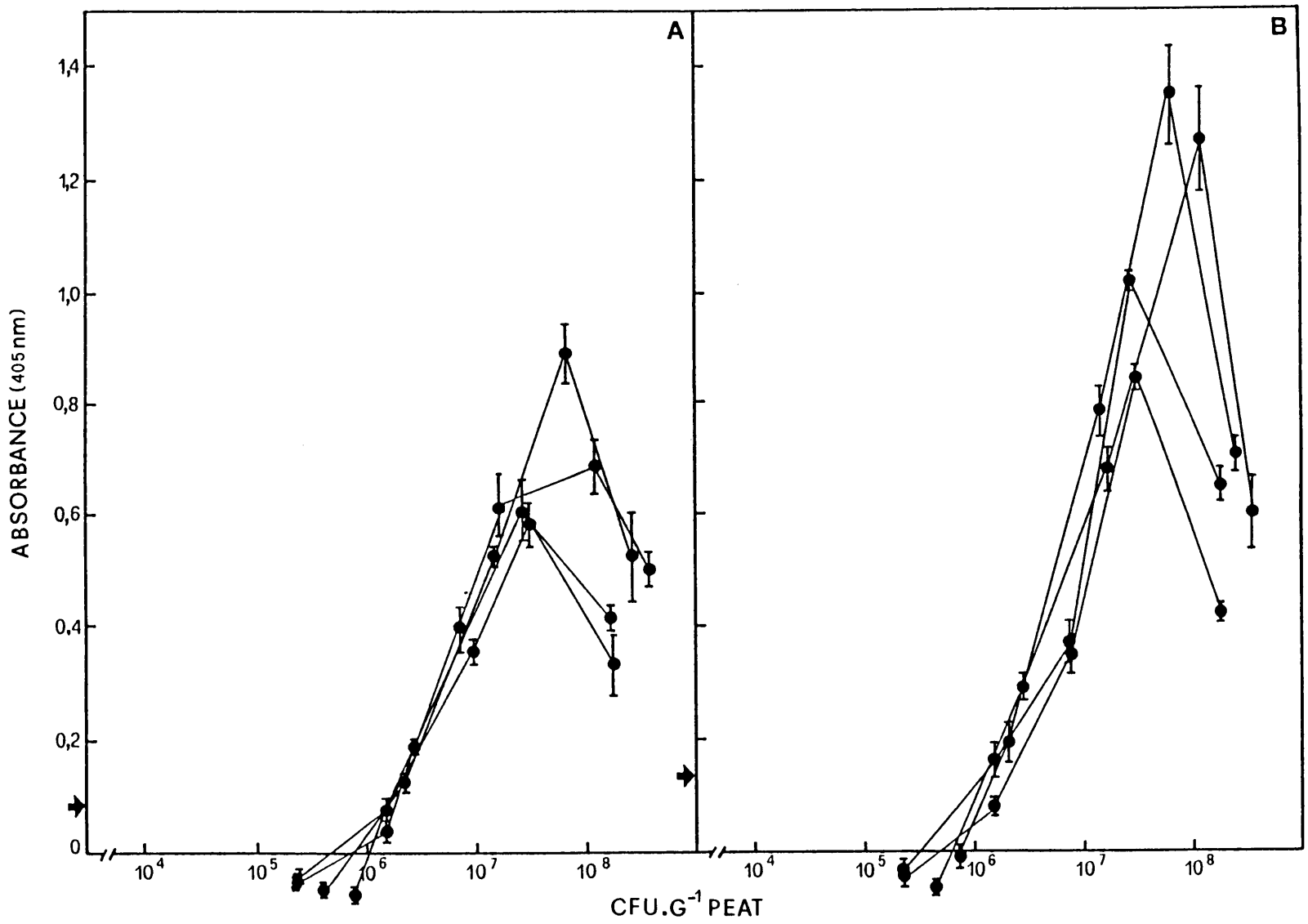


Fig. 9 DAS ELISA A_{405} values and viable cell counts obtained from serial dilutions of each of three *Lotus pedunculatus* inoculants immediately after suspension and after peat particles had settled

A - Five-fold dilutions were made immediately after each inoculant had been suspended in PBS

B - Five-fold dilutions of the supernatant were made after peat particles had been allowed to settle for 12 h

Concentration of XHT1 coating IgG was 2 $\mu g.cm^{-3}$. Dilution of XHT1-specific conjugate was 1:400. A_{405} values and viable cell counts are the means of three replicates. An arrow represents the highest average A_{405} value obtained with the uninoculated peat control. Bars represent the standard deviation.

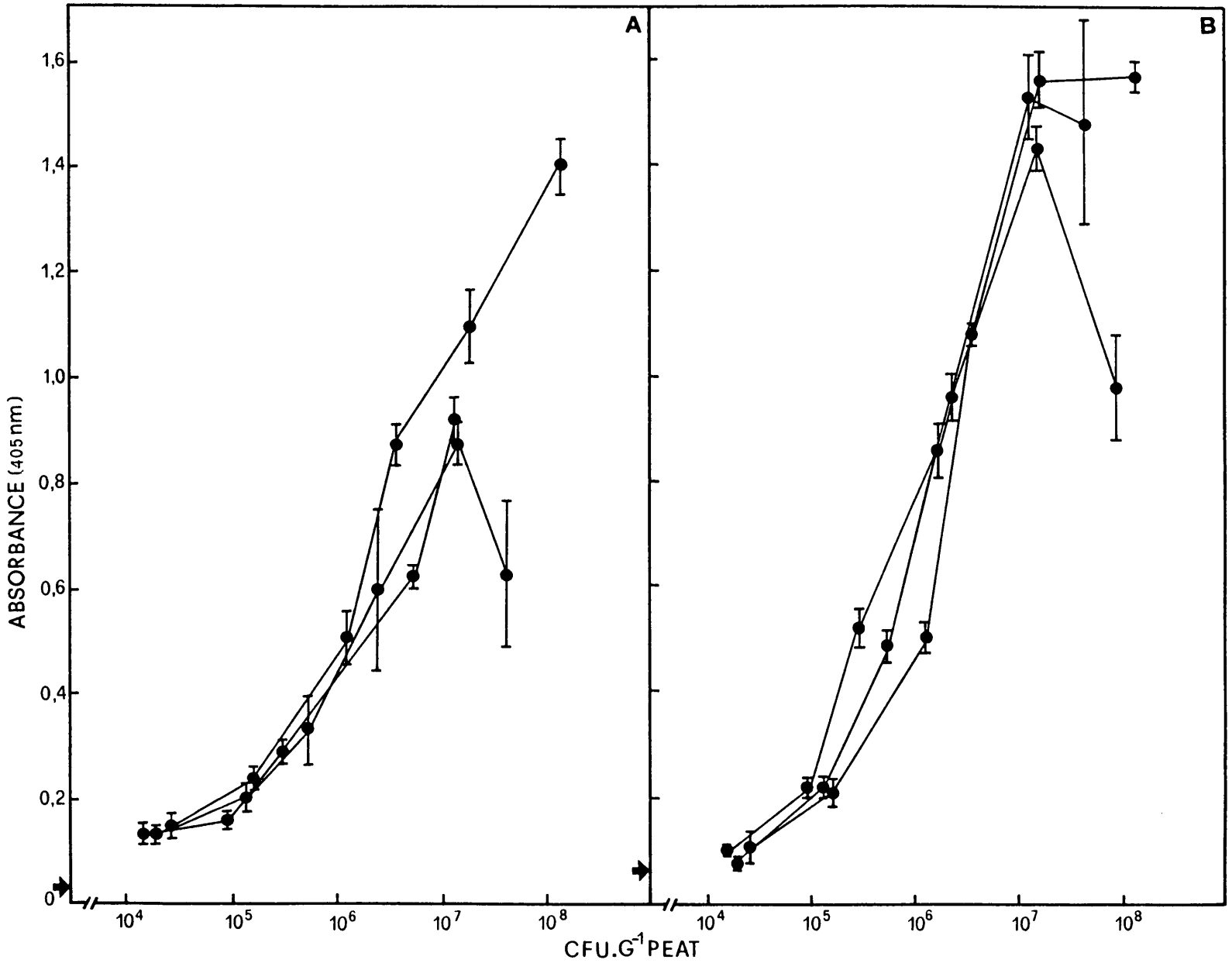
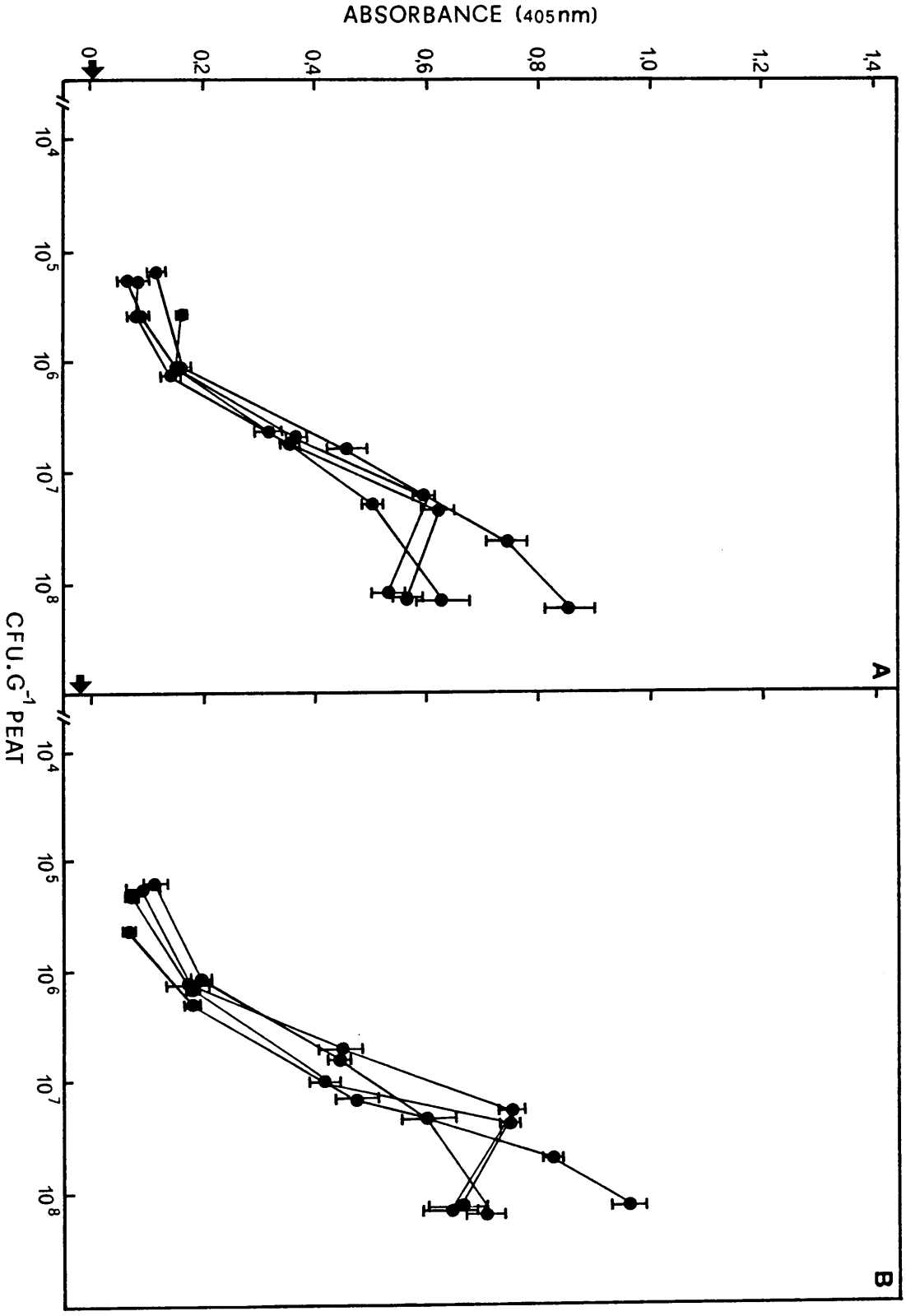


Fig. 10 DAS ELISA A_{405} values and viable cell counts obtained from serial dilutions of each of four groundnut inoculants immediately after suspension and after peat particles had settled

A - Five-fold dilutions were made immediately after each inoculant had been suspended in PBS

B - Five-fold dilutions of the supernatant were made after peat particles had been allowed to settle for 12 h

Concentration of XBL6 coating IgG was 4 $\mu g.cm^{-3}$. Dilution of XBL6-specific conjugate was 1:200. A_{405} values and viable cell counts are the means of three replicates. An arrow represents the highest average A_{405} value obtained with the uninoculated peat control. Bars represent the standard deviation.



between $2,5 \times 10^7$ and $4,5 \times 10^8$ cfu.g^{-1} moist peat at A_{405} = 0,7 (Fig. 8 B). The degree of inhibition differed among suspensions prepared from different packets of the same inoculant, as well as from different inoculants, and was inconsistent. As this phenomenon was most pronounced in the case of soybean inoculant, the strain WB1 antibody-antigen combination was selected for subsequent experiments.

When attempting to detect rhizobial antigen in the peat carrier by DAS ELISA, problems with weak, less specific conjugates were encountered, as shown in Fig. 11. Due to the high background A_{405} value of 0,38 for the uninoculated peat control, the A_{405} value of 0,57 in the inoculant suspension with $9,5 \times 10^7$ strain SR4 cfu.g^{-1} peat, could not be interpreted as positive. Only A_{405} values of twice the negative control, i.e. $A_{405} \geq 0,76$ were taken as positive. As non-specific background reactions decreased with dilution of the uninoculated peat control, $1,5 \times 10^5$, $7,5 \times 10^5$, $3,5 \times 10^6$ and $2,1 \times 10^7$ cfu.g^{-1} peat could be detected. No fast-growing strain was used in subsequent experiments, as the fast-growing strain-specific conjugates exhibited weak and less specific DAS ELISA reactions (See also Fig. 5).

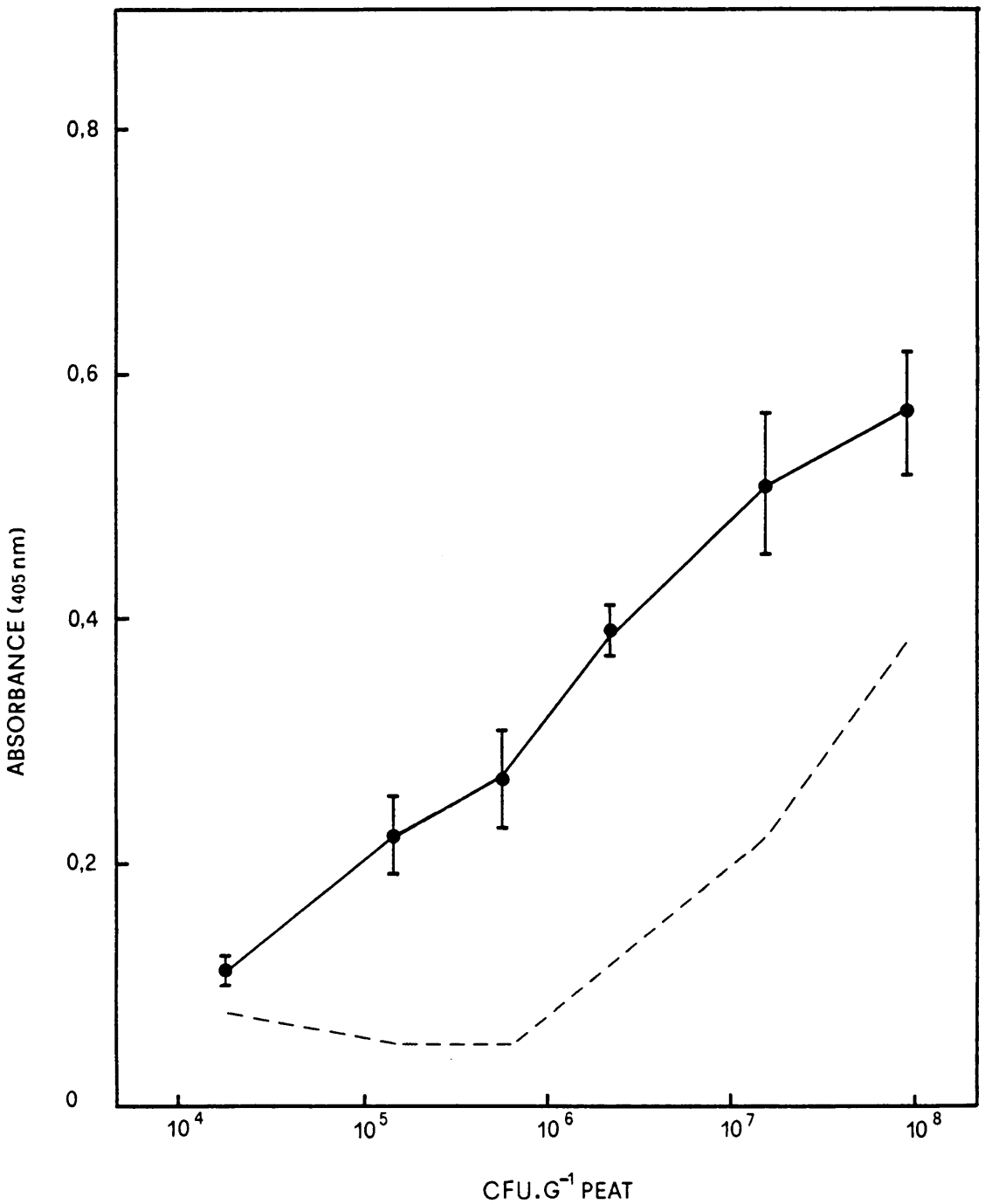
4.6 Reduction of inhibition

Fig. 11 DAS ELISA A_{405} values and viable cell counts obtained from serial dilutions of a clover inoculant immediately after suspension in PBS

●—● Peat inoculant suspension

---- Uninoculated peat suspension

A_{405} values and viable cell counts are the means of four replicates. Concentration of the SR4 coating IgG was 8 $\mu g.cm^{-3}$. SR4-specific conjugate was diluted 1:200. Bars represent the standard deviations.



An attempt was made to reduce or eliminate the factor responsible for interfering with DAS ELISA reactions in the lowest dilutions of inoculant suspensions tested. A high amount of peat particles were present in the lowest dilutions of inoculant suspension, and might have caused the inhibition. The effect of reducing the amount of peat particles in suspension on DAS ELISA is shown in Fig.12 and Fig. 13. The amount of peat particles in suspension were reduced by three different treatments of the soybean and groundnut inoculant suspensions, i.e. sedimentation, centrifugation and filtration. Marked increases in DAS ELISA values occurred with the suspensions with less peat particles than the well-mixed inoculant suspension. Non-specific DAS ELISA reactions in uninoculated peat control wells were low. As highest A_{405} values were obtained with inoculant suspensions subjected to sedimentation and centrifugation, these two treatments were further investigated.

The effect of centrifugation of 20 cm^3 portions of soybean inoculant at 121 x g, 3 020 x g, 12 100 x g and 27 000 x g for 10 min on DAS ELISA values is presented in Fig. 14. Suspension centrifuged at 121 x g gave the strongest DAS ELISA reaction. Increasing the speed of centrifugation resulted in progressively weaker DAS ELISA reactions. Very low non-specific DAS ELISA reactions occurred in the uninoculatd peat control wells.

Fig. 15 shows DAS ELISA values and viable vounts obtained

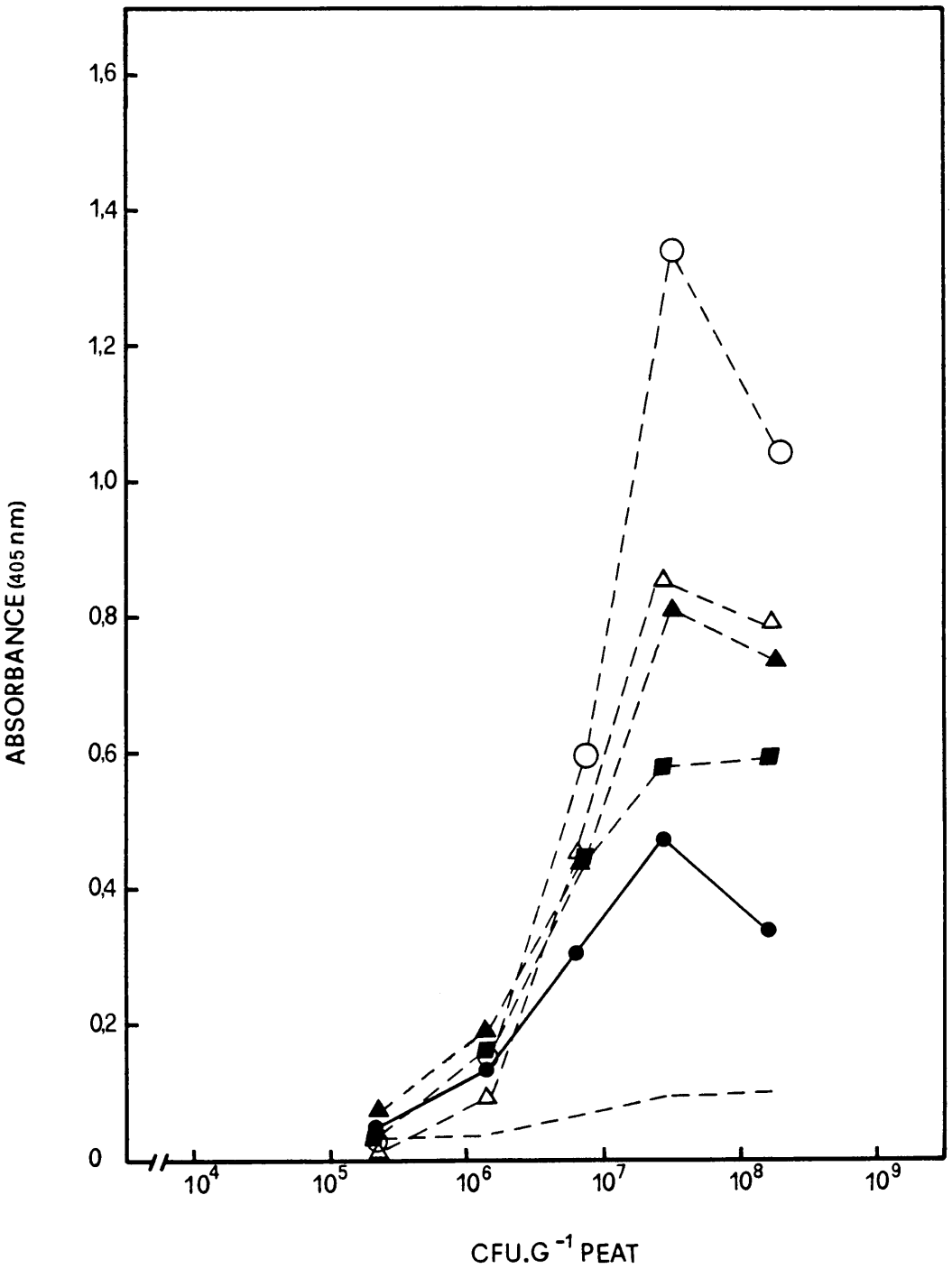


Fig. 13 Viable cell counts of groundnut inoculant suspension and DAS ELISA A_{405} values obtained after five different treatments of the suspension

○--○ Allowed to settle for 24 h

△--△ Centrifuged at 121 x g for 10 min

▲--▲ Centrifuged at 3 020 x g for 10 min

■--■ Filtered through filter paper with large pores (Ederol nr. 11, øm 12,5)

●—● Suspension sampled immediately after mixing

---- Uninoculated peat suspension sampled immediately after mixing

A_{405} values and viable cell counts are the means of three and four replicates respectively. Concentration of XBL6 coating IgG was 4 $\mu g.cm^{-3}$. Dilution of XBL6-specific conjugate was 1:200. Serial five-fold dilutions of inoculant suspension were tested.

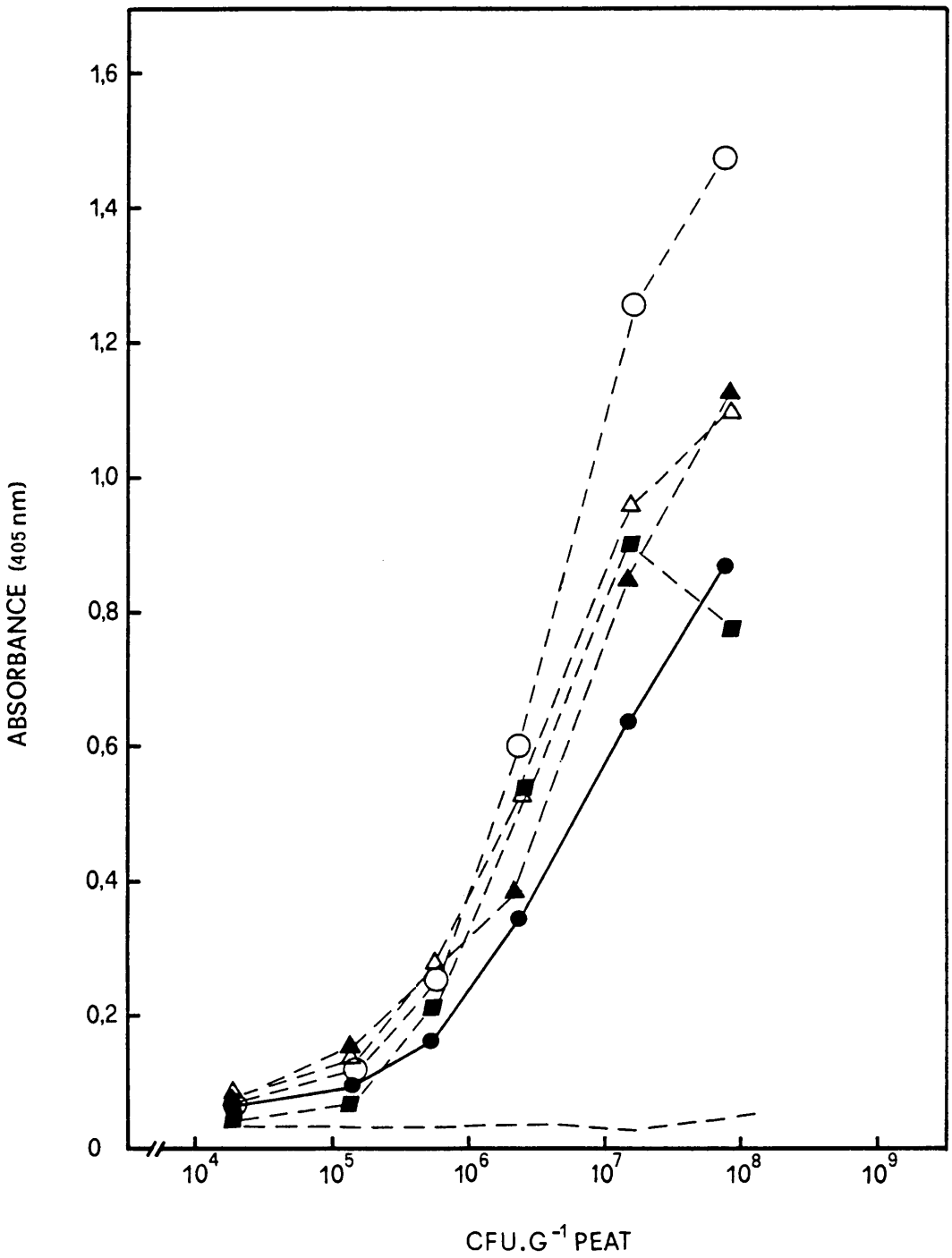


Fig. 14 Viable cell counts of a soybean inoculant suspension and DAS ELISA A_{405} values obtained from the supernatants of the suspension after centrifugation at four different speeds

△--△ 121 x g for 10 min

▲--▲ 3 020 x g for 10 min

◆--◆ 12 100 x g for 10 min

◇---◇ 27 000 x g for 10 min

---- Uninoculated peat suspension centrifuged at 121 x g for 10 min

Concentration of WB1 coating IgG was 4 $\mu g.cm^{-3}$. Dilution of WB1-specific conjugate was 1:600. A_{405} values and viable cell counts are the means of four replicates. Serial five-fold dilutions of inoculant suspension were tested.

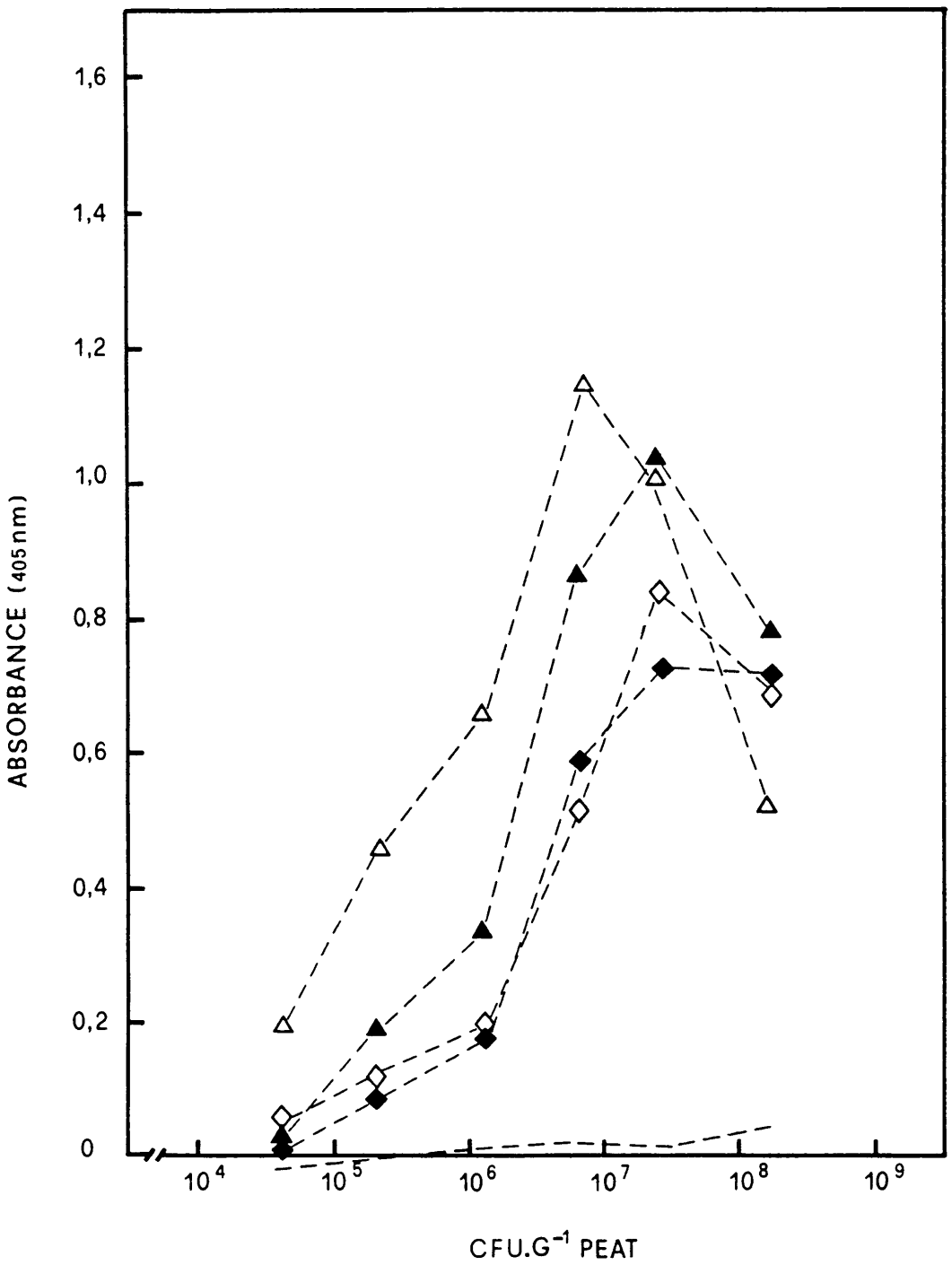
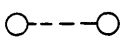
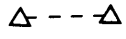
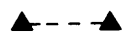


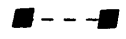
Fig. 15 DAS ELISA A_{405} values obtained with suspension of soybean inoculant in which the peat particles were allowed to settle for various periods


3 h


6 h


9 h

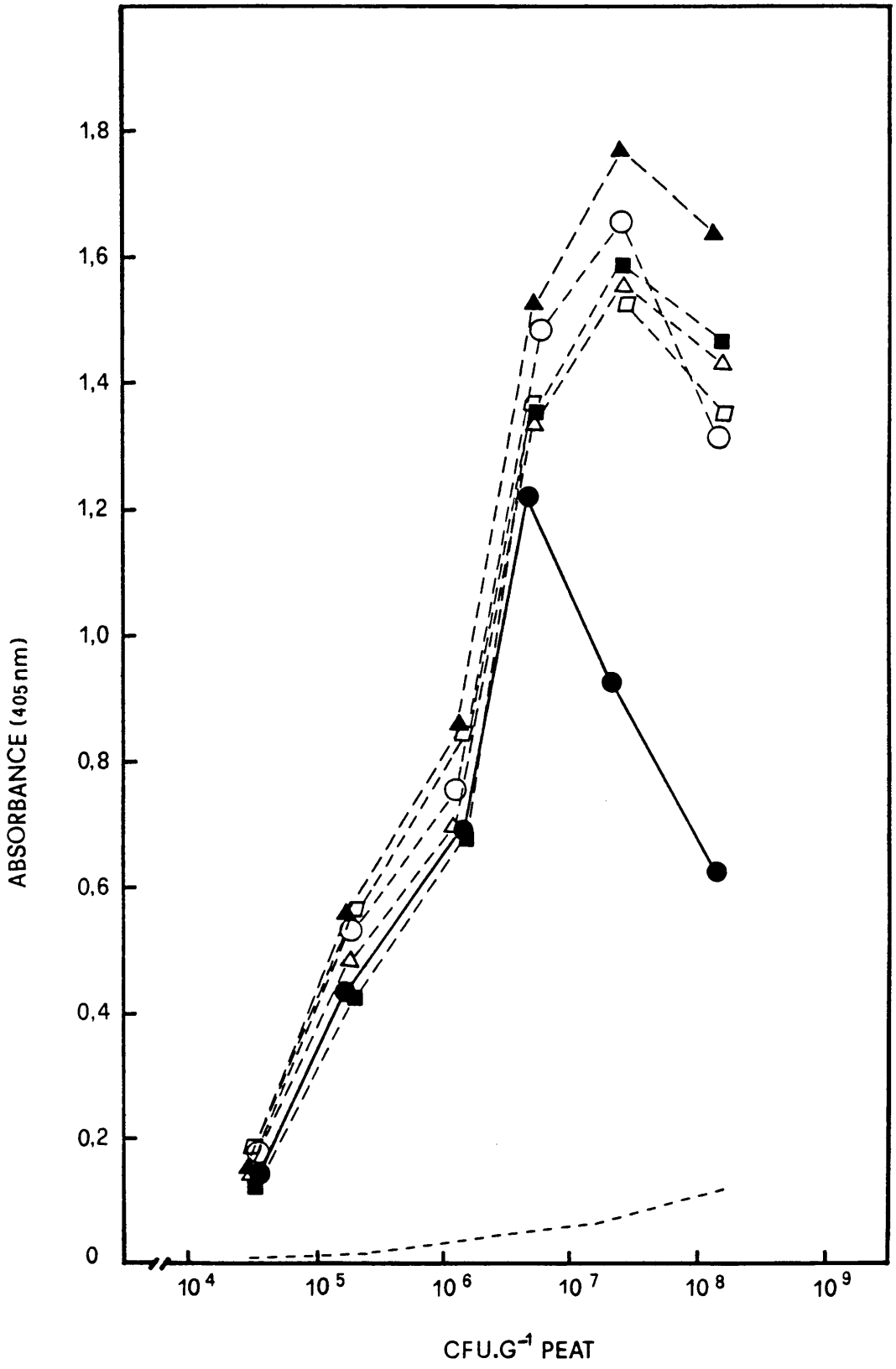

12 h


24 h


0 h

---- Uninoculated peat suspension at 0 h

Concentration of WB1 coating IgG was 2 $\mu g.cm^{-3}$. A_{405} values and viable cell counts are the means of four replicates. Serial five-fold dilutions of inoculant suspension were tested.



with five portions of a soybean inoculant suspension, subjected to sedimentation for 3 h, 6 h, 9 h, 12 h and 24 h respectively. When testing a portion of the well-mixed inoculant suspension in DAS ELISA, marked inhibition of the DAS ELISA reaction occurred at cell concentrations $\geq$ ca. $7,0 \times 10^{6}$ cfu.g^{-1} peat. In contrast, all the suspensions, subjected to different periods of sedimentation, inhibited the DAS ELISA colour reactions at cell concentrations $\geq$ ca. $4,0 \times 10^{7}$ cfu.g^{-1} peat to a lesser degree. Portions of inoculant suspension, subjected to each of the five different periods of sedimentation, performed more or less equally well in DAS ELISA. A sedimentation period of 3 h was considered the most suitable for inclusion in a routine method.

In the final screening for the most successful treatment to reduce inhibition, three treatments were compared, i.e. the well-mixed inoculant suspension and two 8 cm^{3} portions subjected to sedimentation for 3 h and centrifugation at 121 x g for 10 min respectively. The effect of these treatments of soybean inoculant suspension on DAS ELISA values is shown in Fig. 16. Non-specific background reactions in the uninoculated peat control wells were low. More or less equally high A_{405} values were obtained in DAS ELISA with portions of inoculant suspension, subjected to sedimentation for 3 h and centrifugation at 121 x g for 10 min. Relatively high A_{405} values were obtained and less inhibition occurred with low dilutions of these suspensions. The well-mixed inoculant suspension

Fig. 16 DAS ELISA A_{405} values obtained after three pre-treatments of soybean inoculant suspension

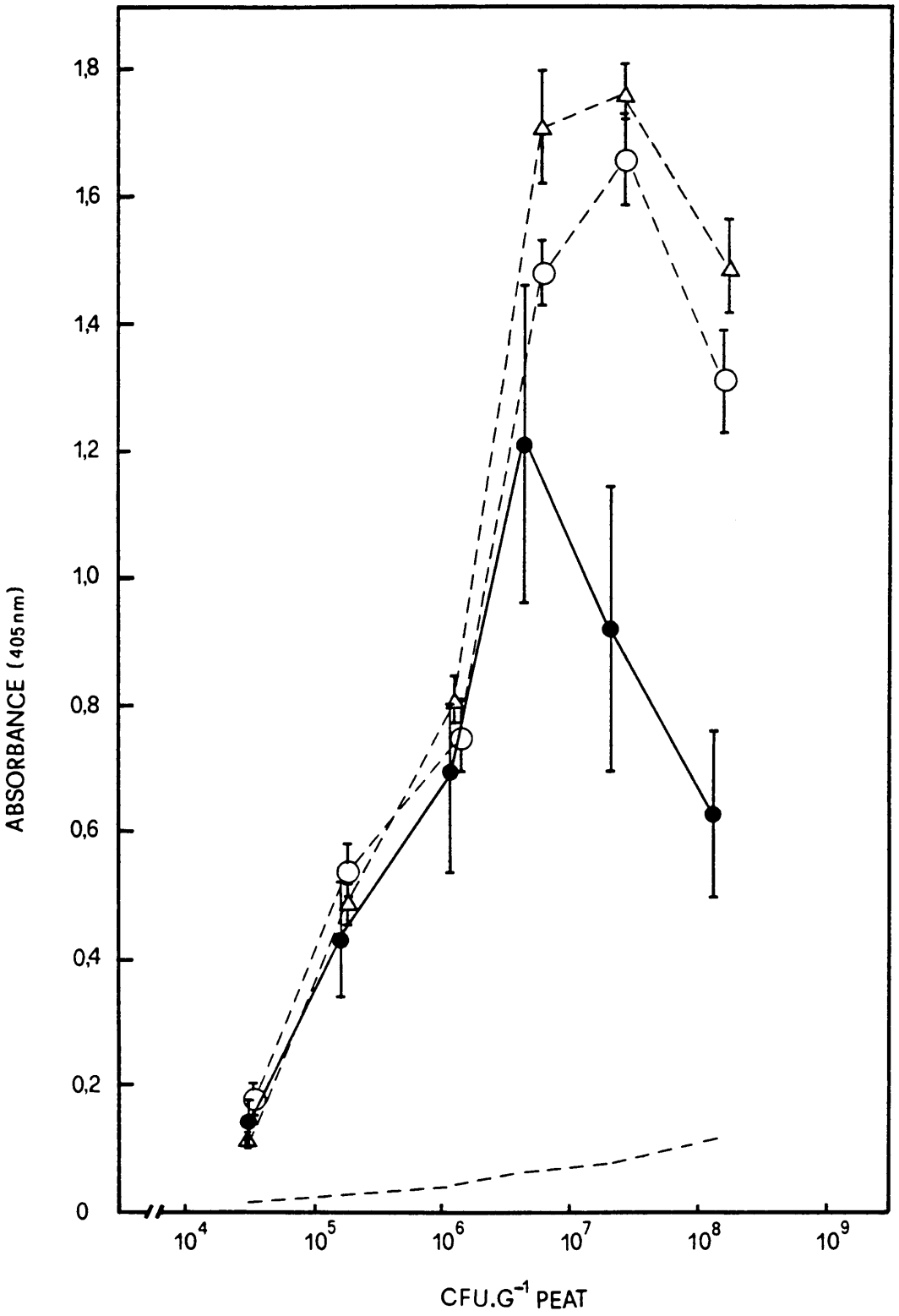
○--○ Allowed to settle for 3 h

△--△ Centrifuged at 121 x g for 10 min

●—● Suspension sampled immediately after mixing

---- Uninoculated peat suspension sampled immediately after mixing

Concentration of WB1 coating IgG was 2 $\mu g.cm^{-3}$. Dilution of WB1-specific conjugate was 1:500. A_{405} values and viable cell counts are the means of four replicates. Bars represent standard deviations.



could not be used due to the strong inhibition of the DAS ELISA reaction in low dilutions, and the lower A_{405} values obtained relative to the other suspensions. A sedimentation period of 3 h was selected to prepare inoculant suspensions for routine laboratory tests.

4.7 Estimates of the number of strain WB1 cfu in the peat by DAS ELISA

4.7.1 Use of laboratory produced soybean inoculant

Plate counts and DAS ELISA were conducted simultaneously on inoculant suspensions of three soybean inoculants prepared from steam sterilized peat, and two inoculants prepared from gamma-irradiated peat. The inoculants were prepared in the laboratory in order to obtain *ca*. $5,0 \times 10^9$, $1,0 \times 10^9$ and $5,0 \times 10^8$ strain WB1 cfu.g^{-1} peat respectively, as these numbers are of interest in quality control tests. Regression lines were calculated for data obtained with three replicate dilution series of each inoculant suspension. Points outside the linear reading range of *ca*. $1,0 \times 10^5$ to $1,0 \times 10^8$ cfu.g^{-1} peat were subjectively omitted before calculations were made. A better fit was obtained between A_{405} values and log$_{10}$ number of cfu.g^{-1} peat than between A_{405} values and the number of cfu.g^{-1}

peat directly. For each regression line calculated, a highly significant linear relationship was found between A_{405} values and the log $_{10}$ number of strain WB1 cfu.g^{-1} peat. As covariance analysis indicated that regression lines for the three replicates of each inoculant suspension did not differ significantly, data obtained for each inoculant could be considered as a single group. Regression lines calculated for data of each of the three inoculants, prepared from steam sterilized peat, differed significantly from each other (Fig. 17 A). Regression lines calculated for data of the two inoculants, prepared from gamma-irradiated peat, also differed significantly (Fig. 17 B). When a single regression line was computed for data of inoculants prepared from steam sterilized peat, and compared with the regression line fitted to the data of inoculants prepared from gamma-irradiated peat, they differed significantly. These results indicated that laboratory produced inoculant could not be used to compile a standard curve.

4.7.2 Use of commercially produced soybean inoculant

In order to compile a standard curve, needed when estimating the number of strain WB1 cfu in commercially produced soybean inoculant, DAS ELISA and plate counts were conducted on 18 packets of

Fig. 17 Linear regression lines of DAS ELISA A$_{405}$ values and the number of B. japonicum strain WB1 cfu.g^{-1} peat for five laboratory produced soybean inoculants

A Inoculants produced from steam sterilized peat

●—● $3,9 \times 10^{9}$ cfu.g^{-1} peat (initial count)
$y = -4,199 + 0,456x$, $r^2 = 0,96$

⊙—⊙ $1,9 \times 10^{9}$ cfu.g^{-1} peat (initial count)
$y = -3,330 + 0,593x$, $r^2 = 0,95$

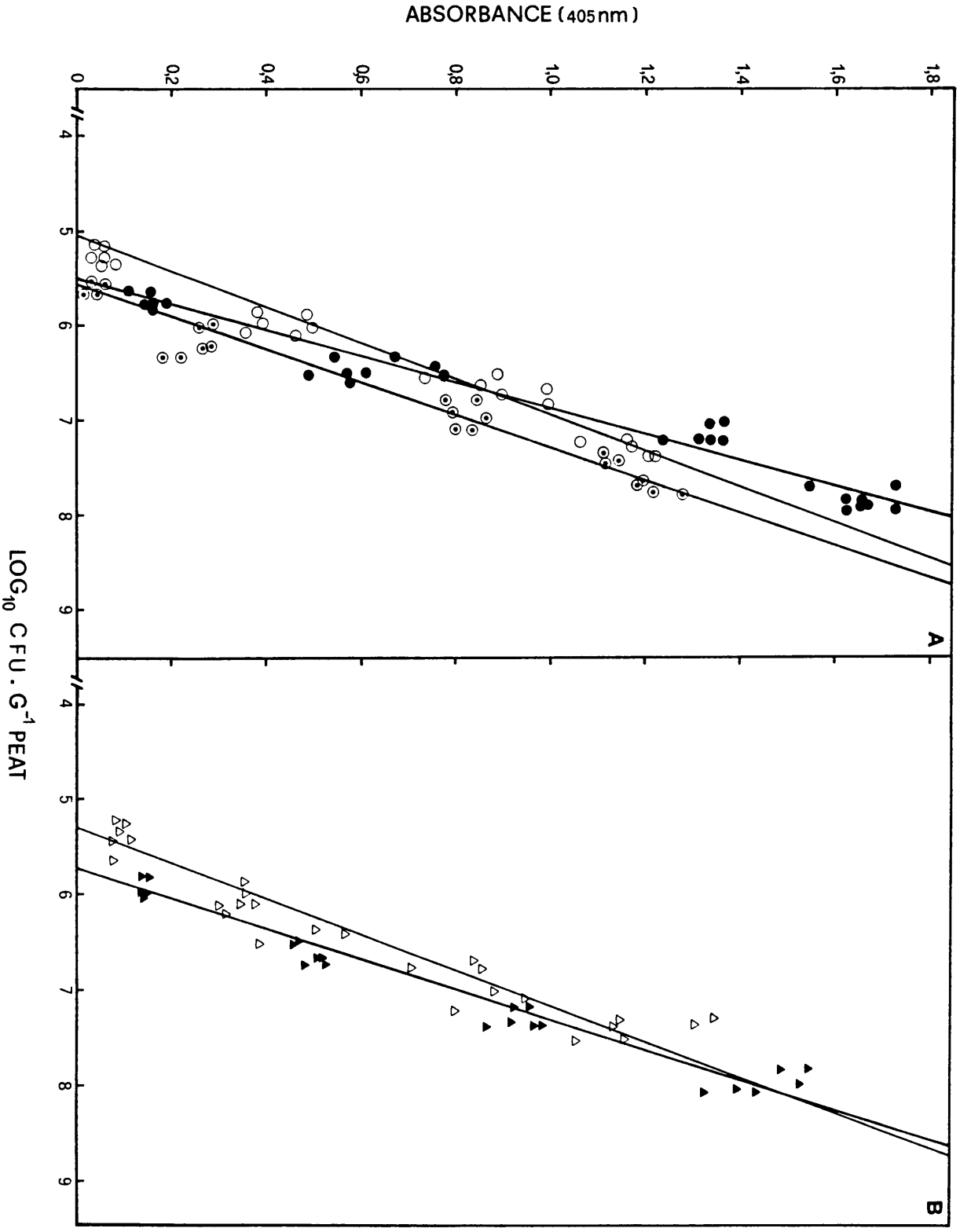
○—○ $2,1 \times 10^{8}$ cfu.g^{-1} peat (initial count)
$y = -2,699 + 0,532x$, $r^2 = 0,97$

B Inoculants produced from gamma-irradiated peat

▲—▲ $5,8 \times 10^{9}$ cfu.g^{-1} peat (initial count)
$y = -3,562 + 0,617x$, $r^2 = 0,97$

△—△ $1,5 \times 10^{9}$ cfu.g^{-1} peat (initial count)
$y = -2,782 + 0,523x$, $r^2 = 0,92$

Concentration of WB1 coating IgG was 4 µg.cm^{-3}.
Dilution of the WB1-specific conjugate was 1:700



soybean inoculant. A scatter diagram showing the relationship between the log $_{10}$ number of viable B. japonicum WB1 cfu.g^{-1} moist peat and A$_{405}$ values, is given in Fig. 18. As the two parameters had a significant correlation coefficient (r) of 0,95, the linear regression equation y = 6,126 + 1,156x was fitted to the observed points. Analysis of variance indicated that the straight line was statistically a very good fit. 89,8% variation in A$_{405}$ values was due to changes in the number of cfu.g^{-1} peat (r^2 = 0,90). As a slightly systematic trend in deviation of observed points from the regression line was observed, i.e. points deviated more from the linear line at higher A$_{405}$ values, the model $y = -35,003 + 8,614x - 0,522x^2$ was also fitted to the data. As r^2 = 0,94, the fit of this model to the data was better than the linear model. The 95% and 99% confidence limits calculated for the second model are shown in Fig. 19. At high cell numbers the interval between the confidence limits increased.

4.8 Comparison of the number of B. japonicum strain WB1 cfu.g^{-1} peat as estimated by DAS ELISA and plate counts

The assumption that the number of strain WB1 cfu.g^{-1} moist peat, as estimated by DAS ELISA, will agree with numbers of cfu.g^{-1} determined by

Fig. 18 The relation between DAS ELISA A_{405} values and viable cell numbers of B. japonicum strain WB1 in peat inoculant

Individual values obtained from each of 18 packets of soybean inoculant were used to compile the standard curve: $y = 6,125 + 1,156x$, $r^2 = 0,90$ (——). DAS ELISA tests used a WB1 IgG concentration of 4 $\mu g.cm^{-3}$ and a WB1 - specific conjugate dilution of 1:700. A_{405} values and viable cell counts are the means of four replicates.

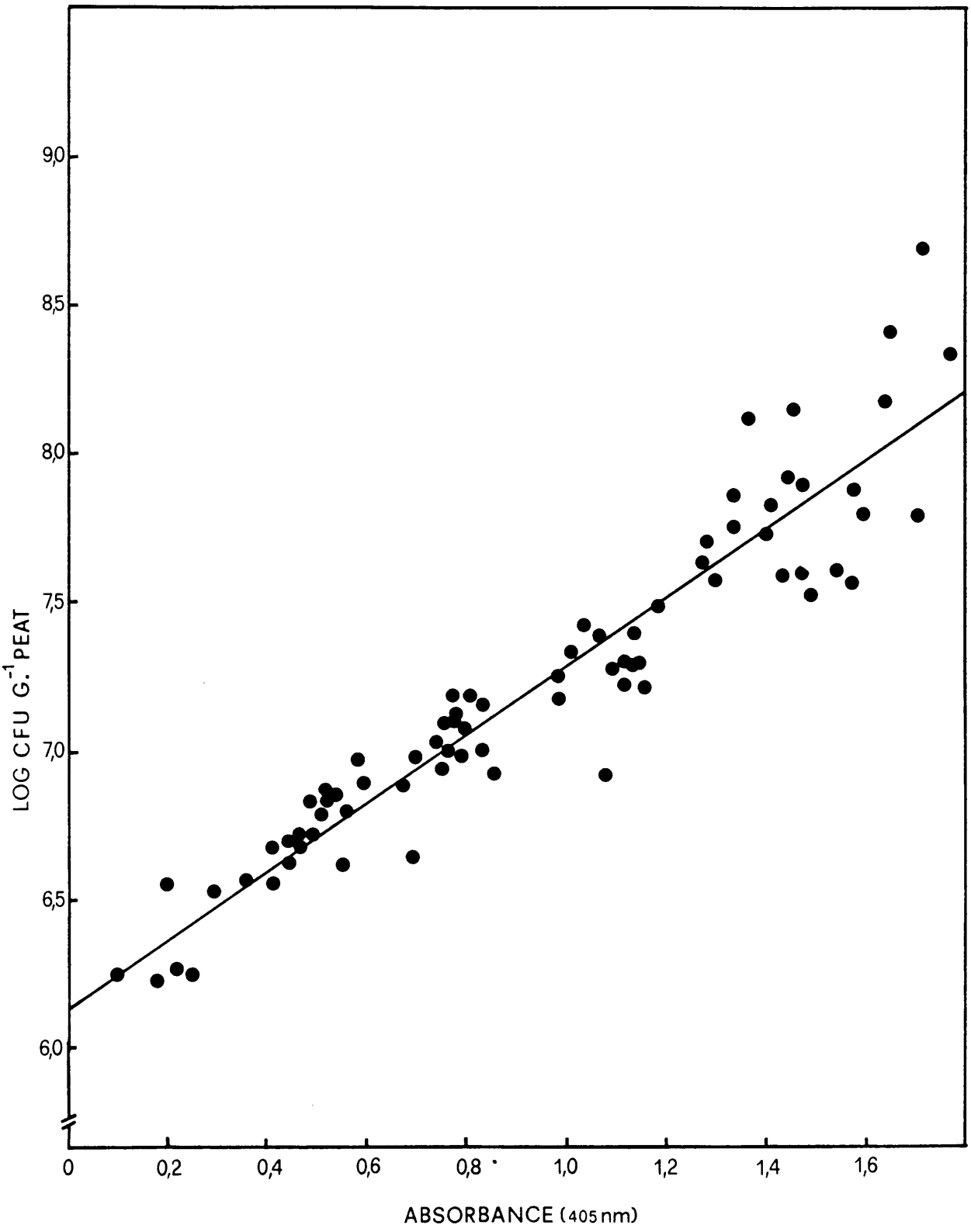


Fig. 19 The relation between DAS ELISA A_{405} values and viable cell numbers of B. japonicum strain WB1 in peat inoculant

▲ Confidence limits at P = 0,05

● Confidence limits at P = 0,001

Individual values of each of 18 packets of soybean inoculant were used to compile the standard curve: $y = -35,003 + 8,614x - 0,522x^2$ (—●——●—). Concentration of WB1 coating IgG was 4 $\mu g.cm^{-3}$. Dilution of WB1-specific conjugate was 1:700. A_{405} values and viable cell counts are the means of four replicates.

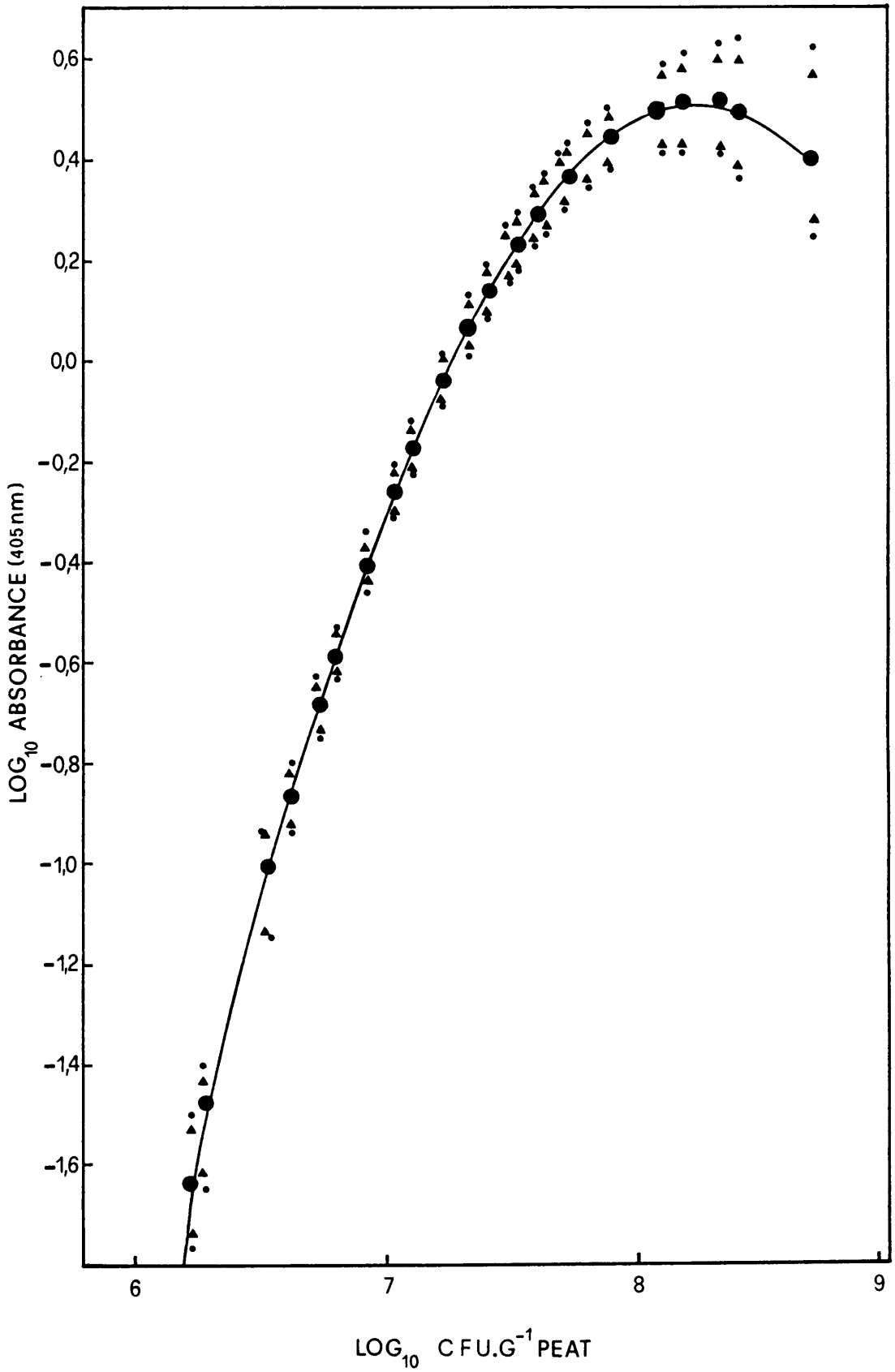


plate counts, was checked by testing 43 packets of soybean inoculant by DAS ELISA as well as by plate counts. On the basis of plate counts, 100% of the soybean inoculants, produced from gamma-irradiated peat were approved for marketing whereas DAS ELISA estimates indicated that 93,5% of the packets were of acceptable quality. Only 6,5% would have been rejected for marketing due to DAS ELISA estimates less than $5,0 \times 10^{8}$ cfu.g^{-1} moist peat (Table 3). On the basis of plate counts, 83,3% of the soybean inoculants produced from steam sterilized peat were not approved for marketing. In contrast, DAS ELISA estimates indicated that all these packets contained numbers $> 5,0 \times 10^{8}$ cfu.g^{-1} moist peat (Table 4). Although the cause for the low viable counts in soybean inoculant, produced from steam sterilized peat, was unknown, B. japonicum strain WB1 appeared to be extremely sensitive to peat carriers subjected to steam sterilization for 1 h at 124 °C.

Regression analysis of data on the number of B. japonicum strain WB1 cfu.g^{-1} for the 43 packets, determined by plate counts and DAS ELISA, showed that 51,6% of the variation in the number of cfu.g^{-1} peat estimated by DAS ELISA, could be explained by changes in the numbers obtained with plate counts. Thus, 48,4% of the variation in numbers, estimated by DAS ELISA, remained unexplained. In order to show the extent of uncertainty in the estimates made, and to provide an interval very likely to contain the true value of the number of cfu.g^{-1} peat being estimated by DAS ELISA, 95% confidence limits were calculated for the

TABLE 3 Estimates of the number of Bradyrhizobium japonicum strain WB1 cfu in gamma-irradiated peat inoculant by DAS ELISA and the plate count technique

Inoculant no[a]	DAS ELISA estimate[b] cfu.g^{-1}	Plate count[b] cfu.g^{-1}
347A	$5,0 \times 10^{8}$	$5,9 \times 10^{8}$
347B	$1,7 \times 10^{9}$	$2,2 \times 10^{9}$
350A	$3,3 \times 10^{9}$	$2,4 \times 10^{9}$
350B	$4,4 \times 10^{9}$	$2,5 \times 10^{9}$
351A	$6,8 \times 10^{8}$	$1,0 \times 10^{9}$
351B	$1,0 \times 10^{9}$	$2,3 \times 10^{9}$
353A	$2,3 \times 10^{9}$	$3,9 \times 10^{9}$
353B	$1,9 \times 10^{9}$	$2,5 \times 10^{9}$
354A	$1,4 \times 10^{9}$	$1,0 \times 10^{8}$
354B	$1,3 \times 10^{9}$	$8,2 \times 10^{8}$
355A	$1,5 \times 10^{9}$	$9,8 \times 10^{8}$
355B	$1,8 \times 10^{9}$	$1,7 \times 10^{9}$
356A	$3,7 \times 10^{9}$	$2,3 \times 10^{9}$
358A	$2,4 \times 10^{8}$	$5,3 \times 10^{8}$
358B	$6,1 \times 10^{9}$	$8,8 \times 10^{9}$
359A	$1,5 \times 10^{9}$	$2,3 \times 10^{9}$
359B	$2,6 \times 10^{8}$[c]	$4,1 \times 10^{8}$
360A	$2,4 \times 10^{8}$	$6,1 \times 10^{9}$
360B	$7,3 \times 10^{9}$	$2,2 \times 10^{9}$
361A	$1,7 \times 10^{9}$	$4,3 \times 10^{9}$
361B	$1,6 \times 10^{8}$[c]	$2,4 \times 10^{8}$
362A	$3,3 \times 10^{9}$	$8,0 \times 10^{9}$
362B	$1,4 \times 10^{9}$	$4,3 \times 10^{9}$
4A	$2,6 \times 10^{8}$	$5,8 \times 10^{9}$
4B	$7,1 \times 10^{8}$	$1,9 \times 10^{9}$
7A	$6,0 \times 10^{9}$	$1,1 \times 10^{9}$
7B	$2,1 \times 10^{9}$	$5,1 \times 10^{9}$
16A	$6,7 \times 10^{9}$	$6,3 \times 10^{9}$
16B	$5,8 \times 10^{9}$	$4,2 \times 10^{9}$
17A	$1,2 \times 10^{9}$	$2,2 \times 10^{9}$
17B	$2,8 \times 10^{9}$	$1,9 \times 10^{9}$

[a] A and B denote packets from the same inoculant batch.

[b] Each value is the average of four replicates.

[c] Inoculants containing < $5,0 \times 10^{8}$ cfu.g^{-1} are not sold.

$F = 34,13$ (highly significant at $P = 0,001$)

$r = 0,72$

TABLE 4 Estimates of the number of Bradyrhizobium japonicum strain WB1 cfu in steam sterilized peat inoculant by DAS ELISA and the plate count technique

Inoculant no[a]	DAS ELISA estimate[b] cfu.g^{-1}	Plate count[b] cfu.g^{-1}
3A	$8,3 \times 10^{8}$	$< 5,0 \times 10^{8}$ [c]
3B	$9,6 \times 10^{8}$	$< 5,0 \times 10^{8}$ [c]
5A	$1,8 \times 10^{9}$	$3,7 \times 10^{9}$
5B	$3,0 \times 10^{9}$	$2,8 \times 10^{9}$
6A	$7,6 \times 10^{8}$	$< 5,0 \times 10^{8}$ [c]
6B	$1,0 \times 10^{9}$	$< 5,0 \times 10^{8}$ [c]
8A	$2,1 \times 10^{9}$	$< 5,0 \times 10^{8}$ [c]
8B	$5,8 \times 10^{8}$	$< 5,0 \times 10^{8}$ [c]
9A	$1,0 \times 10^{9}$	$< 5,0 \times 10^{8}$ [c]
9B	$1,4 \times 10^{9}$	$< 5,0 \times 10^{8}$ [c]
13A	$2,0 \times 10^{9}$	$< 5,0 \times 10^{8}$ [c]
13B	$2,1 \times 10^{9}$	$< 5,0 \times 10^{8}$ [c]

[a] A and B denote packets from the same inoculant batch.

[b] Each value is the average of four replicates.

[c] Inoculants containing $< 5 \times 10^{8}$ cfu.g^{-1} may not be sold

data in Table 3, and are shown in Fig. 20. The confidence limits became larger with increased A_{405} values, and indicated that the extent of uncertainty in estimates made at high A_{405} values was large.

4.9 Screening methods to determine if B. japonicum strain WB1 cells in peat are viable

Results presented in Section 4.8 indicated that viable as well as non-viable strain WB1 cells were detected by DAS ELISA in soybean inoculant produced from steam sterilized peat. An easy method was thus required to ensure that the counts estimated by DAS ELISA consisted mainly of viable cells (cfu). Fig. 21 shows an CR-YM agar plate with sparsely distributed soybean inoculant particles and colonies developed after an incubation period of 10 d at 28 °C. An obvious difference was observed between plates where soybean inoculant with low viable counts and high viable counts were tested. Contamination in the inoculant could also be assessed with this method. Distinction between soybean inoculants with e.g. $2,0 \times 10^8$ and $5,0 \times 10^8$ cfu.g^{-1} moist peat was not obvious with this method.

Results of a semi-quantitative estimate of the number of strain WB1 cfu.g^{-1} moist peat by a

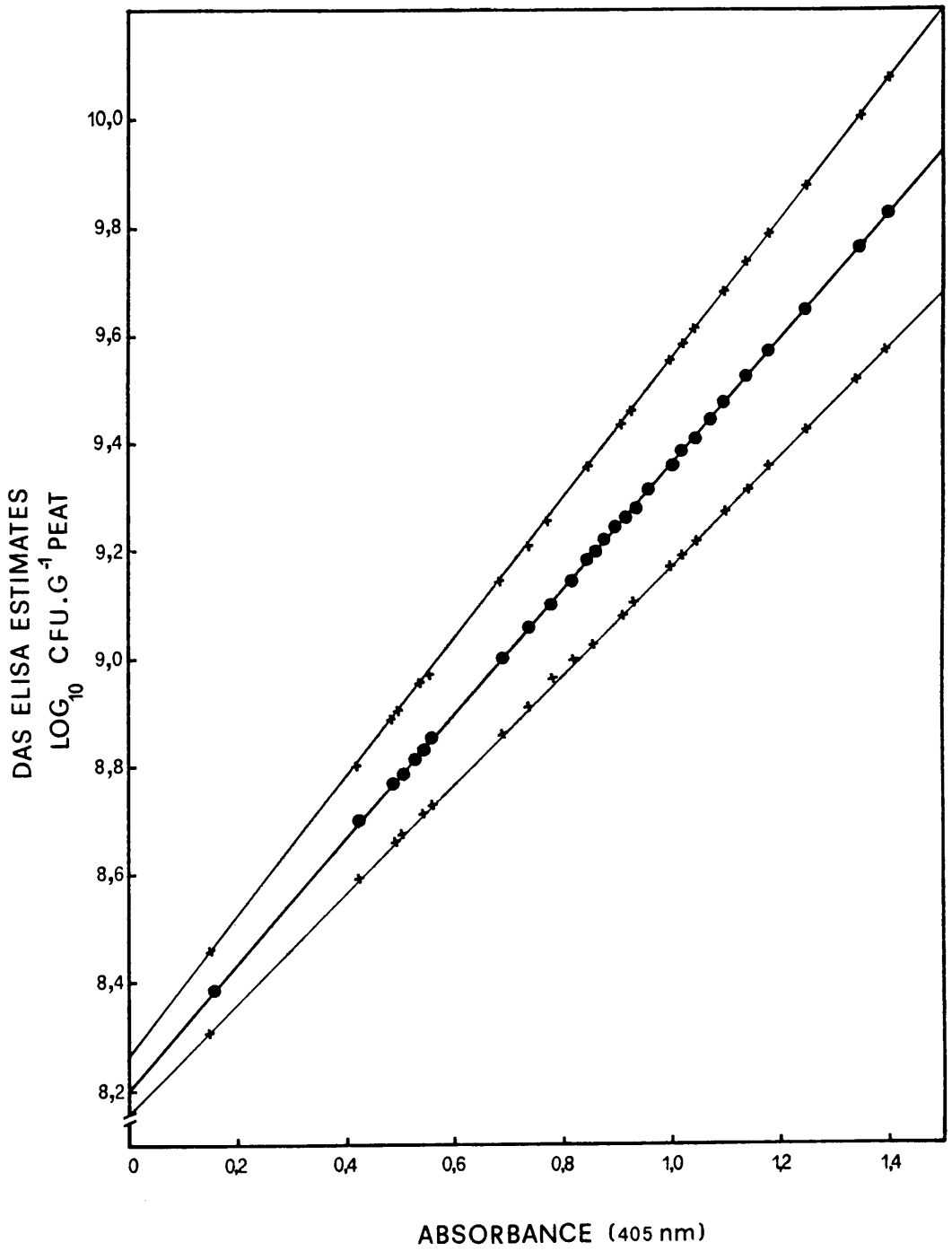
Fig. 20 Confidence limits (P = 0,05) for viable cell numbers estimated from DAS ELISA data from 43 packets of commercialy produced soybean inoculant

Cell numbers were calculated from the linear regression equation y = 6,124 + 1,156x

⟷ Confidence limits (P = 0,05)

⟷ Relation between viable cell numbers and DAS ELISA estimated viable cell numbers

A values are the means of four replicates. 405
Concentration of WB1 coating IgG was 4 µg.cm^{-3}.
Dilution of WB1-specific conjugate was 1:700.



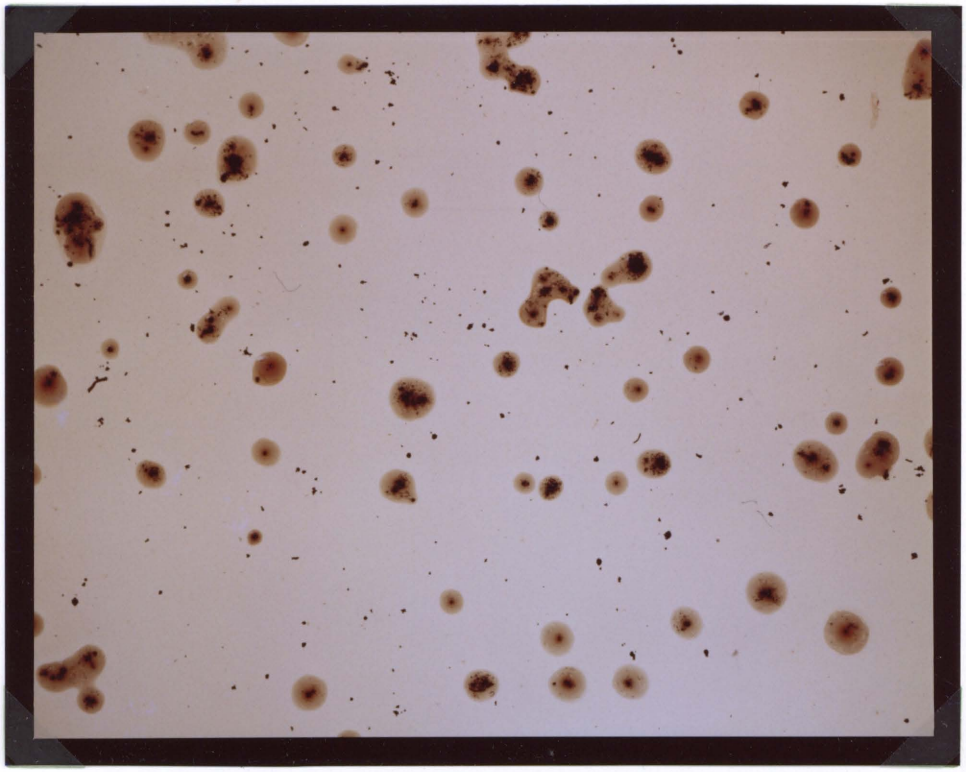


Fig. 21 Particles of a high quality soybean inoculant distributed sparsely onto a CR-YM agar plate and colonies developed after incubation.

The plate was incubated for 10 d at 28 °C. Inoculant particles are black. The colonies are probably all of B. japonicum strain WB1.

modification of the Miles and Misra drop plate technique are shown in Fig. 22. The level of contamination in each sample could be assessed. Soybean inoculant with a relativley high level of contamination and ca. $1,0 \times 10^{8}$ cfu.g^{-1} peat (Fig. 22 A) was not approved for marketing whereas soybean inoculant shown in Fig. 22 B, with a lower level of contamination and ca. $9,0 \times 10^{8}$ cfu.g^{-1} peat was of acceptable quality.

4.10 Comparison of the numbers of B. japonicum strain WB1 cfu in peat as determined by plate counts and the Miles and Misra drop plate technique

The purpose of this experiment was to determine whether estimates of the numbers of strain WB1 cfu obtained by the Miles and Misra drop plate technique agreed with numbers determined by plate counts. The Miles and Misra drop plate technique, in addition to routine plate counts, was conducted on 32 packets of soybean inoculant. Results are shown in Table 5. A highly significant correlation existed between the number of cfu.g^{-1} peat determined by plate counts and the Miles and Misra drop plate technique respectively. For $r = 0,92$, 84% of the variation in the number of cfu.g^{-1} peat, estimated by Miles and Misra drop plate counts, could be explained by changes in counts of the the plate count technique.

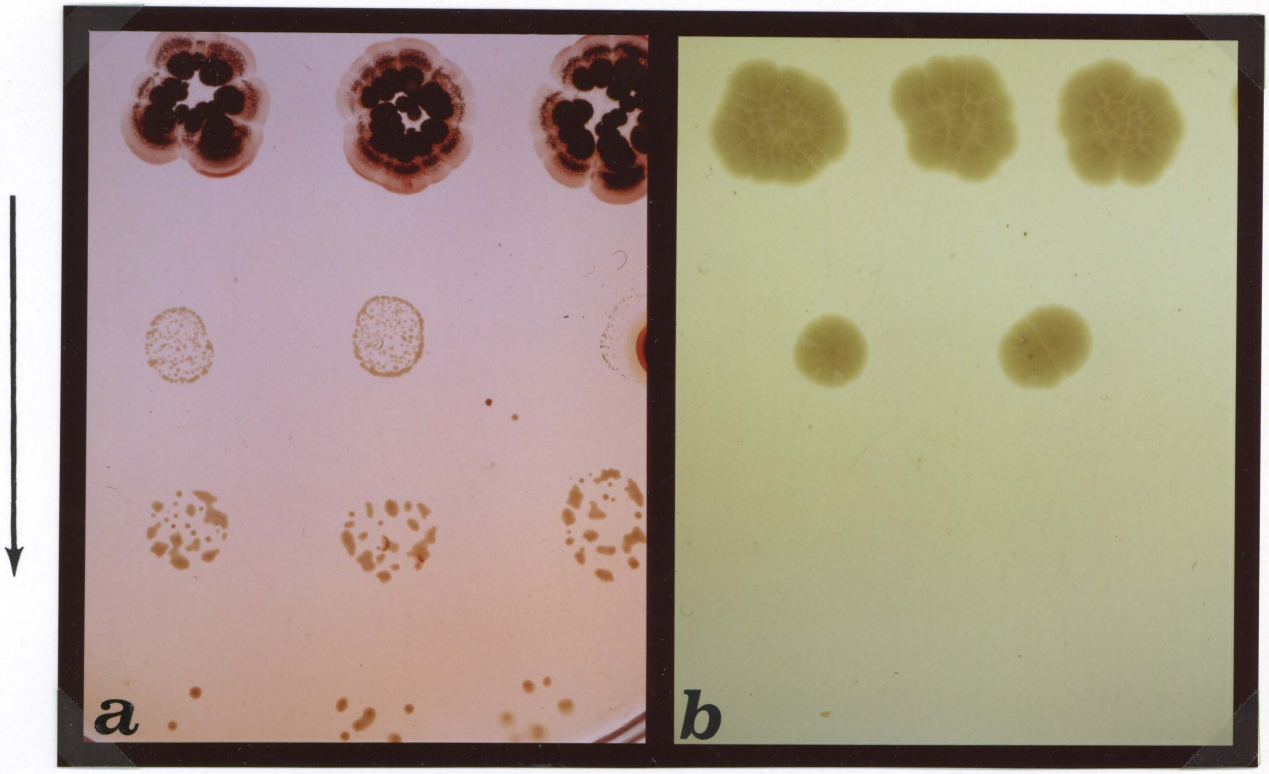
Fig. 22 Colonies developed from drops of two serially diluted soybean inoculant suspensions on agar plates

A - Colonies from soybean inoculant with a high number of viable B. japonicum WB1 cells and a low level of contamination

B - Colonies from soybean inoculant with a low number of viable B. japonicum WB1 cells and a high level of contamination

CR-YM agar (a). All colonies on nutrient agar (b) are contaminants. Plates were incubated for 8 d at 28 °C. The arrow indicates the direction of application of drops from higher dilutions.

A



B

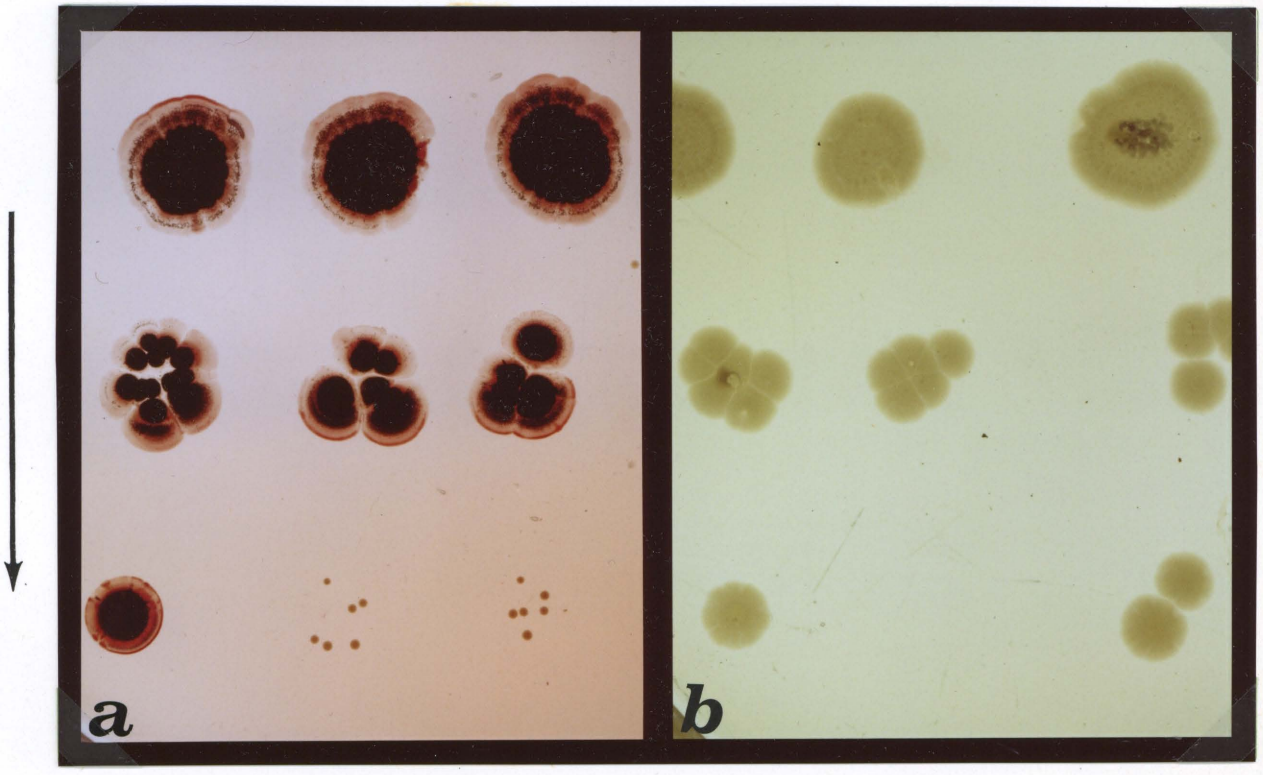


TABLE 5 Estimates of *Rhizobium japonicum* strain WB1 cfu in peat inoculants by the plate count and Miles and Misra drop plate techniques

Inoculant no	Plate count[a] cfu.g^{-1}	Miles and Misra drop plate count[a] cfu.g^{-1}
1	$5,9 \times 10^9$	$4,4 \times 10^9$
2	$3,8 \times 10^9$	$3,1 \times 10^9$
3	$3,1 \times 10^9$	$2,4 \times 10^9$
4	$4,0 \times 10^9$	$2,1 \times 10^9$
5	$7,0 \times 10^9$	$5,8 \times 10^9$
6	$3,5 \times 10^9$	$2,6 \times 10^9$
7	$6,9 \times 10^9$	$2,6 \times 10^8$
8	$1,9 \times 10^9$	$5,1 \times 10^9$
9	$3,9 \times 10^9$	$6,0 \times 10^9$
10	$2,9 \times 10^9$	$2,8 \times 10^9$
11	$4,1 \times 10^9$	$4,7 \times 10^9$
12	$5,3 \times 10^9$	$6,9 \times 10^9$
13	$5,4 \times 10^9$	$5,4 \times 10^9$
14	$5,0 \times 10^9$	$5,7 \times 10^9$
15	$1,4 \times 10^9$	$2,4 \times 10^9$
16	$4,6 \times 10^9$	$5,7 \times 10^9$
17	$6,0 \times 10^9$	$7,4 \times 10^9$
18	$4,6 \times 10^9$	$8,3 \times 10^9$
19	$2,9 \times 10^9$	$2,9 \times 10^9$
20	$4,4 \times 10^9$	$5,5 \times 10^9$
21	$5,8 \times 10^9$	$7,9 \times 10^9$
22	$5,1 \times 10^9$	$8,1 \times 10^9$
23	$1,2 \times 10^9$	$1,1 \times 10^8$
24	$9,7 \times 10^8$	$7,8 \times 10^9$
25	$4,5 \times 10^9$	$3,2 \times 10^9$
26	$4,3 \times 10^9$	$5,5 \times 10^9$
27	$1,7 \times 10^9$	$1,9 \times 10^9$
28	$2,6 \times 10^9$	$3,7 \times 10^9$
29	$2,4 \times 10^9$	$1,9 \times 10^9$
30	$2,9 \times 10^8$[b]	$2,9 \times 10^8$[b]
31	$3,4 \times 10^8$	$4,4 \times 10^8$
32	$6,5 \times 10^8$	$8,8 \times 10^8$

[a] Each value is the average of four replicates.

[b] Inoculants containing < $5,0 \times 10^8$ cfu.g^{-1} may not be sold.

$F = 149,26$ (highly significant at $P = 0,001$)

$r = 0,92$

4.11 The effect of non-viable Bradyrhizobium cells on DAS ELISA

This experiment was aimed at establishing the effect of sterilization on soybean inoculant antigen in terms of damage done to antigenic groups that could influence detection by DAS ELISA. Three soybean inoculants, produced from steam sterilized peat, and three soybean inoculants produced from gamma-irradiated peat were used. Plate counts as well as DAS ELISA were conducted on the inoculants before and after sterilization. Results of the three inoculants treated by steam sterilization are given in Table 6. Counts determined before sterilization indicated that DAS ELISA detected both viable and non-viable cells, as viable counts were low and A_{405} values high. After sterilization, no viable cells were detected by plate counts, whereas A_{405} values of the three suspensions decreased by ca. 50%, i.e. $\leq$ 0,632, 0,471 and 0,553 respectively. The A_{405} values were still high enough at a 1:200 dilution of soybean inoculant suspension to indicate that a fair number of cells were present in the carrier. Table 7 shows viable cell counts and A_{405} values obtained before and after gamma-irradiation of the soybean inoculants. For inoculants with viable counts of $2,6 \times 10^9$, $2,9 \times 10^9$ and $9,0 \times 10^8$ cfu.g^{-1} peat respectively, the high A_{405} values obtained by DAS ELISA

TABLE 6 Comparison of viable cell counts and DAS ELISA values (A_{405}) obtained with three soybean inoculants before and after steam sterilization[a]

Inoculant no	Before sterilization[b]		After sterilization[b]	
	Viable cell count WB1 cfu.cm^{-3}	DAS ELISA[c] A_{405}	Viable cell count WB1 cfu.cm^{-3}	DAS ELISA[c] A_{405}
1	$6,8 \times 10^{3}$	1,421	0	0,701
	$3,4 \times 10^{3}$	1,209	0	0,632
	$1,7 \times 10^{3}$	1,124	0	0,597
	$8,5 \times 10^{2}$	0,868	0	0,556
	$4,3 \times 10^{2}$	0,673	0	0,492
2	$5,1 \times 10^{3}$	1,352	0	0,579
	$2,6 \times 10^{3}$	1,168	0	0,471
	$1,3 \times 10^{3}$	0,989	0	0,419
	$6,4 \times 10^{2}$	0,846	0	0,389
	$3,2 \times 10^{2}$	0,770	0	0,364
3	$2,3 \times 10^{3}$	1,337	0	0,653
	$1,2 \times 10^{3}$	1,135	0	0,553
	$5,8 \times 10^{2}$	0,982	0	0,541
	$2,9 \times 10^{2}$	0,862	0	0,478
	$1,5 \times 10^{2}$	0,685	0	0,410
Uninoculated peat control	0	0,029	0	0,037

[a] Each value is the average of three replicates.

[b] Viable cell counts and DAS ELISA values were determined from a series of two-fold dilutions of each inoculant.

[c] Cell numbers could not be estimated from these A_{405} values because a standard curve was not available. Concentration of WB1 coating IgG was 4 µg.cm^{-3}; strain WB1-specific conjugate was diluted 1:8 000.

TABLE 7 Comparison of viable cell counts and DAS ELISA values (A_{405}) obtained with three soybean inoculants, before and after gamma-irradiation[a]

Inoculant no	Before sterilization[b]		After sterilization[b]	
	Viable cell count WB1 cfu.cm^{-3}	DAS ELISA[c] A_{405}	Viable cell count WB1 cfu.cm^{-3}	DAS ELISA[c] A_{405}
1	$4,3 \times 10^{7}$	1,213	0	0,496
	$2,8 \times 10^{7}$	1,170	0	0,464
	$1,4 \times 10^{7}$	1,001	0	0,378
	$7,2 \times 10^{6}$	0,964	0	0,298
	$3,6 \times 10^{6}$	0,963	0	0,207
2	$4,8 \times 10^{7}$	1,116	0	0,411
	$3,2 \times 10^{7}$	1,061	0	0,353
	$1,6 \times 10^{7}$	0,987	0	0,220
	$8,1 \times 10^{6}$	0,925	0	0,180
	$4,0 \times 10^{6}$	0,837	0	0,097
3	$1,5 \times 10^{7}$	1,041	0	0,449
	$9,8 \times 10^{6}$	0,984	0	0,315
	$4,9 \times 10^{6}$	0,912	0	0,138
	$2,4 \times 10^{6}$	0,834	0	0,103
	$1,2 \times 10^{6}$	0,850	0	0,081
Uninoculated peat control	0	0,052	0	-0,027

[a] Each value is the average of three replicates.

[b] Viable cell counts and DAS ELISA values were determined from a series of two-fold dilutions of each inoculant.

[c] Cell numbers could not be estimated from these A_{405} values because a standard curve was not available. Concentration of WB1 coating IgG was 4 µg.cm^{-3}; strain WB1 specific conjugate was diluted 1:8 000.

confirmed that the three inoculants were of high quality. After gamma-irradiation, viable WB1 cells could not be detected. At the 1:180 dilution of the three soybean inoculant antigen supensions, A_{405} values were reduced to $\leq$ 0,378, 0,220 and 0,138 respectively. The more noticeable decrease in A_{405} values thus occurred with inoculant subjected to gamma-irradiation rather than to steam sterilization.

CHAPTER 5

DISCUSSION

Recent reports indicated that the enzyme-linked immunosorbent assay (ELISA) can be used to estimate the population density of rhizobia effectively in peat without a time-consuming plate count or plant infection count (Kishinevsky et al., 1982; Martensson & Gustafsson, 1985; Nambiar & Anjaiah, 1985). It was therefore feasible to study the application of the ELISA technique in the quality control of legume inoculants in South Africa.

The quality of antiserum and conjugates prepared and their reactivity in ELISA were evaluated, as accurate enumeration of rhizobia in peat by ELISA could not be expected when immunoreactants of inferior quality were used.

The high somatic agglutination titres of eight of the nine antisera tested, indicated that high quality antisera against strains of rhizobia were produced. Strain RF14 did not elicit a high agglutination titre and was not used in subsequent tests, as specific antisera with high antibody titres were essential for the ELISA technique (Kishinevsky & Bar-Joseph, 1978).

The value of the tube agglutination test to indicate antiserum quality for ELISA could be questioned, as results indicated that strong ELISA reactions were not necessarily

obtained with antisera with high agglutination titres. For example, the strain RF6 antibody-antigen combination performed weakly in ELISA, whereas the somatic agglutination titre of strain RF6 antiserum was 12 800. In contrast, the strain WB1 antibody-antigen combination performed well in ELISA and high A_{405} values were obtained. The somatic agglutination titre of WB1 antiserum was 1 600. Kishinevsky & Bar-Joseph (1978) and Kishinevsky et al. (1982) also observed that serological specificity of ELISA generally agreed with that of somatic agglutination tests, but that differences in A_{405} values for each strain- antibody combination occurred. The discrepancy in the behaviour of the same antiserum in agglutination and ELISA tests might have been caused by the ratio between IgM and IgG in the antiserum, as mainly antibodies of the IgG-class are measured by the ELISA technique (Engvall & Perlmann, 1972) and IgM is classed a good agglutinin but a poor precipitin (Humphrey & Vincent, 1973).

Cross-reaction could be demonstrated between strain RF14 antiserum and RF6 antigen by tube agglutination tests. Heterologous reactions that occurred in DAS ELISA, i.e. between XHT1 IgG and VK10 antigen, and RF6 IgG and RF14 antigen, were not detected by agglutination tests, probably because the ELISA technique is more sensitive than the agglutination technique (Kishinevsky & Gurfel, 1980).

Experience with each of six conjugates in ELISA suggested that the quality of enzyme-linked antibodies was vital to

the ELISA technique. The protein A conjugate performed excellent in indirect ELISA in terms of good detection of antigens at high dilutions of reactants, thus permitting economical use of reagents. Non-specific interactions did not occur in assays with cell suspensions of rhizobia and this conjugate. These findings were in agreement with those of Barbara & Clark (1982) and Kishinevsky & Maoz (1983) with protein A conjugates in indirect ELISA. With enzyme-linked antibodies prepared against the slow-growing strains, good detection of antigen was obtained in DAS ELISA and non-specific reactions were low. The WB1-specific and XHT1-specific conjugates were superior to the XBL6-specific conjugate, as they permitted more economical use of reagents than the XBL6-specific conjugate. Conjugates specific to fast-growing strains were of poor quality in terms of high non-specific background reactions and resulted in less economical use of reagents. Due to the low reactivity of fast-growing strains and the high background reactions encountered in DAS ELISA, only slow-growing strains were used.

Low reactivity of Rhizobium strains in DAS ELISA was presumably caused indirectly by extracellular polysaccharides. Of relevance is the ability of the faster-growing strains of rhizobia to produce moderate to large amounts of extracellular polysaccharides under certain growth conditions (Vincent, 1970; Ghai, Hisamatsu, Amenura & Harada, 1981). Production of these polysaccharides increased viscosity of culture media

(Fraser, 1975; Meade et al., 1985), and the increased viscosity led to inefficient separation of cells from culture media by centrifugation (Elsworth, 1962). The inoculum prepared from these cells probably contained complex antigenic components from the culture media, which could have resulted in the production of antiserum of low specificity. Antibodies of low specificity linked to enzymes (the conjugates) could have caused high background reactions in DAS ELISA, as the linked enzyme has an amplifying effect in ELISA (O'Sullivan et al., 1979). This could explain the high background reactions encountered in DAS ELISA with fast-growing strains. Ahmad et al. (1981) found that strains producing small amounts of extracellular polysaccharides were highly reactive in ELISA, whereas strains producing large amounts of extracellular polysaccharides tended to be of low reactivity. These authors considered mucilage a physical barrier to antigen-antiserum reactions, but washing of the cells by centrifugation did not increase reactivity. In the case of fast-growing strains studied by Kishinevsky & Gurfel (1980), highest A_{405} values were obtained after ultrasonic and mechanical disruption of the cells. Pre-treatment of cells of fast-growing strains by these two procedures might have improved weak positive ELISA reactions. However, improvement of the ELISA reactivity of fast-growing strains by altering the growth medium, immunization schedule employed, pre-treatment of Rhizobium cells etc. was considered beyond the scope of this investigation.

For practical reasons, the number of strains used to evaluate the potential of the indirect and DAS ELISA methods to count rhizobia in peat, were reduced to three slow-growing strains, i.e. WB1, XBL6 and XHT1. Strains WB1 and XHT1 were selected as they were important strains in commercial inoculant manufacture. Strain XBL6 was chosen because it could possibly replace the groundnut strain presently used for inoculant production (C.J. Otto, pers comm). As the ELISA reactivity of strains XCV14, RF6 and SR4 were low and non-specific reactions relatively high, these strains were not considered for subsequent use. Strains XCT9 and VK10 were not selected, because strain XCT9 is atypical of the rhizobia (Vincent, 1982) and strain VK10 is a very slow grower and thus laborious to handle (P.L. Steyn, pers comm).

Antigen used in the ELISA technique was prepared by heat treatment of cells and inoculant suspensions in boiling water for 30 min, as this destroys flagellar but not somatic antigen. The surface somatic antigens of rhizobia are the most strain specific (Vincent, 1982). In addition, heated cells of rhizobia gave higher A_{405} values in ELISA than unheated cells (Kishinevsky & Bar-Joseph, 1978; Kishinevsky & Gurfel, 1980; Fuhrmann & Wollum II, 1985).

Positive detection of two Bradyrhizobium strains in peat by the indirect ELISA method was unsuccessful. This indicated that the indirect ELISA method could not be used to identify these strains in legume inoculants. Failure to

detect the two strains in peat might have been caused by insufficient adsorption of their antigens to the polystyrene surfaces of microtitre plates, as antigens are adsorbed to plastic polymer surfaces by weak, mainly physical forces and are not covalently linked (Van Oss & Singer, 1966). Peat particles might have interfered with these weak forces and were therefore removed from suspension by centrifugation. After this treatment, weak but inconsistent indirect ELISA reactions were obtained with soybean inoculant suspensions. Inconsistency might have been caused by loose antigen or differential antigen detachment during incubation, as Lehtonen & Viljanen (1980) reported that as many as six washes were necessary to remove all loose antigen from polystyrene surfaces. A total leakage of antigen of 20 to 30% could take place from polystyrene surfaces during the assay (Lehtonen & Viljanen, 1980). Reliable identification of Bradyrhizobium strains in peat should therefore be possible, provided that peat particles are removed from inoculant suspension before conducting the indirect ELISA test and that the way of adsorbing antigen to polystyrene surfaces is modified. In fact, by evaporating cell suspensions in the wells at elevated temperatures, Olsen & Rice (1984) overcame problems to detect passively adsorbed culture antigen by the indirect ELISA method. The indirect ELISA technique provides major logistical advantages over the DAS ELISA technique (Kishinevsky & Maoz, 1983), especially when a number of different strains of rhizobia are involved, and thus warrants further investigation.

As Martensson et al. (1985) did not report any problems in determining the relative amounts of two R. leguminosarum biovar trifolii strains in soil with their modification of the indirect ELISA technique, not only detection, but also enumeration of rhizobia in peat ought to be possible with the indirect ELISA technique. This was not the case with the indirect ELISA method used here. It seemed that the surface concentration and leakage of antigen had to be controlled and standardized if the ELISA method was to be used for quantitative measurement of antigen. Lehtonen & Viljanen (1980) recommended covalent coupling of antigen to the solid phase for quantitative antibody detection. Ehlers & Paul (1984) coated plates with 3-(triethoxysilyl)-propylamine and coupled virus particles covalently to the plate surface by using glutaraldehyde. The use of the $F(ab')_2$-fraction (Barbara & Clark, 1982; Nambiar & Anjaiah, 1985) to by-pass the antigen coupling problem could also be investigated.

Contrary to the indirect ELISA method, Bradyrhizobium strains could be detected in peat by the DAS ELISA method. Plate counts as well as DAS ELISA were conducted on inoculant suspensions containing each of three Bradyrhizobium strains. Inhibition of the DAS ELISA colour reaction occurred in the lowest dilution of inoculant suspensions tested, but a colour gradient was obtained with higher dilutions. It seemed probable that peat was responsible for the inhibitory effect, as inhibition of DAS ELISA colour reactions decreased at higher dilutions and as

higher A_{405} values were obtained with the supernatant of inoculant suspensions than with the well-mixed suspensions. A possible explanation is that the sediment of finely ground Putfontein peat formed a physical barrier which interfered with the binding of antigen to antigen binding sites on the antibodies in the wells. Putfontein peat might also have contained substances with antigens common to some of the rhizobial antigen which could have competed for antigen binding sites on the antibodies in the wells. This was not supported by the low background reactions of the uninoculated peat controls. However, the cause of the inhibitory effect remained a matter of conjecture, as Kishinevsky *et al*. (1982) and Nambiar & Anjaiah (1985) did not encounter inhibition of DAS ELISA colour reactions when estimating the number of *Bradyrhizobium* sp. (*Arachis*) cfu in peat by DAS ELISA.

The necessity to remove, or reduce the inhibition of DAS ELISA colour reactions in the lowest dilutions of inoculant suspensions tested was obvious, as inhibition was inconsistent and differed when testing inoculant suspensions that contained the same or different strains. Experiments to reduce or eliminate inhibition of the DAS ELISA reaction were conducted using *B. japonicum* strain WB1, as inhibition was most prominent with inoculant produced from this strain. Soybean inoculant suspensions, sedimented for five different periods, centrifuged at four different speeds and filtered gave all rise to higher A_{405} values than the well-mixed suspensions. Of the two

treatments that appeared most suitable for inclusion in a routine method, i.e. centrifugation at 121 x g for 10 min and sedimentation for 3 h, sedimentation was preferred as it was less laborious than centrifugation. No delay was caused by this extra step to prepare inoculant suspensions, as the first step in the DAS ELISA method also involved a 3 h incubation period with IgG. Hence, by allowing peat particles in the first dilution of soybean inoculant suspensions to settle for 3 h, inhibition was markedly reduced and preparation of inoculant suspensions standardized.

The lowest number of rhizobia significantly detected above peat control differed for each of three Bradyrhizobium strains tested. If A_{405} values of twice the A_{405} value of the uninoculated peat control were taken as positive readings, ca. $4{,}0 \times 10^{6}$ cfu.g^{-1} peat of strain WB1, $6{,}0 \times 10^{4}$ cfu.g^{-1} peat of strain XHT1 and $4{,}0 \times 10^{5}$ cfu.g^{-1} peat of strain XBL6 could be detected. Kishinevsky et al. (1982) were able to detect $5{,}0 \times 10^{6}$ cfu.g^{-1} peat when using the DAS ELISA method. Using fluorescent ELISA, Renwick & Jones (1985) were able to detect $2{,}0 \times 10^{5}$ cfu.g^{-1} soil. The sensitivity of DAS ELISA seemed to differ for different strains, batches and concentrations of conjugates and IgG used.

In preliminary experiments, a linear relationship was observed for a narrow range of B. japonicum strain WB1 numbers at a given enzyme-conjugate dilution. This was in accordance with the results of Nambiar & Anjaiah (1985).

In order to express the relationship between the number of *B. japonicum* strain WB1 cfu.g^{-1} peat and A_{405} values mathematically, the plate count and DAS ELISA were conducted simultaneously on laboratory produced soybean inoculants. These inoculants were prepared in the laboratory in order to obtain predetermined numbers of cfu.g^{-1} peat covering the range usually encountered in quality tests. Statistical analysis indicated a poor linear correlation between A_{405} values and the number of cfu.g^{-1} peat. Nambiar & Anjaiah (1985) ascribed the poor correlation between A_{405} values and the number of cfu.g^{-1} peat to the dependence of the intensity of the colour developed on the amount of reagents (antiserum, antibody, conjugate) bound and equilibrium reached during each incubation step in DAS ELISA, rather than to the concentration of individual reagents added. Vincent (1970) recommended counts to be converted to logarithms (base 10) for statistical calculations. The semi-log transformation was therefore employed to express the relationship between the number of cfu.g^{-1} peat and A_{405} values mathematically.

Regression lines calculated for each of the five laboratory produced soybean inoculants were compared statistically. Covariance analysis showed that differences among regression lines fitted for each of the three replicates of an inoculant were not significant. Reproducible results could therefore be obtained for each inoculant tested. Significant differences in slopes of regression lines fitted for each of three inoculants produced

from steam sterilized peat, showed that the relationship between viable counts and A_{405} values was not the same in all three cases. This was also the case with inoculants produced from gamma-irradiated peat. A possible explanation for these differences was that viable counts (plate counts) were related to the total cell count (DAS ELISA), as DAS ELISA can detect viable as well as non-viable cells (Renwick & Jones, 1985). Due to the incubation period of only 14 h, fluctuations in viable numbers could possibly have occurred. The number of B. japonicum strain WB1 cfu could have needed a longer incubation period to stabilize, as was shown for R. meliloti strain U45 (Strijdom & Deschodt, 1976). Suspensions of different inoculum densities, injected into sterile peat, could have resulted in different ratios of viable to non-viable cells during growth in peat, as shown by studies of Meade et al. (1985). They found that viability of cells in the peat declined rapidly when suspensions of high cell densities ($> 10^{11}$ cfu.g^{-1}) and peat were mixed. During subsequent incubation, no nett increase in numbers of viable cells occurred in peat that received ca. $8,6 \times 10^{9}$ and $4,0 \times 10^{10}$ cfu.g^{-1} respectively. Peat supported growth and viability of cells when suspensions with low initial cell densities were administered to the peat. Accordingly, it was assumed that differences observed in the relationship between viable counts and DAS ELISA values among packets of inoculant resulted from the laboratory produced inoculant used. Therefore, commercially manufactured inoculant were

used to compile a standard curve.

In order to estimate the number of Bradyrhizobium strain WB1 cfu.g^{-1} peat from DAS ELISA readings, a standard curve that related the number of WB1 cfu.g^{-1} peat and A_{405} values was compiled, using commercially produced inoculant. Data from 18 packets were used. A log-log transformation, with the number of cfu.g^{-1} peat as independent variable, best expressed the relationship between the two parameters. Confidence limits, calculated for the model $y = -35,003 + 8,614x - 0,522x^2$, became larger at high A_{405} values. This observation suggested that the factor interfering with the DAS ELISA reaction was still present, but to a lesser degree. Because of computational ease, the semi-log transformation, i.e. a linear regression equation, was chosen to approximate the number of cfu.g^{-1} from A_{405} readings. It fitted reasonably well over the cfu number range and A_{405} values involved. Nambiar & Anjaiah (1985) also used the semi-log transformation to estimate the number of cfu of strains of Bradyrhizobium sp. (Arachis) in peat by DAS ELISA. The $\log_{10}$ number of cfu.g^{-1} peat could directly be calculated with this equation, as A_{405} values were used as the independent variables.

In practice a close agreement was obtained between viable counts and DAS ELISA estimates in the quality control of commercially produced soybean inoculants. On the basis of plate counts, all the inoculants produced from gamma-

irradiated peat were approved for marketing. Plate counts confirmed quality control by means of DAS ELISA for 93,5% of the inoculants. Only 6,5% of the inoculants would have been rejected on the basis of DAS ELISA estimates. An additional 6,5% rejection of inoculants could therefore occur when applying DAS ELISA as quality control test, and manufacturers will probably sustain economical losses. The less accurate quality control exerted by DAS ELISA than by plate counts was unaccountable.

In accordance with results of Nambiar & Anjaiah (1985), estimates by the DAS ELISA procedure were close to values obtained from the plate count. However, statistical analysis indicated a 51,6% correlation only. The discrepancy between statistical predictions and actual values was unexpected and unexplicable. Therefore, the extent of uncertainty in DAS ELISA estimates made was demonstrated by calculating 95% confidence limits. Satisfactory estimates could probably be made at low A_{405} values, as confidence limits were relatively small. It seemed that the extent of uncertainty in DAS ELISA estimates was larger at high A_{405} values, as the 95% confidence limits became progressively larger with increased A_{405} values. This suggested that estimates of the number of cfu.g^{-1} peat by DAS ELISA at high A_{405} values were of little use. The inhibitory effect observed with the lowest dilutions of inoculant suspensions in DAS ELISA reactions, might have been responsible for the large

95% confidence limits at high A_{405} values. Hence, it remains doubtful if reliable estimates of the number of B. japonicum strain WB1 cfu in peat could be obtained with a single DAS ELISA test. Further research is necessary to remove factors responsible for variation and to narrow 95% confidence limits.

Results obtained with soybean inoculants produced from steam sterilized peat demonstrated that loss of viability of Bradyrhizobium cells in peat interfered with reliable enumeration of viable cells by DAS ELISA. Whereas the ten inoculants were not approved for marketing due to low viable counts, DAS ELISA estimates were $> 5,0 \times 10^{8}$ cfu.g^{-1} peat. This is in direct contrast to results of Nambiar & Anjaiah (1985) who found that that loss of viability in peat did not interfere with enumeration of viable rhizobia by DAS ELISA. This observation indicated that viable as well as non-viable cells could be detected by DAS ELISA, as was also experienced by Renwick & Jones (1985). DAS ELISA could overestimate the number of viable B.japonicum strain WB1 cfu in peat, and could therefore not be used as the only means to assess inoculant quality.

Findings with the ten packets of soybean inoculant also showed that the assumption that most of the Bradyrhizobium cells were viable 15 d after the inoculum had been administered to the sterile peat carrier, did not hold true in all cases. DAS ELISA indicated that an inoculum of sufficient size was administered to each packet. An

estimated 10-fold increase in cell numbers probably took place, but due to an unknown factor, e.g. a toxic substance, cell numbers declined rapidly within 15 d of incubation. It seemed likely that steam sterilization rendered the peat unsuitable for growth and survival of strain WB1 cells because most of the batches of soybean inoculant, manufactured from gamma-irradiated peat from the same source, were approved for marketing. This was in accordance with the view upheld by Strijdom & Jansen van Rensburg (1981), i.e. primarily sterilization practise, and not manufacturer, was responsible for differences in inoculant quality. As B. japonicum strain WB1 proved to be sensitive to steam sterilized peat, soybean inoculant will in future be manufactured only with gamma-irradiated peat (P.L. Steyn, pers comm).

If DAS ELISA is ever to be applied in quality control tests, an additional test will be required to ensure that strain WB1 cells in the peat carrier are viable. The presence of viable cells in the peat carrier could be shown by distributing inoculant particles on agar surfaces and by incubating the plates. More information was obtained with a modification of the Miles and Misra drop plate technique. As results showed that a highly significant correlation existed between viable counts of the the plate count and Miles and Misra drop plate techniques, the Miles and Misra drop plate technique was chosen as additional test to the DAS ELISA test. This technique permitted approximate viable counts to be made, and at the same time

indicated whether inoculants were contaminated. Use of the Miles and Misra drop plate count, in order to complement DAS ELISA estimates, would prolong the time required to obtain final results, but the 6 to 8 d required for soybean inoculants still compared favourable with the 10 d required for plate counts. When used only to detect contamination, final results could be obtained within 4 d.

In practice inoculants rejected because of viable counts $< 5,0 \times 10^{8}$ cfu.g^{-1} peat are often resterilized and re-used for inoculant production. In such cases the DAS ELISA method would be unsuitable as quality control test, if it is incapable of distinguishing between viable and dead cells. For this reason it was necessary to investigate whether DAS ELISA could still detect WB1 antigen in resterilized soybean inoculant. Results indicated that some damage was done to WB1 antigens by sterilization as A_{405} values were lower after sterilization of the soybean inoculant. Antigens bombarded by gamma-rays were probably damaged to a greater extent than antigens steam sterilized for 1 h at 124 °C, as A_{405} values decreased to a lesser extent after steam sterilization than after gamma- irradiation. However, DAS ELISA could still detect sufficient WB1 antigen in the peat carrier to render the test unreliable under these conditions. This would probably also be the case when

gamma-irradiated rejected inoculants were reused for inoculant production. This problem can be overcome if inoculant producers are not allowed to inoculate resterilized rejected inoculant with the strain previously used for inoculant production.

The most important findings of this study were a) the failure to detect and enumerate rhizobia effectively in peat by indirect ELISA, b) the success achieved with DAS ELISA to detect strains of rhizobia in peat, c) the adequate reduction of the inhibitory effect exerted by the peat in DAS ELISA, d) the apparent unsuitability of DAS ELISA for estimating numbers of cfu of Rhizobium strains in peat, e) the fact that a single DAS ELISA test cannot safely replace the plate count for estimating the number of Bradyrhizobium strain WB1 cfu in legume inoculants, f) the necessity that an additional test be included when DAS ELISA is used as quality control test, g) the necessity of a precautionary measure in cases where inoculants are produced from rejected, resterilized inoculants and h) the discrepancy between statistical predictions and actual counts obtained when comparing viable counts with the DAS ELISA estimates of cfu in legume inoculants.

Finally, in this study DAS ELISA, in conjunction with the inexpensive Miles and Misra drop plate count had provided sufficient information to allow for reliable quality control. In fact, inoculant manufacturers as well as the

P.P.R.I. will benefit economically if the plate count and serological strain identification tests would in future be replaced by these two tests.

CHAPTER 6

SUMMARY

Samples of each batch of legume inoculant manufactured in South-Africa, are tested by the Plant Protection Research Institute for the number of viable Rhizobium and Bradyrhizobium cells per gram peat, strain identity and possible contamination before marketing. Possible replacement of plate counts and serological identification tests by a single indirect or DAS ELISA test, to assess the quality of legume inoculants, was investigated.

Six of the nine strains of rhizobia investigated, are currently used for inoculant production. Somatic agglutination titres of most antisera, measured by tube agglutination tests, were high. Use of these antisera did not always lead to effective ELISA systems, as low reactivity in ELISA were obtained with some antisera with high somatic agglutination titres. Due to low reactivity of the four Rhizobium strains in ELISA, only Bradyrhizobium strains were used.

Optimal concentrations of immunoreactants differed for each antigen-antibody combination tested in ELISA. Consequently, optimal ELISA concentrations had to be determined for each new batch of immunoglobulin, antigen and conjugate prepared.

The plate count, as well as the indirect ELISA test ,were conducted on inoculant suspensions containing each of two Bradyrhizobium strains. The indirect ELISA tests were negative, but centrifuged suspensions gave rise to weak, but inconsistant indirect ELISA reactions.

Colour gradients were obtained in DAS ELISA with higher dilutions of inoculant suspension, containing each of three Bradyrhizobium strains. In the lowest dilutions tested, inhibition of the DAS ELISA reaction occurred. By allowing peat particles in the first dilution to settle for 3 h, the inhibition effect was markedly reduced. The lowest concentration of rhizobia significantly detected above the peat control differed for the three Bradyrhizobium strains and ranged from $6,0 \times 10^{4}$ to $4,0 \times 10^{6}$ colony forming units per gram ($cfu.g^{-1}$) peat.

Reproducible results were obtained with suspensions of each laboratory produced inoculant tested by the plate count technique and DAS ELISA. Statistical analysis indicated that the relationship between A_{405} values and the number of $cfu.g^{-1}$ peat differed among packets tested.

Using data obtained with 18 packets of commercially produced soybean inoculant, a highly significant linear relationship was obtained between the $\log_{10}$ number of viable $cfu.g^{-1}$ peat and A_{405} values. With another eight packets a discrepancy between low viable counts and high DAS ELISA A_{405} values was found, which would have led to the marketing of inferior inoculants. The

inexpensive Miles and Misra drop plate count was therefore evaluated as a complementary test to DAS ELISA, and was successful. Although results of the DAS ELISA method agreed well with the viable count method when tested on commercially produced soybean inoculants, statistical analysis indicated a 51,6% correlation only.

Damage to WB1 antigens were caused by sterilization, but DAS ELISA could still detect WB1 antigen in sterilized inoculant. Hence, it is possible that inoculant of inferior quality, prepared from rejected, resterilized inoculant, could be approved for marketing on the basis of DAS ELISA estimates.

A single indirect or DAS ELISA test therefore cannot replace both plate counts and serological identification tests in the quality control of legume inoculants in South Africa. DAS ELISA, complemented by Miles and Misra viable cell counts, will provide all the required information for reliable quality control.

SAMEVATTING

Monsters van elke lot peulplantentstof vervaardig in Suid-Afrika, word deur die Navorsingsinstituut vir Plantbeskerming getoets ten opsigte van lewende Rhizobium en Bradyrhizobium getalle, rasidentiteit en moontlike kontaminasie voor bemarking. Vervanging van plaattellings en serologiese identifikasie-toetse met 'n enkele indirekte of DAS ELISA- toets vir gehaltebepaling van peulplant-entstowwe is ondersoek.

Ses van die nege Rhizobium- en Bradyrhizobium-rasse wat in die studie gebruik is, word ook gebruik vir entstofproduksie. Somatiese agglutinasietiters van meeste van die antisera, getoets met behulp van buisagglutinasie-toetse, was hoog. Gebruik van hierdie antisera het nie altyd doeltreffende ELISA-sisteme tot gevolg gehad nie, aangesien lae reaktiwiteit in ELISA verkry is met van die antisera met hoë agglutinasietiters. As gevolg van die lae reaktiwiteit van die vier Rhizobium-rasse in ELISA, is slegs die Bradyrhizobium-rasse gebruik.

Optimale konsentrasies van immunoreaktante het verskil vir elke antigeen-teenliggaam- kombinasie getoets in ELISA. Gevolglik moes optimale ELISA-kondisies gestandaardiseer vir elke nuuutbereide immunoglobulien-, antigeen- en konjugaatlot.

Plaattellings, sowel as die indirekte ELISA toets, is

uitgevoer op entstofsuspensies wat elk een van twee Bradyrhizobium-rasse bevat het. Indirekte ELISA-toetse was negatief. Na sentrifugasie van entstofsuspensies is swak, maar veranderlike, indirekte ELISA-reaksies verkry.

Kleurgradiënte is verkry in DAS ELISA met hoër verdunnings van entstofsuspensies, wat elk een van drie Bradyrhizobium-rasse bevat het. Inhibisie van die DAS ELISA- kleurreaksies het voorgekom in die laagste verdunnings getoets. Die inhibisie-effek is merkbaar verminder deur veenpartikels in die laagste verdunnings vir 3 h te laat sedimenteer. Die laagste konsentrasie van rhizobiums betekenisvol bo die veenkontrole waargeneem, het verskil vir die drie Bradyrhizobium-rasse getoets, en het gevarieer van $6,0 \times 10^{4}$ tot $4,0 \times 10^{6}$ kolonievormende eenhede per gram ($kve.g^{-1}$) veen.

Herhaalbare resultate is verkry met suspensies van elke pakkie laboratoriumbereide entstof wat terselfdertyd met plaattellings en DAS ELISA getoets is. Statistiese analises het aangedui dat die verwantskap tussen A_{405}-waardes en die aantal $kve.g^{-1}$ veen verskil het vir pakkies getoets.

'n Hoogs betekenisvolle lineêre verwantskap is verkry tussen die $\log_{10}$ aantal kiembare $selle.g^{-1}$ veen en A_{405}-waardes met data van 18 pakkies kommersieel vervaardigde sojaboonentstof. Met agt ander pakkies is teenstrydige resultate aangedui deur lae kiembare tellings en hoë A_{405} DAS ELISA-waardes wat sou lei tot bemarking

van entstowwe van minderwaardige gehalte. Die goedkoop Miles en Misra-drupplaattegniek is dus geëvalueer as ondersteuningstoets vir DAS ELISA, en is suksesvol bevind. Hoewel resultate van die DAS ELISA-metode goed met die plaattellingsmetode ooreengestem het vir kommersieel-vervaardigde entstof, het statistiese analises slegs 51,6% korrelasie aangedui.

WB1-antigene is beskadig tydens sterilisering, maar WB1-antigeen kon steeds m.b.v. DAS ELISA in gesteriliseerde entstof waargeneem word. Dit is dus waarskynlik dat entstof van swak gehalte, wat vervaardig is met afgekeurde, hergesteriliseerde entstof, goedgekeur kan word vir bemarking op grond van DAS ELISA-skattings.

'n Enkele indirekte of DAS ELISA-toets kan dus nie plaattellings sowel as serologiese identifikasietoetse vervang in die gehaltebeheer van peulplantentstowwe in Suid-Afrika nie. DAS ELISA, aangevul deur Miles en Misra-kiembare tellings, sal al die nodige inligting vir gehalte-beheer verskaf.

CHAPTER 7

REFERENCES

AHMAD. M.H.. EAGLESHAM, A.R.J. & HASSOUNA, S. (1981). Examining serological diversity of "cowpea" rhizobia by the ELISA technique. Arch. Microbiol., 130, 281-287.

AKKERMANS, A.D.L., ABDULKADIR, S. & TRINICK, M.J. (1978). Nitrogen-fixing root nodules in Almaceae. Nature, London, 274, 190.

ALLEN, O.N. (1959). Experiments in soil bacteriology. Minneapolis: Burgess Publishing Co.

ALLEN, ETHYL K. & ALLEN O.N. (1958). Biological aspects of symbiotic nitrogen fixation. In: Encyclopedia of Plant Physiology. Springer-Verlag, Berlin: Ruhland. Vol 8, pp. 48-118.

ALLEN, ETHYL K. & ALLEN, O.N. (1961). The scope of nodulation in the Leguminosae. In: Recent advances in botany. Toronto & New York: University of Toronto Press, pp. 585-588.

ALLEN, ETHYL K. & ALLEN O.N. (1981). The Leguminosae, a source book of characteristics, uses and nodulation. University of Wisconsin Press.

ALMON, L. & BALDWIN, I.L. (1933). The stability of cultures of Rhizobium. J. Bact., 26, 229-250.

AVRAMEAS, S. (1969). Coupling of enzymes to proteins with glutaraldehyde. Immunochemistry, 6, 43-52.

AYANABA, A. & DART, P.J. (1977). Biological nitrogen fixation in farming systems of the tropics: Papers presented at a symposium held at the International Institute of Tropical Agriculture, Ibadan, Nigeria, October 1975. Chichester, New York, Brisbane, Toronto: J. Wiley & Sons.

BARBARA, D.J. & CLARK, M.F. (1982). A simple indirect ELISA using $F(ab')_2$ fragments of immunoglobulin. J. gen. Virol., 58, 315-322.

BARRET, J.T. (1970). Textbook of Immunology. St Louis, U.S.A.: The C V Mosley Company.

BARTHOLOMEW, P.E. (1971). Inenting en verpilling van peul-gewassaad. Boerdery S A, Julie, 28-31.

BERGER, J.A., MAY, S.N., BERGER, L.R. & BOHLOOL, B.B. (1979). Colorimetric enzyme-linked immunosorbent assay for the identification of strains of Rhizobium in culture and in nodules of lentils. Appl. environ. Microbiol., 37, 642-646.

BOHLOOL, B.B. & SCHMIDT, E.L. (1970). Immunofluorescent detection of Rhizobium japonicum in soils. Soil Sci., 110, 229-236.

BOORSMA, D.M. & KALSBEEK, G.L. (1975). A comparative study of horseradish peroxidase conjugates prepared with a one-step and two-step method. J. Histochem. Cytochem., 23, 200-207.

BROCKWELL, J. (1963). Accuracy of a plant-infection technique for counting populations of Rhizobium trifolii. Appl. Microbiol., 11, 377-383.

BROCKWELL, J. (1971). Selection of Rhizobium meliloti strains for inoculation of Medicago rugosa Desr. cv. Paragosa. Aust. CSIRO Div. Plant Ind. Field Stn. Rec. No. 10, 51-58.

BROCKWELL, J. (1977). Application of legume seed inoculants. In: A treatise on dinitrogen fixation, Section IV, Agronomy and ecology. Edited by R.W.F. Hardy & A.H. Gibson. New York, London, Sydney, Toronto: J. Wiley & Sons, pp. 277-309.

BROCKWELL, J. (1980). Experiments with crop and pasture legumes. Principle and practice. In: Methods for evaluating biological nitrogen fixation. Edited by F.J. Bergerson. Chichester: J. Wiley & Sons, pp. 417-488.

BROCKWELL, J., DUDMAN, W.F., GIBSON, S.H., HELY, F.W. & ROBINSON, A.C. (1968). An integrated programme for the improvement of legume inoculant strains. In: Trans 9th Intern Cong. Soil Sci, Adelaide, Australia. Edited by J.W. Holmes. Sydney, Australia: Angus & Robertson, Vol 2, pp. 103-144.

BROCKWELL, J., SCHWINGHAMER, E.A. & GAULT, R.R. (1977). Ecological studies of root nodule bacteria introduced into field environments. 5. A critical examination of the stability of antigenic and streptomycin-resistance markers for identification of strains of Rhizobium trifolii. Soil Biol. Biochem., 9, 19-24.

BURTON, J.C. (1965). The Rhizobium-legume association. In: Microbiology and soil fertility. Edited by G.M. Gilmour & O.N. Allen. Corvallis: Oregon State University Press, pp. 107-134.

BURTON, J.C. (1976). Methods of inoculating seeds and their effect on survival of rhizobia. In: Symbiotic nitrogen fixation in plants. Edited by P.S. Nutman. Cambridge, London, New York, Melbourne: Cambridge University Press, pp. 175-190.

BURTON, J.C. (1979). Rhizobium species. In: Microbial technology, 2nd ed. Edited by H.J. Peppler & D. Perlmann. New York, N V, USA: Academic Press, pp. 29-58.

BURTON, J.C. (1982). Modern concepts in legume inoculation. In: Biological nitrogen fixation, technology for tropical agriculture. Edited by P.H. Graham & Susan C. Harris. Cali, Colombia: Centro Internacional de Agricultura Tropical, pp. 105 - 112.

BUSHNELL, O.A.& SARLES, W.B. (1939). Investigations upon the antigenic relationships of the root-nodule bacteria of the soybean, cowpea and lupine cross-inoculation groups. J. Bact., 38, 401-410.

CALDWELL, B.E. & VEST, G. (1968). Nodulation interactions between soybean genotypes and serogroups of Rhizobium japonicum. Crop Sci., 8, 680.

CALDWELL, B.E. & WEBER, D.F. (1970). Distribution of Rhizobium japonicum serogroups in soybean nodules as affected by planting dates. Agron. J., 62, 12-14.

CHASE, M.W. (1967). Production of antiserum. Section A. Preparation of immunogens. In: Methods in immunology and immunochemistry, vol 1. Edited by C.A. Williams & M.W. Chase. New York, London: Academic Press, pp. 197-209.

CLARK, M.F. & ADAMS, A.N. (1977). Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. J. gen. Virol., 34, 475-483.

CLOONAN, M.J. (1963). Black nodules in Dolichos. Aust. J. Science, 26, 121.

CLOONAN, M.J. & HUMPHREY, BEVERLY A. (1976). A new method for strain identification of Rhizobium trifolii in nodules. J. appl. Bact., 40, 101-107.

DAMIRGI, S.M., FREDERICK, L.R. & ANDERSON, I.C.(1967). Serogroups of Rhizobium japonicum in soybean nodules as affected by soil types. Agron. J., 59, 10.

DATE, R.A. (1976). Principles of Rhizobium strain selection. In: Symbiotic nitrogen fixation in plants. Edited by P.S. Nutman. Cambridge, London, New York, Melbourne: Cambridge University Press, pp. 137-150.

DATE, R.A. & ROUGHLEY, R.J. (1977). Preparation of legume seed inoculants. In: A treatise on dinitrogen fixation, Sec IV, Agronomy and ecology. Edited by R.W.F. Hardy & A.H. Gibson. New York, London, Sydney, Toronto: J. Wiley & Sons, pp. 243-276.

DATE, R.A. & DECKER, A.M. (1965). Minimal antigenic constitution of 28 strains of Rhizobium japonicum. Can. J. Microbiol., 11, 1-8.

DESCHODT, C.C. & STRIJDOM, B.W. (1976). Effective nodulation of Aspalathus linearis spp. linearis by rhizobia from other Aspalathus species. Phytophylactica,

8, 103-104.

DIATLOFF, A. (1977). Ecological studies of root-nodule bacteria introduced into field environments. 6. Antigenic and symbiotic stability in Lotononis rhizobia over a 12-year period. Soil Biol. Biochem., 9, 85-88.

DUDMAN, W.F. (1964). Immune diffusion analysis of the extracellular soluble antigens of two strains of R. meliloti. J. Bact., 88 (3), 782-794.

DUDMAN, W.F. (1968). Capsulation in Rhizobium species. J. Bact., 95 (3), 1200-1201.

DUDMAN, W.F. (1971). Antigenic analysis of Rhizobium japonicum by immunodiffusion. Appl. Microbiol., 21, 973-985.

DUDMAN, W.F. (1977). Serological methods and their application to dinitrogen-fixing organisms. In: A treatise on dinitrogen fixation, Sec IV, Agronomy and ecology. Edited by R.W.F. Hardy & A.H. Gibson. New York, London, Sydney, Toronto: J. Wiley & Sons, pp. 487-508.

DUDMAN, W.F. & BROCKWELL, J. (1968). Ecological studies of root-nodule bacteria introduced into field environments. I. A survey of field performance of clover inoculants by gel immune diffusion serology. Aust. J. agric. Res., 19, 739-747.

DUNHAM, D.H. & BALDWIN, I.L. (1931). Double infection of leguminous plants with good and poor strains of rhizobia. Soil Sci., 32, 235-248.

EHLERS, U. & PAUL, H.L. (1984). Binding of viruses from crude plant extracts to glutaraldehyde-treated plates for indirect ELISA. J. virol. Meth., 8, 217-224.

ELSWORTH, R. (1962). Phase separation of suspensions by centrifuge. New Jersey: New Brunswick Sci. Co.

ENGVALL, EVA (1978). Preparation of enzyme-labelled staphylococcal Protein A and its use for detection of antibodies. Scand. J. Immunol., 8 (7), 25-31.

ENGVALL, EVA, JONSSON, KARIN & PERLMANN, P. (1971). Enzyme-linked immunosorbent assay. II. Quantative assay of protein antigen, immunoglobulin G, by means of enzyme-labelled antigen and antibody-coated tubes. Biochem. Biophys. Acta, 251, 427-434.

ENGVALL, EVA & PERLMANN, R. (1971). Enzyme-linked immunosorbent assay (ELISA). Quantitative assay of immunoglobulin G. Immunochemistry, 8, 871-874.

ENGVALL, EVA & PERLMANN, P. (1972). Enzyme-linked immunosorbent assay, ELISA. III. Quantification of specific antibodies by enzyme-labelled anti-immunoglobulin in antigen-coated tubes. J. Immunol., 109, 129-135.

FRASER, M.E. (1975). A method of culturing Rhizobium meliloti on porous granules to form a pre-inoculant for lucerne seed. J. appl. Bact. 39, 345-351.

FRED, E.B., BALDWIN, I.L. & McCOY, ELIZABETH (1932). Root nodule bacteria and leguminous plants. Madison: Univ. Wisconsin, Studies in Sci.

FUHRMANN, H. & WOLLUM II, A.G. (1985). Simplified enzyme-linked immunosorbent assay for routine identification of Rhizobium japonicum antigens. Appl. environ. Microbiol., 49 (4), 1010-1013.

GHAI, S.K., HISAMATSU, M., AMENURA, A. & HARADA, T. (1981). Production and chemical composition of extracellular polysaccharides of Rhizobium. J. gen. Microbiol., 122, 33-40.

GIBSON, A.H., DUDMAN, W.F., WEAVER, R.W., HORTON, J.C. & ANDERSON, I.C. (1971). Variation within serogroup 123 of Rhizobium japonicum. Plant and Soil, Special vol, 33-37.

GRAHAM, P.H. (1963). Antigenic affinities of the root-nodule bacteria of legumes. Antonie van Leeuwenhoek, J. Microbiol. Serol., 29, 281-291.

GRAHAM, P.H. (1976). Identification and classification of root nodule bacteria. In: Symbiotic nitrogen fixation in plants. Edited by P.S. Nutman. Cambridge, London, New

York, Melbourne: Cambridge Univ. Press, pp. 99-112.

GRAHAM, P.H. & HARRIS, SUSAN C. (1982). Biological nitrogen fixation. Technology for tropical agriculture: Papers presented at a workshop held at CIAT, March 9-31, 1981. Cali, Colombia: Centro Internacionalde Agricultura Tropical.

GROBBELAAR, N. & STRIJDOM, B.W. (1971). The extent of biological nitrogen fixation in the Republic of South Africa. In: Proteins and food supply in the Republic of South Africa. Edited by J.W. Claassens & H.J. Potgieter. Cape Town: Balkema Publ., pp. 291-305.

HAHN, N.J. (1966). The Congo-red reaction in bacteria and its usefulness in the identification of bacteria. Can. J. Microbiol., 35, 725-733.

HALLIDAY, W.J. (1971). Immunological paralysis of mice with pneumococcal polysaccharide antigens. Bacteriol. Rev., 35, 267-289.

HARDING, N. (1982). Throwing light on enzyme immunoassay. Laboratory Equipment Digest, 20, 89-97.

HERRIDGE, D.F. & ROUGHLEY, R.J. (1975). Variation in colony characteristics and symbiotic effectiveness of Rhizobium. J. appl. Bact., 38, 19-27.

HODGSON, A.L.M. & WAID, J.S. (1981). Use of enzyme-linked immunosorbent assay (ELISA) to identify bacteriocinogenic strains of Rhizobium in nodules following a mixed strain inoculation. In: Current perspectives in nitrogen fixation. Edited by A.H. Gibson & W.F. Newton. Canberra: Australian Academy of Science, p.430.

HOLLAND, A.A. (1966). Serologic characteristics of certain root-nodule bacteria of legumes. Antonie van Leeuwenhoek, J. Microbiol. Serol., 32, 410.

HUGHES, D.Q. & VINCENT, J.M. (1942). Serological studies of the root-nodule becteria. III. Tests of neighbouring strains of the same spp. Proc. Linn. Soc. N.S.W., 67, 142-152.

HUMPHREY, BEVERLY A. & VINCENT, J.M. (1963). Polysaccharide fractions and transformations in Rhizobium. Nature, 199, 927.

HUMPHREY, BEVERLY A. & VINCENT, J.M. (1965). The effect of calcium nutrition on the production of diffusible antigens by Rhizobium trifolii. J. gen. Microbiol., 41, 109-118.

HUMPHREY, BEVERLY A. & VINCENT, J.M. (1973). Differential antibody response to the somatic antigenic determinants of Rhizobium trifolii. Microbios , 7, 87-93.

JANSEN VAN RENSBURG, H. & STRIJDOM, B.W. (1969). Strains of Rhizobium japonicum and inoculant production in South Africa. Phytophylactica, 1, 201-204.

JANSEN VAN RENSBURG, H. & STRIJDOM, B.W. (1974). Quality control of Rhizobium inoculants produced from sterilized and non-sterile peat in South Africa. Phytophylactica, 6, 307-310.

JANSEN VAN RENSBURG, H. & STRIJDOM, B.W. (1985). Effectiveness of Rhizobium strains used in inoculants after their introduction into soil. Appl. environ. Microbiol., 49 (1), 127-131.

JANSEN VAN RENSBURG, H., STRIJDOM, B.W. & KRIEL, MARTIE M. (1976). Necessity for seed inoculation of soybeans in South Africa. Phytophylactica, 8, 91-96.

JOHNSON, H.W.& MEANS, URA M. (1963). Serological groups of Rhizobium japonicum recovered from nodules of soybeans (Glycine max) in field soils. Agron. J., 55, 269-271.

JOHNSON, H.W. & MEANS, URA M. (1964). Selection of competitive strains of soybean nodulating rhizobia. Agron. J., 56, 60-62.

JOHNSON, H.W., MEANS, URA M. & WEBER, C.R. (1965). Competition for nodule sites between strains of Rhizobium japonicum applied as inoculum and strains in soil.

Agron. J., 57, 179.

JOHNSTON, A.W.B. & BERINGER, J.E. (1975). Identification of the Rhizobium strains in pea root nodules using genetic markers. J. gen. Microbiol., 87, 343-350.

JONES, D.G. & BROMFIELD, E.S.P. (1978). A study of the competitive ability of streptomycin and spectinomycin mutants of Rhizobium trifolii using various marker techniques. Ann. appl. Biol., 88, 448-450.

JONES, D.G. & MORLEY, SUSAN J. (1981). The effect of pH on plant "preference" for strains of Rhizobium trifolii using fluorescent ELISA for strain identification. Ann. appl. Biol., 97, 183-190.

JONES, D.G. & RUSSELL, P.E. (1972). The application of immunofluorescence techniques to host plant nodule bacteria selectivity experiments using Trifolium repens. Soil Biol. Biochem., 4, 277-282.

JONES, R.M., STRIJDOM, B.W. & THERON, E.P. (1974). The indigenous South African clovers (T. africanum Ser and T. burchellianum Ser) and their potential as pasture legumes. Tropical Grasslands, 8, 7-16.

JOSEY, D.P., BEYNON, J.L., JOHNSTON, A.W.B. & BERINGER, J.E. (1979). Strain identification in Rhizobium using intrinsic antibiotic resistance. J. appl. Bact., 46, 343-350.

KAUSHIK, B.D., DADARWAL, K.R. & VENKATRAMAN, G.S. (1973). Serological properties of mutants of Rhizobium. Curr. Sci., 42, 508-509.

KISHINEVSKY, B. & BAR-JOSEPH, M. (1978). Rhizobium strain identification in Arachis hypogea nodules by enzyme-linked immunosorbent assay (ELISA). Can. J. Microbiol., 24, 1537-1543.

KISHINEVSKY, B. & GURFEL, DEBORA (1980). Evaluation of enzyme-linked immunosorbent assay (ELISA) for serological identification of different Rhizobium strains. J. appl. Bact., 79, 517-526.

KISHINEVSKY, B., GURFEL, DEBORA, LOBEL, RINA & NEMAS, CHAYA (1982). Use of the enzyme-linked immunosorbent assay (ELISA) as a serological screening test in studies of peanut Rhizobium strains. Israel J. Botany, 31, 119-130.

KISHINEVSKY, B. & MAOZ, A. (1983). ELISA identification of Rhizobium strains by use of enzyme labelled-Protein A. Current Microbiol., 9, 45-49.

KISHINEVSKY, B., MAOZ, A., GURFEL, DEBORA & NEMAS, CHAYA (1984). A comparative evaluation of direct and indirect enzyme-linked immunosorbent assay (ELISA) in serological studies of Rhizobium strains. In: Symbiotic nitrogen fixation I. Edited by B.S. Ghai. Ludhiana: USG Publishers & Distributors, pp. 127-139.

KLECZKOWSKA, J. (1950). A study of phage resistant mutants of Rhizobium trifolii. J. gen. Microbiol., 4, 298-310.

KLECZKOWSKA, J. & THORNTON, H.G. (1944). A serological study of root-nodule bacteria from pea and clover inoculation groups. J. Bact., 48, 661-672.

KNOOTZ, F.P. & FABER, J.E. (1961). Somatic antigens of Rhizobium japonicum. Soil Sci., 91, 228-232.

KOENIG, RENATE (1978). ELISA in the study of homologous and heterologous reactions of plant viruses. J. gen. Virol., 40, 309-318.

KREMER, R.J. & WAGNER, G.H. (1978). Detection of soluble Rhizobium japonicum antigens in soil by immunodiffusion. Soil Biol. Biochem., 10, 247-255.

KRONVALL, G., SEAL, U.S., FINSTAD, JOANNE & WILLIAMS, R.C. (1970). Phylogenetic insight into evolution of mammalian Fc fragment of γG globulin using staphylococcal protein A. J. Immunol., 104, 140-147.

LEHTONEN, O.P. & VILJANEN, M.K. (1980). Antigen attachment in ELISA. J. immunol. Methods, 34, 61-70.

LINDEMANN, W.C., SCHMIDT, E.L. & HAM, G.E. (1974). Evidence for double infection within soybean nodules. Soil Sci., 118, 274-279.

LOOS, M.A. & LOUW, H.A. (1964). A study of the clover root-nodule bacteria in soils of the George district. II. Symbiotic characteristics. S. Afr. J. agric. Sci., 7, 447-456.

LORKIEWICZ, Z. & DUSINSKI, M. (1963) cited by Dudman, W.F. (1977). Serological methods and their application to dinitrogen-fixing organisms. In: A treatise on dinitrogen fixation, Sec IV, Agronomy and ecology. Edited by R.W.F. Hardy & A.H. Gibson. New York, London, Sydney, Toronto: J. Wiley & Sons, pp. 487-508.

LORKIEWICZ, Z. & RUSSA, R. (1971). Immunochemical studies of Rhizobium mutants. Plant and Soil, Special volume, 105-109.

MAOLINI, R. & MASSEYEFF, R. (1975). A sandwich method for enzyme immunoassay. I. Application to rat and human alpha-fetoprotein. J. immunol. Methods, 8, 223-234.

MARTENSSON, ANNA M. & GUSTAFSSON, J.G. (1985). Competition between Rhizobium trifolii strains for nodulation, during growth in a fermenter, and in soil-based inoculants, studied by ELISA. J. gen. Microbiol., 131, 3077-3082.

MARTENSSON, ANNA M., GUSTAFSSON, J.G. & LJUNGGREN, H.D. (1984). A modified highly sensitive enzyme-linked immunosorbent assay (ELISA) for Rhizobium meliloti strain identification. J. gen. Microbiol., 130, 247-253.

MEADE, J., HIGGINS, P. & O'GARA, F. (1985). Production and storage of Rhizobium leguminosarum cell concentrates for use as inoculants. J. appl. Bact., 58, 517-524.

MEANS, URA M., JOHNSON, H.W. & DATE, R.A. (1964). Quick serological method of classifying strains of Rhizobium japonicum in nodules. J. Bact., 87 (3), 547-553.

MORLEY, SUSAN J. & JONES, D.G. (1980). A note on a highly sensitive modified ELISA technique for Rhizobium strain identification. J. appl. Bact., 49, 103-109.

NAMBIAR, P.T.C. & ANJAIAH, V. (1985). Enumeration of rhizobia by enzyme-linked immunosorbent assay (ELISA). J. appl. Bact., 58, 187-193.

NEL, P.C. (1962). The inoculation of Lupins on Elsenburg soils with special reference to the effectiveness of different wetting media. S Afr. J. agric. Sci., 5, 537-546.

NORRIS, D.O. (1958). A red strain of Rhizobium from Lotononis bainesii Barker. Aust. J. agric. Res., 9, 629-639.

NUTMAN, P.S. (1976). IBP field experiments on nitrogen fixation by nodulated legumes. In: Symbiotic Nitrogen Fixation in plants (IBP Handbook no. 7). Edited by P.S. Nutman. Cambridge: Cambridge University Press, pp.

211-237.

OKON, Y., ESHEL, Y. & HENIS, Y. (1972). Cultural and symbiotic properties of Rhizobium strains isolated from nodules of Cicer arietinum L. Soil Biol. Biochem., 4, 165-170.

OLSEN, P.E. & RICE, W.A. (1984). Minimal antigenic characterization of eight Rhizobium meliloti strains by indirect enzyme-linked immunosorbent assay (ELISA). Can. J. Microbiol., 30, 1093-1099.

OLSEN, P.E., RICE W.A., STEMKE, G.W. & PAGE, W.J. (1981). Serological identification of Canadian selected Rhizobium meliloti strains in commercial inoculants. In: Proceedings of the 8th North American Rhizobium Conference, 3-7 September, 1981, pp. 456-467.

OLSEN, P.E., RICE, W.A., STEMKE, G.W. & PAGE, W.J. (1983). Strain specific serological techniques for the identification of Rhizobium meliloti in commercial alfalfa inoculants. Can. J. Microbiol., 29, 225-230.

O'SULLIVAN, M.J., BRIDGES, J.W. & MARKS, V. (1979). Enzyme immunoassay, a review. Ann. clin. Biochem., 16, 221-240.

PARKER, C.A. & GROVE, P.L. (1970). The rapid serological identification of rhizobia in small nodules. J. appl. Bact., 33, 248-252.

PARKER, C.A., TRINICK, M.J. & CHATEL, D.L. (1977). Rhizobia as soil and rhizosphere inhabitants. In: A treatise on dinitrogen fixation, section IV. Agronomy and ecology. Edited by R.W.F. Hardy & A.H. Gibson. New York, London, Sydney, Toronto: J. Wiley & Sons, pp. 311-352.

PETERSON, H.L. & LOYNACHAN, T.E. (1981). The significance and application of Rhizobium in agriculture. International Review of Cytology, supplement 13, 311-331.

PHILIPS, E.P. (1951). Botanical survey of South Africa. Memoir no 25. Pretoria: Government Printer.

PIKE, R.M. (1967). Antibody heterogeneity and serological reactions. Bacteriol. Rev., 31 (1), 157-174.

POSTGATE, J.R. (1969). Viable counts and viability. In: Methods in Microbiology, vol 1. Edited by J.R. Norris & D.W. Ribbons. London and New York: Academic Press, pp. 611-628.

PURCHASE, H.F., VINCENT, J.M. & WARD, L.M. (1951). Serological studies of the root-nodule bacteria. IV. Further analysis of isolates from Trifolium and Medicago. Proc. Linn. Soc. N.S.W., 76, 1.

READ, MARGARET P. (1953). The establishment of serologically identifiable strains of Rhizobium trifolii in field soils in competition with the native microflora. J. gen. Microbiol., 9, 1-14.

RENWICK, ANNABEL & JONES, D.G. (1985). A comparison of the fluorescent ELISA and antibiotic resistance identification techniques for use in ecological experiments with Rhizobium trifolii. J. appl. Bact., 58 (2), 199-206.

RICE, W.A., OLSEN, P.E. & PAGE, W.J. (1984). ELISA evaluation of the competitive abilities of two Rhizobium meliloti strains. Can. J. Microbiol., 30, 1187-1190.

ROUGHLEY, R.J. (1968). Some factors influencing the growth and survival of root nodule bacteria in peat culture. J. appl. Bact., 31, 259-265.

ROUGHLEY, R.J. (1970). The preperation and use of legume seed inoculants. Plant and Soil, 32, 675-701.

ROUGHLEY, R.J. (1976). The production of high quality inoculants and their contribution to legume yield. In: Symbiotic nitrogen fixation in plants. Edited by P.S. Nutman. Cambridge, London, New York, Melbourne: Cambridge University Press, pp.125-136.

ROUGHLEY, R.J. (1982). The storage, quality control, and use of legume seed inoculants. In: Biological nitrogen fixation technology for tropical agriculture. Edited by P.H. Graham & Susan C. Harris. Cali, Colombia: Centro Internacional de Agriculture Tropical, pp. 115-126.

ROUGHLEY, R.J. & VINCENT, J.M. (1967). Growth and survival of Rhizobium spp. in peat culture. J. appl. Bact. 36, 263-272.

ROWE, D.S. (1970). Nomenclature of immunoglobulins. Nature (London), 228, 509.

SALONEN, E.M. & VAHERI, A. (1979). Immobilization of viral and mycoplasma antigens and of immuno-globins on polystyrene surface for immunoassays. J. immunol. Methods, 30, 209-218.

SCHEFFLER, J.G. & LOUW, H.A. (1967). The serological characteristics of the clover rhizobia in the soils of the Stellenbosch districts. S. Afr. J. agric. Sci., 10, 161-174.

SCHMIDT, E.L. (1974). Quantitative autecological study of microorganisms in soil by immunofluorescence. Soil Science, 188 (3), 141-149.

SCHMIDT, E.L., BANKOLE, R.O. & BOHLOOL, B.B. (1968). Fluorescent-antibody approach to the study of rhizobia in soil. J. Bact., 95 (6), 1987-1992.

SCHWINGHAMER, E.A. & DUDMAN, W.F. (1973). Evaluation of spectinomycin resistance as a marker for ecological studies with Rhizobium spp. J. appl. Bact., 36, 263-272.

SCHWINGHAMER, E.A. & DUDMAN, W.F. (1980). Methods for identifying strains of diazotrophs. In: Methods for evaluating biological nitrogen fixation. Edited by F.J. Bergersen. Chichester, New York, Brisbane, Toronto: J. Wiley & Sons, pp. 337-365.

SCHWINGHAMER, E.A. & REINHARDT, D.J. (1963). Lysogeny in Rhizobium leguminosarum and R. trifolii. Aust. J. Biol. Sci., 16, 597-605.

SINHA, R.C. & PETERSON, E.A. (1980). Homologous serological analysis of R. meliloti strains by immunodiffusion. Can. J. Microbiol., 26, 1157-1161.

SKRDLETA, V. (1965). Somatic sero-groups of Rhizobium japonicum. Plant and Soil, 23 (1), 43-48.

SKRDLETA, V. (1969 a). Serological analysis of eleven strains of Rhizobium japonicum. Antonie van Leeuwenhoek, 5, 77-83.

SKRDLETA, V. (1969 b). Application of immunopresipitation in agar gel for the serological typing of soybean root-nodules. Folia Microbiol. (Praque), 14, 32-35.

SKRDLETA, V. (1973). Relationship between soybean cultivars and Rhizobium japonicum serotypes with single- and multi-strain inoculants. I. Greenhouse pot experiments. Zentralbl. Bakteriol. Parasitenk. Infektionskr. Abt II, 128, 543.

SNEDECOR, G.W. & COCHRAN, W.G. (1967). Statistical methods, 6th ed., Ames: Iowa State Univ. Press.

STAPHORST, J.L. & STRIJDOM, B.W. (1972). The effect of yeast extract concentration in media on strains of Rhizobium meliloti. Phytophylactica, 4, 29-32.

STEEL, R.G.D. & TORRIE, J.H. (1960). Principles and procedures of statistics with special references to the biological sciences. New York: Mc Graw-Hill Book Co.

STEINBORN, JULIA & ROUGHLEY, R.J. (1975). Toxicity of sodium and chloride ions to Rhizobium spp. in broth and peat culture. J. appl. Bact., 39, 133-138.

STEVENS, J.W. (1923). Can all strains of a specific organism be recognized by agglutination? J. infect. Diseases, 33, 557 - 566.

STEVENS, J.W. (1925). A study of various strains of Bacillus radicicola from nodules of alfalfa and sweet clover. Soil Sci., 20, 45-66.

STRIJDOM, B.W. (1977). Biologiese stikstofbinding: Navorsing in Suid-Afrika en die benutting van die proses in die landbou. Tydskrif vir Natuurwetenskappe, 17 (3), 64-85.

STRIJDOM, B.W. & DESCHODT, C.C. (1976). Carriers of rhizobia and the effects of prior treatment on the survival of rhizobia. In: Symbiotic nitrogen fixation in plants. Edited by P.S. Nutman. Cambridge, London, New York, Melbourne: Cambridge University Press, pp. 151-168.

STRIJDOM, B.W. & JANSEN VAN RENSBURG, H. (1981). Effect of steam sterilization and gamma - irradiation of peat on quality of Rhizobium inoculants. Appl. environ. Microbiol., 41 (6), 1344-1347.

STRIJDOM, B.W. & WASSERMAN, V.D. (1984). Huidige en potensiele bydraes deur biologiese stikstofbinding-sisteme tot die Suid-Afrikaanse landbou. In: Tegniese mededeling no 187, Handelinge stikstofsimposium Mei, 1982, pp. 80-86.

TCHAN, Y.T. (1982). Application of some newer serological techniques. In: Nitrogen fixation in legumes, Edited by J.M. Vincent. London: Academic Press, pp. 27-32.

THOMPSON, J.A. (1980). Production and quality control of legume inoculants. In: Methods for evaluating biological nitrogen fixation. Edited by F.J. Bergerson. New York: J. Wiley & Sons, pp.489-534.

TRINICK, M.J. (1969). Identification of legume nodule bacteroids by the fluorescent antibody reaction. J. appl. Bact., 32, 181-186.

TRINICK, M.J. (1973). Symbiosis between Rhizobium and the non-legume Trema aspera. Nature, London, 244, 459.

VAN DER MERWE, S.P. & STRIJDOM, B.W. (1973). Serological specificity of rhizobia from nodules of groundnuts cultivated in South African soils. Phytophylactica, 5, 119-122.

VAN DER MERWE, S.P., STRIJDOM, B.W. & JANSEN VAN RENSBURG, H. (1972). Use of the fluorescent antibody technique to detect a bacterial contaminant in nodule squashes of leguminous plants. Phytophylactica, 4, 97-100.

VAN DER MERWE, S.P., STRIJDOM, B.W. & UYS, C.J. (1974). Groundnut response to seed inoculation under extensive agricultural practices in South African soils. Phytophylactica, 6, 295-302.

VAN OSS, C.J. & SINGER, J.M. (1966) cited by Lehtonen, O.P. & Viljanen, M.K. (1980). Antigen attachment in ELISA.

J. immunol. Meth., 34, 61-70.

VAN WEEMEN, B.K. & SCHUURS, A.H.W.M. (1971). Immuno-assay using antigen-enzyme conjugates. F.E.B.S. Letters, 15, 232-236.

VINCENT, J.M. (1941). Serological studies of the root-nodule bacteria. I. Strains of Rhizobium meliloti. Proc. Linn. Soc. N.S.W., 66, 145-154.

VINCENT, J.M. (1942). Serological studies of the root-nodule bacteria. II. Strains of Rhizobium trifolii. Proc. Linn. Soc. N.S.W., 67, 82-86.

VINCENT J.M. (1944). Variation of the nitrogen-fixing property of Rhizobium trifolii. Nature, London, 153, 496-497.

VINCENT, J.M. (1954). The root-nodule bacteria of pasture legumes. Proc. Linn. Soc. N.S.W., 79, 1.

VINCENT, J.M. (1970). A manual for the practical study of root-nodule bacteria. I.B.P. Handbook no. 15, Oxford: Blackwell Scientific Publishers.

VINCENT, J.M. (1974). In: The biology of nitrogen fixation, North-Holland Research Monographs: Frontiers of Biology, vol. 33. Edited by A. Quispel. North-Holland, Amsterdam, pp. 265-314.

VINCENT, J.M. (1977). Quality control of inoculants. In: Exploiting the legume-Rhizobium symbioses in tropical agriculture. Edited by J.M. Vincent, A.S. Whitney & J. Bose. College of Tropical agriculture Miscellaneous Publication 145, Department of Agronomy and Soil Science, University of Hawaii, pp.447-456.

VINCENT, J.M. (1982). Serology. In: Nitrogen fixation, vol. 2, Rhizobium. Edited by W.J. Bergerson. Oxford: Clarendon Press, pp.235-273.

VINCENT, J.M. & HUMPHREY, BEVERLY, A. (1968). Modification of the antigenic surface of Rhizobium trifolii by a deficiency of calcium. J. gen. Microbiol., 54, 397-405.

VINCENT, J.M. & HUMPHREY, BEVERLY A. (1970). Taxonomically significant group antigens in Rhizobium. J. gen. Microbiol., 63, 379-382.

VINCENT, J.M., HUMPHREY, BEVERLY A. & SKRDLETA, V. (1973). Group antigens in slow-growing rhizobia. Arch. Microbiol., 89, 79-82.

VINCENT, J.M. & WATERS, L.M. (1953). The influence of the host on competition amongst clover root-nodule bacteria. J. gen. Microbiol., 9, 357-370.

VINCENT J.M. & WATERS, L.M. (1954). The root-nodule bacteria as factors in clover establishment in the red basaltic soils of the Lismore district, New South Wales. Aust. J. agric. Res., 5, 61-76.

VINCENT, J.M., WHITNEY, A.S.& BOSE, J. (1977). Exploiting the legume-Rhizobium symbiosis in tropical agriculture. Proceedings of a workshop held at Kahului, Maui, Hawaii, August 23-28, 1976. College of Tropical Agriculture Miscellaneous Publication 145. Department of Agronomy and Soil Science, University of Hawaii.

VINTIKOVA, H., SROGL, M. & SKRDLETA, V. (1961). A contribution to the serological typization of the rhizobia. Folia Microbiol. (Prague), 6, 243.

VOLLER, A., BIDWELL, D.E. & BARTLETT, ANNE (1973). Microplate enzyme immunoassays for the immunodiagnosis of virus infections. In: Manual of clinical immunology. Edited by N.R. Rose, H. Friedman. Amer. Soc. Microbiol., Washington D C, pp. 506-512.

VOLLER, A., BIDWELL, D.E. & BARTLETT, ANNE (1976a). Enzyme immunoassays in diagnostic medicine: theory and practice. Bull, W.H.O., 53, 55-56.

VOLLER, A., BIDWELL, D.E. & BARTLETT, ANNE (1976b). The enzyme-linked immunosorbent assay (ELISA). Practical microplate enzyme-immunoassays. In: Protides of the

biological fluids. Edited by H. Peeters. Oxford: Pergamon Press, 24, pp. 751-758.

VOLLER, A., BIDWELL, D.E., & BARTLETT, ANNE (1977).The enzyme-linked immunosorbent assay (ELISA). A review with a bibliography of microplate applications. Guernsey, Europe: Flow Line Publications, pp. 1-48.

WILLIAMS, C.A. & CHASE, M.W. (1967). Methods in immunology and immunochemistry, vol 1. Preparation of antigens and antibodies. New York & London: Academic Press.

WILSON, N.H.M., HUMPHREY, BEVERLY A. & VINCENT, J.M. (1975). Loss of agglutinating specificity in stock cultures of Rhizobium meliloti. Arch. Microbiol., 103, 151-154.

WRIGHT, W.H. (1925). The nodule bacteria of soybeans. I. Bacteriology of strains. Soil Sci., 20, 95-129.

WRIGHT, W.H., SARLES, W.B. & HOLST, E.G. (1930). A study of Rhizobium japonicum isolated from various soils. J. Bact., 19, 39.