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THE APPLICATION OF ENZYME-LINKED IMMUNOSORBENT ASSAYS (ELISA) TO STRAIN IDENTITY DETERMINATIONS OF *RHIZOBIUM MELILOTI*

BY

© Perry E. Olsen

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

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ABSTRACT

Antisera were prepared against six Rhizobium meliloti strains including three strains which had been selected for Canadian soil and climate conditions and are currently used in the production of commercial alfalfa (Medicago spp.) inoculants. The antisera were highly cross-reactive in agglutination tests, but strain specificity was obtained by repeated massive adsorptions of the antisera with cells of cross-reacting strains. Only two of the strains were found to be serologically identical, thus five serotypes were found among the six strains examined.

Immunoglobulin G was purified from adsorbed antisera by salt fractionation followed by affinity and gel chromatography and was then conjugated with alkaline phosphatase by a one-step glutardialdehyde proecedure. Antibody-enzyme conjugates were purified by gel chromatography and gave strain specific reactivity with homologous cells in the double antibody sandwich enzyme-linked immunosorbent assay (ELISA).

The adsorbed antisera were used in micro-agglutination tests and in the ELISA procedure to demonstrate the presence of the Canadian developed strains in their respective commercial peat-base inoculants at levels of 10^8 to 10^9 viable cells per g. An ELISA determination of the identities of two strains inhabiting over three hundred nodules was accomplished in a study designed to elicit information about possible competitive nodulation effects between two commercial strains

when applied as a mixed inoculum in varying numerical ratios. Both strains formed single-strain inhabited nodules according essentially to the relative proportion of inoculating numbers. Approximately 13% of the nodules were found to be inhabited by both strains. The dual-strain inhabited nodules occurred predominantly when one strain had considerable numerical advantage in terms of the inoculating population applied, suggesting possible symbiotic differences between the strains. The serological techniques described allow for the strain identity determination of small quantities of cell-bound Rhizobium meliloti antigen; no serological differences were found between rhizobial nodule antigen and that of the corresponding strain when vegetatively grown.

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ABBREVIATIONS

DEAE	-	diethylaminoethyl
EIA	-	enzyme immunoassay
ELISA	-	enzyme-linked immunosorbent assay
HIM	-	host induced modification
LPS	-	lipopolysaccharide
NRG	-	Northern Research Group
PBS	-	phosphate buffered saline
PNPP	-	paranitrophenyl phosphate
PPS	-	phosphate peptone solution
YEMA	-	yeast extract mannitol agar
YEMB	-	yeast extract mannitol broth

1. INTRODUCTION

Life on earth depends on the photosynthetic conversion of carbon dioxide to organic substances. The growth of green organisms possessing photosynthetic apparatus in turn depends on a wide variety of essential substances. The most widely limiting of these is combined nitrogen. Because nitrogen enters biological systems only when it is combined or "fixed" with another element, usually hydrogen or oxygen, it is both physically abundant (N_2) and nutritionally scarce (NO_3^-, NH_4^+). Biological nitrogen fixation has supported the growth of plants for a lengthy evolutionary period, yet as a genetic capacity is limited to procaryotic organisms in the form of a few genera of bacteria (including cyanobacteria). No higher organisms (aside from industrial man) have developed mechanisms for the fixation of atmospheric nitrogen, but many plants share directly in the procaryotic capability by forming symbiotic associations with nitrogen fixing bacteria. The most widely studied of these symbiotic associations exists between higher plants of the family Leguminosae and a heterogeneous grouping of bacteria currently classified as the genus Rhizobium. In natural ecosystems, and under the deliberate manipulation of man since at least the time of Christ, the Rhizobium-legume symbiosis has restored plant available nitrogen to the soil from the abundant, but inert, reservoir in the atmosphere.

Recent economic emphasis of the limited nature of fossil fuels has placed premiums upon research leading to increased knowledge of the processes and mechanisms of natural biological nitrogen fixation.

It is reasonable to hope that such knowledge will lead to intelligent manipulations and modifications of existing fixation systems allowing decreased dependence upon the energy costly chemical production of nitrogenous fertilizers. The Rhizobium-legume symbiotic association is only one of several biological nitrogen fixing systems of ecological importance, yet it is currently the most significant in North American agriculture. The established agricultural practice of inoculating legume seed to improve crop production provides the basis for selection of Rhizobium strains particularly adapted to specific host cultivars and environmental conditions. The increasingly recognized variability amongst strains of a rhizobial species has led to the concept that the Rhizobium-legume interaction may be influenced at the strain-cultivar level and that the relationship is affected by a wide variety of ecological conditions. Rhizobium strain characterization and selection thus offers rapid and practical potential for agricultural nitrogen fixation gains.

The requirement of investigators to work with strains within a Rhizobium species and the particular need for studies of the competitive aspect of interactions between strains requires that investigators possess reliable and efficient methods of distinguishing amongst them. This problem of strain identification within a mixed strain population is the major barrier to experiments designed to elucidate relationships and interactions which may exist. The work described in this thesis was undertaken to investigate the possibility of establishing a strain identification system for commercial Canadian strains of Rhizobium

meliloti. Specifically, the work was designed to: (a) determine if sufficient antigenic diversity exists to allow serological strain identification of commercial *R. meliloti* strains; (b) establish a serological assay system sensitive enough to identify the strains directly as individual colonies and as bacteroids in nodules; and (c) apply the serological assay in studies of ecological or quality control significance.

2. LITERATURE REVIEW OF STRAIN IDENTIFICATION TECHNIQUES IN GENUS *RHIZOBIUM*

2.1 The Root-Nodule Bacteria

The literature pertaining to the legume-*Rhizobium* symbiosis has been recently described as "overwhelming" (Bal and Wong 1982), yet despite the fact that the botanical family *Leguminosae* includes 700 genera and 14,000 species (Graham 1976), most of the study devoted to *Rhizobium*-legume interactions has been conducted with hosts of agricultural significance. This has resulted in the widely used, but increasingly challenged, taxonomic classification of *Rhizobium* which arranges the bacteria into species on the basis of the type of legume host nodulated. This classification stems largely from the work of Baldwin and Fred (1929), Eckhardt, Baldwin and Fred (1931), and Fred, Baldwin and McCoy (1932). Jordan and Allen (1974), in the eighth edition of *Bergey's Manual of Determinative Bacteriology*, divide the genus *Rhizobium* into six species distinguished chiefly by differences in legume host nodulated. In addition to the six specified *Rhizobium* species, an ill-defined group of organisms termed *Rhizobium* spp. is recognized whose common characteristic is the ability to nodulate within an extensive assortment of legumes collectively called the cowpea miscellany (Table 1).

Symbiotic promiscuity (cross-infection between host groups) and the existence of effectiveness subgroups within compatible legume-*Rhizobium* groupings has tended to erode the legume host infectivity

Table 1. *Rhizobium* species and host nodulated

Species	Growth rate	Host nodulated	Host common name
R. trifolii	fast	*Trifolium*	Clover
R. leguminosarum	fast	*Pisum*, *Lens*, *Vicia*, *Lathyrus*	Pea
R. phaseoli	fast	*Phaseolus vulgaris*	Bean
R. meliloti	fast	*Melilotus*, *Medicago*, *Trigonella*	Alfalfa, Sweetclover, Fenugreek
R. japonicum	slow	*Glycine max*	Soybean
R. lupini	slow	*Lupinus*, *Ornithopus*	Lupin
Rhizobium spp.	variable	*Vigna*, *Desmodium*, *Arachis*, etc.	Cowpea Miscellany

basis of classical rhizobial taxonomy. Nevertheless, for practical purposes it seems unrewarding to supersede the deeply ingrained terminology of classical Rhizobium taxonomy until such time as the processes of infectivity and effectivity have been defined and correlated with such information as may be gained from DNA hybridization studies and genetic analysis of the interrelating roles of both host and bacterium in symbiosis. Furthermore, a very great number of légumes and symbionts remain altogether uninvestigated and it seems appropriate for reclassification to await the collection of comparative data from these interactions (Jordan and Allen 1974).

2.2 Non-Serological Techniques for Rhizobium Strain Identification

2.2.1 Technique Applicability

A variety of approaches and techniques have been applied to the identification of Rhizobium strains, ranging from simple recognition of unique strain characteristics to relatively complex electrophoretic analysis of protein patterns. Norris (1958), for example, reported a Rhizobium strain with a unique red pigment and Cloonan (1963) described a strain forming black nodules on Dolichos lablab. On the other hand, Roberts et al. (1980) used two dimensional polyacrylamide gel electrophoresis to identify strains on the basis of pattern analysis of the differential distribution of numerous strain proteins.

Whereas a distinctive colony or nodule color associated with a particular strain is easily recognized, such characteristics are so rare as to be of limited value and, in any case, limit the investigator to particular strains and hosts. Highly complex identification schemes such as electrophoretic protein pattern analysis are not readily adaptable to identity determinations of a large number of samples of varying size and age such as nodules obtained in a field or greenhouse experiment. Generally, the applied Rhizobium worker selects the most expedient of strain identification techniques consistent with accuracy, sensitivity, and experimental design. Techniques yet devised tend to fall short in respect of one extreme or the other. This review of Rhizobium strain identification techniques is confined to those of general applicability to field and greenhouse studies of nodulation patterns.

2.2.2 Antibiotic Resistance

Mutant marker characteristics, particularly resistance to one or more antibiotics, have been widely used for Rhizobium strain identification (Johnston and Beringer 1975; Cooper 1979; Schwinghamer and Dudman 1973; Pinto et al. 1974; Jones and Bromfield 1978; Obaton 1973; Brockwell et al. 1977). The stable, single step mutation of Rhizobium strains to high level resistance to antibiotics such as streptomycin, spectinomycin, or rifampicin is ordinarily readily achieved, without the aid of mutagens, by simply growing the organisms

on antibiotic containing media (Schwinghamer and Dudman 1980). The use of strains resistant to different combinations of antibiotics allows the detection of each through replicate plating of a nodule squash onto each appropriate antibiotic containing medium. The selective, contaminant suppressing nature of antibiotic media has made the use of antibiotic resistant strains particularly popular in the design of nodulation studies conducted in field soils. Unfortunately, it is not safe to assume that an antibiotic resistant mutant is otherwise identical to the parent strain (Jones and Bromfield 1978; Josey et al. 1979; Zelazna-Kowalska 1971). The problem is emphasized in the case of mutants selected for high level resistance to a number of antibiotics (Schwinghamer and Dudman 1980). Rhizobium mutants to amino-glycosidic antibiotics such as streptomycin and spectinomycin have, however, been found to be less likely to have associated symbiotic defects than mutants to antibiotics having effects on cell membranes or walls (Schwinghamer and Dudman 1980).

One approach to circumventing the problem of unseen strain modifications in antibiotic resistant mutants has been to avoid the use of mutants altogether and measure the "intrinsic" antibiotic resistance patterns of the parent strains (Josey et al. 1979). These patterns of intrinsic resistance to low levels of a spectrum of antibiotics differ sufficiently between strains to allow identification. The requirement, however, for extreme laboratory care concerning media and, in particular, the age, number, and growth

phase of the cells to be tested presents problems to the routine application of this technique.

2.2.3 Symbiotic Markers

A Rhizobium strain will occasionally be identified which has some symbiotic character allowing it to be differentiated from other strains. Such a character can be a selective nodulation among a group of cultivars (Diatloff 1977), a distinctive nodulation morphology (Cloonan 1963), or differing levels of N_2 fixation effectiveness (Brockwell 1980). The use of a symbiotic marker as a strain identification technique implicitly requires a relatively long period of time for the identifying phenotype to develop as the legume grows. Where the marker is defined as a differential phenotype between a pair or group of cultivars the amount of work required to make the identification is concomitantly increased. Brockwell and Diatloff concluded that symbiotic markers, even when highly specific, were tedious and time consuming as means of strain identification (Brockwell 1980).

2.2.4 Bacteriophage Typing

Interest in the Rhizobium-bacteriophage interaction has been divided between the demonstration of Rhizobium parasitization by

phage resulting in poor nodulation (Demelon and Dunez 1936; Barnet 1972, 1979) and the use of specific phage to type Rhizobium species (Staniewski 1970b) and strains (Staniewski 1970a; Lesley 1982). For strain typing, both Staniewski and Lesley worked with R. meliloti. Staniewski subdivided 71 strains into 10 groups according to susceptibility differences to 7 phages whereas Lesley obtained 80 groups from 350 strains using 15 typing phages. Lesley (1982) emphasized the requirement for careful control of variables including culture media and both cell and phage concentrations to obtain reproducible results.

Host-induced modification (HIM) of phage has been observed in Rhizobium (Lesley 1982) in which the strain specificity of the bacteriophage was controlled by the Rhizobium strain on which the phage was last vegetatively grown. The use of such a phage for strain typing could develop from the modification of the phage into a variety of strain specific typing stocks (Schwinghamer and Dudman 1980).

2.3 Serological Techniques for Rhizobium Strain Identification

2.3.1 Rhizobium Antigenicity

Serological techniques have provided the most adaptable and specific methods for the recognition of diazotrophic microorganisms

at the strain level (Dudman 1977) and such techniques have been applied extensively to the genus Rhizobium (Schwinghamer and Dudman 1980). Antigenic analysis of Rhizobium species and strains by several groups working independently has indicated that the composition of rhizobial lipopolysaccharide (LPS) differs as significantly between strains of a single host nodulating group (species) as between members of different host nodulating groups (Carlson et al. 1978; Russa et al. 1981; Zajac et al. 1975; Zevenhuizen et al. 1980; Humphrey and Vincent 1969). Since rhizobial strain antigens are sometimes shared, sometimes specific, sometimes dominant, sometimes minor, and the whole issue further confused by the fact that different workers employ techniques designed to detect different types of antigen (LPS, flagellar, "internal", diffusing, non-diffusing, etc.), no group of investigators has reported a serological capability to categorically identify all members of genus Rhizobium at the "species" level. All Rhizobium species, however, as defined by host nodulation grouping, contain serologically distinct strains (Dudman 1977), and for purposes of identifying strains within a microbiologically defined system serological success has been reported for each of the host nodulating groups (for review see Dudman 1977). Of the several Rhizobium species, R. meliloti has been singled out as the most frustrating to identify serologically at the strain level (Sinha and Peterson 1980; Humphrey and Vincent 1975; Lesley 1982).

The cell wall lipopolysaccharide antigens of Rhizobium appear to be the locus of greatest antigenic specificity as both flagellar

and internal protein antigens are cross-reactive between strains and, in some cases, between species (Vincent 1941; Vincent and Humphrey 1970; Vincent and Waters 1953). The water soluble exopolysaccharide "slime" of R. meliloti did not show antigenic activity in an immunodiffusion analysis of eight strains (Humphrey and Vincent 1975).

2.3.2 Agglutination

Of the several serological techniques which have been applied to the strain identification of Rhizobium the agglutination reaction was the earliest and is still widely used. The evolution of agglutination techniques has been in the direction of allowing strain identifications to be made with smaller and smaller sample volumes, but as a basic requirement cell concentrations need to be in the range of 10^8 to 10^9 cells per mL. The demonstration that soybean nodules contain sufficient bacterial antigen for individual strain identifications (Means et al. 1964) led to attempts to directly identify the bacterial component of smaller nodules from legumes such as clover, alfalfa and birdsfoot trefoil. Parker and Grove (1970) performed successful microagglutination identifications of small nodules in microtiter plates although antigen volume and concentration limited the testing to a single antiserum per nodule. Microagglutination testing in microtiter plates can be reliably

performed with as little as 50 uL of 10^8 to 10^9 cells per mL of test antigen suspension (Olsen et al. 1981), but competition studies involving the presence of two or more inoculating strains require that each nodule be tested against antisera specific to each inoculating strain, preferably in replicate fashion. This requirement provided the impetus for development of techniques more sensitive than agglutination for the identification of Rhizobium strains directly from legume nodules.

2.3.3 Fluorescent Antibody

Immunofluorescence techniques provide a microscopic approach to strain identifications in which even single microbial cells may be examined and identified. Immunofluorescence has been used to identify both vegetatively grown cells (Jones and Russell 1972) and bacteroids (Trinick 1969) and attempts have been made to make the technique quantitative (Fliermans and Hazen 1980). Dual strain inhabitation of single nodules has been detected with immunofluorescence techniques (Lindeman et al. 1974). Immunofluorescence techniques are of unquestioned value in strain identification and more particularly in elucidating specific events in the microbial attachment and nodulation process (Dazzo and Brill 1979). Immunofluorescence and it's effective quantitation require sophisticated equipment and considerable skill is required in both application of the technique

and interpretation of results (Schmidt 1973). Immunofluorescence data obtained from ecological studies has generally been "quantitated" by subjective comparisons of homologous and heterologous fluorescence (Bohlool and Brock 1974). Fluorescent photometric techniques now available should see increasing use of fluorescent antibody techniques in ecological studies of Rhizobium strains.

2.3.4 Gel Immunodiffusion and Immunoelectrophoresis

Gel immunodiffusion has been widely used for antigenic analysis of Rhizobium strains (Dudman 1964, 1971; Vincent 1970; Vincent and Humphrey 1970; Humphrey and Vincent 1975) due to the capability of the technique to resolve separate antigen-antibody reactions. While immunodiffusion offers analytical resolution between antigens, it is one of the least sensitive of serological methods for the identification of antigen (Cloonan and Humphrey 1976). Since immunodiffusion involves the detection of soluble diffusing antigen, it is not suitable for the detection of particulate antigen such as lipopolysaccharide bound to cell walls of whole cells. This fact has occasionally been overlooked, and in at least one case has led to the erroneous conclusion that strains of R. meliloti are not serologically distinguishable (Sinha and Peterson 1980). Immunoelectrophoresis further increases the analytical resolving power of gel immunodiffusion,

but has the same limiting feature of requiring relatively high concentrations of diffusable antigens for analysis.

2.3.5 Enzyme-linked Immunosorbent Assay (ELISA)

The most recent of serological techniques to be applied to the serological identification of Rhizobium strains is the enzyme-linked immunosorbent assay (ELISA). The sensitivity of serological reactions can be greatly increased by the use of either radio- or enzyme-labeled antibody allowing the detection of minute amounts of antibody bound to small quantities of antigen. Since radio-immunoassay techniques require special precautions due to the health hazards of radioactive isotopes (usually I^{125} or I^{131}) and because of the short life span of radio-conjugated antibodies, the ELISA technique has become widely used in recent years, particularly in medical diagnostics. Once prepared, ELISA conjugated antibodies are extremely stable and economical to use because only small volumes of extremely dilute preparations are required. Both direct and indirect ELISA methods exist, depending (as with fluorescent antibody techniques), on whether the enzyme label is attached to antibodies reacting specifically with the test antigen or to secondary antibodies binding to unlabeled specific antibody reacting with the test antigen. Competitive inhibition versions of the technique exist and soluble, viral, or particulate antigen may be detected.

First efforts at enzyme labeling of antibody were intended to overcome technical limitations of fluorescent antibody techniques (Hildebrand 1979). The first enzyme labeling procedure conjugated acid phosphatase to specific antibody (Ram et al. 1966). This was followed by the use of horseradish peroxidase as labeling enzyme which gave greater stability and more rapid tissue penetration than did acid phosphatase (Nakane and Pierce 1967). Engvall and Perlmann introduced the ELISA technique using alkaline phosphatase conjugated to rabbit IgG (Engvall and Perlmann 1971). An ELISA assay using horseradish peroxidase as labeling enzyme was also introduced in 1971 (van Weeman and Schuurs 1971). ELISA types and applications have proliferated widely in the decade since then (for review see The Enzyme Linked Immunosorbent Assay (ELISA) by Voller, Bidwell and Bartlett 1979).

2.4 Literature Review of ELISA Applications to the Identification of *Rhizobium* Strains

2.4.1 Literature Search

A computer search for literature concerned with the ELISA identification of *Rhizobium* was conducted by the Agriculture Canada library facility in Ottawa during November of 1982. Only six pertinent articles were found. These are individually reviewed below

in chronological order of their literature appearance.

2.4.2 Kishinevsky, B. and M. Bar-Joseph. 1978. Rhizobium strain identification in Arachis hypogaea nodules by enzyme-linked immunosorbent assay (ELISA). Can. J. Microbiol. 24:1537-1543.

The ELISA technique was first used to identify Rhizobium strains in this report by Kishinevsky and Bar-Joseph (1978), working with peanut nodule bacteria (Rhizobium spp.). Strain identification was based on a double antibody sandwich ELISA for the detection of particulate antigen in the form of both vegetative and bacteroid peanut rhizobia. The technique involved the following sequence: (1) non-specific adsorbtion of specific rabbit IgG antibody to plastic wells of microtiter plates; (2) specific binding of test rhizobia to the adsorbed antibody; (3) specific binding of alkaline phosphatase labeled rabbit IgG antibody to the bound rhizobia; (4) detection of the presence of bound alkaline phosphatase by conversion of p-nitrophenyl phosphate substrate to yellow p-nitrophenol and quantitating by colorimetric measurement at 405 nm. The specific IgG preparations used for both sensitizing antibody and specific conjugated antibody were purified from antiserum by diethylaminoethyl-cellulose column chromatography and enzyme-antibody conjugation was accomplished by a one step glutardialdehyde method. The conjugation reaction product was not further purified before use except for dialysis to remove unreacted

glutardialdehyde.

Using the procedure described, Kishinevsky and Bar-Joseph were able to detect homologous reactions with heat treated (100°C, 30 min.) Rhizobium suspensions at dilutions as low as 10^5 cells per mL. They were also able to identify homologous crushed nodule antigen; found that root tissue did not interfere with the test; and confirmed the presence of nodules inhabited simultaneously by two serologically distinct Rhizobium strains. The detection and identification of rhizobia cells at 10^5 cells per mL is a sensitivity factor increase of approximately 10^4 over agglutination techniques and 10^5 over immunodiffusion techniques (Kishinevsky and Bar-Joseph 1978; Dudman and Brockwell 1968; Vincent 1970).

2.4.3 Berger, J.A., May, S.N., Berger, L.R. and Bohlool, B.B. 1979. Colorimetric enzyme-linked immunosorbent assay for the identification of strains of Rhizobium in culture and in the nodules of lentils. Appl. and Environ. Microbiol. 37:642-646.

Berger et al. used an indirect form of the ELISA test to identify strains of Rhizobium leguminosarum. This form of the ELISA had the following sequence of events: (1) non-specific attachment of rhizobial antigen to wells in glass agglutination slides by heat fixing; (2) treatment of attached antigen with bovine gamma globulin to eliminate subsequent non-specific attachment of specific antibodies; (3) specific binding of rabbit anti-Rhizobium antibody to Rhizobium antigen; (4) specific binding of alkaline phosphatase conjugated

sheep anti-rabbit antibodies to anti-Rhizobium rabbit antibodies; (5) detection of bound alkaline phosphatase by conversion of p-nitrophenyl phosphate to yellow p-nitrophenol and quantitating by colorimetric measurement at 400 nm.

Berger et al. indicated complete agreement between ELISA and immunofluorescence results in their experiments which identified both vegetatively grown and bacteriod rhizobia, although their results indicate far less quantitative differences between positive and negative colorimetric ELISA values than were reported for the Kishinevsky and Bar-Joseph experiments. The Berger et al. report does not contain an ecological study of Rhizobium and is confined to a description of technique.

2.4.4 Morley, S.J. and D.G. Jones. 1980. A note on a highly sensitive modified ELISA (enzyme-linked immunosorbent assay) technique for Rhizobium strain identification. J. Appl. Bacteriol. 49:103-109.

This report describes an increase in ELISA sensitivity to Rhizobium strains obtained through the substitution of a fluorescent substrate (3-O-methylfluorescein phosphate) in place of p-nitrophenyl phosphate. Alkaline phosphatase activity was measured as an increase in fluorescence as the substrate was hydrolysed enzymatically to the more fluorescent 3-O-methylfluorescein. Fluorescence was quantitated

with a spectrophotofluorimeter. The sequence of events for this fluorescent ELISA was that of the "double antibody sandwich" described for Rhizobium by Kishinevsky: (1) non-specific attachment of sensitizing antibody to polystyrene wells in microtiter plates; (2) specific attachment of rhizobial antigen; (3) specific attachment of alkaline phosphatase labeled anti-Rhizobium antibody; (4) detection and quantitation of alkaline phosphatase activity by enzyme activity on substrate.

Morley and Jones made quantitative comparisons between the fluorescent and colorimetric substrate sensitivities and reported the fluorescent substrate to be superior in terms of detection sensitivity. They report Rhizobium trifolii detection at 10^4 cells per mL with the fluorescent substrate and at 10^8 cells per mL with the PNPP substrate. All comparisons are based on only one strain-antibody relationship, however, and it should be recalled that Kishinevsky and Bar-Joseph (1978) reported strain detection at 10^5 cells per mL using the PNPP substrate. This Morley and Jones report does not describe an ecological study of Rhizobium and is confined to a technical comparison of ELISA substrates for the detection of alkaline phosphatase.

2.4.5 Kishinevsky, B. and D. Gurfel. 1980. Evaluation of enzyme-linked immunosorbent assay (ELISA) for serological identification of different Rhizobium strains. J. Appl. Bacteriol. 49:517-526.

This ELISA-Rhizobium study by Kishinevsky and Gurfel is an evaluation of the applicability of the "double antibody sandwich" ELISA with PNPP substrate (see Kishinevsky and Bar-Joseph 1978) to strains of R. trifolii, R. leguminosarum, R. meliloti, R. lupini, and R. spp. (peanut rhizobia). The results indicate suitability of the procedure for each Rhizobium species with minimum antigen concentration detection limits of about 10^5 vegetative cells per mL or from 80 to 800 ug of wet nodule tissue per well. No antigen common to all strains was found.

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

Hodgson and Waid used the ELISA method of Berger et al. 1979, (described previously), to identify R. trifolii strains in nodules. Little data was presented in this poster synopsis to support the authors' conclusion that bacteriocinogenic strains have a competitive nodulating advantage over non-bacteriocinogenic strains. While such a suggestion is not illogical, not enough information is presented for the reader to judge. Notably lacking are quantitative data on cross-reactivity of ELISA conjugates, controls, and criteria of nodule strain inhabitancy.

2.4.7 Jones, D.G. and S.J. Morley. 1981. The effect of pH on host plant 'preference' for strains of Rhizobium trifolii using fluorescent ELISA for strain identification. Ann. Appl. Biol. 97:183-190.

Using the fluorescent substrate ELISA technique (Morley and Jones 1980), this study is the first application of ELISA to an ecological study of host nodulation by Rhizobium. The authors describe a three variable experiment in which two R. trifolii strains are examined for competitive nodulation of four clover cultivars growing at three pH levels. The objective of the study was to establish whether or not cultivar differences in "preference" for nodulation by the two strains are affected by the pH of the growth medium. A comparison was also made between Rhizobium growth rates in yeast extract mannitol broth at different pH levels and nodulating ability at the different pH levels.

The authors were able to correlate growth rates of R. trifolii in laboratory media of various pH with nodulating efficiency of the two strains at the various pH levels, but found differences in the effect of pH on nodulation between cultivars. This result indicated that environmental factors (such as pH) may affect the Rhizobium-host recognition process in different ways for different bacterial strains and plant cultivars. The authors conclude that acid tolerant Rhizobium should be sought to achieve effective nodulation in acid soils and that the strain-cultivar relationship must not be overlooked in the selection of Rhizobium strains for inoculant use.

3. MATERIALS AND METHODS

3.1 Buffers

All buffers were prepared shortly before use and ajusted to desired pH with either HCl or NaOH as required. Buffers for all chromatography procedures were deaerated immediately before use by vacuum.

3.1.1 Phsophate Buffered Saline (PBS)

Phosphate buffered saline (pH 7.2) containing 0.02% NaN_3 was used for all cell washings and suspensions (except plant inoculation suspensions) and for all antisera dilutions including antibody-enzyme conjugate dilutions. This buffer was also used for all exclusion gel chromatography. Cells for use in the immunization of rabbits were washed in this buffer omitting NaN_3. With the addition of Tween 20 (0.05%), this buffer was also used for all ELISA wash procedures and for suspension of bacteroids from plant nodule squashes. Constitution of the buffer was, per liter distilled water: NaCl, 7.65 g; Na_2HPO_4, 1.27 g; NaH_2PO_4, 0.10 g; KH_2PO_4, 0.21 g; NaN_3, 0.20 g.

3.1.2 Antibody Sensitization Buffer

An antibody sensitization buffer or "coating buffer" was used to facilitate antibody deposition to the polystyrene wells in the first step of the ELISA double antibody sandwich technique. This was a carbonate-bicarbonate buffer (pH 9.6) containing, per liter distilled water: Na_2CO_3, 1.59 g; $NaHCO_3$, 2.93 g; NaN_3, 0.20 g. It was prepared fresh for each use. Later procedures replaced this buffer with PBS as described in 3.1.1 without affecting results.

3.1.3 DEAE Affi-Gel Blue Buffer

Affinity chromatography buffer (pH 8.0) for elution of IgG from DEAE Affi-Gel Blue consisted of, per liter distilled water: TRIS-HCl, 2.42 g; NaCl, 1.64 g.

3.1.4 ELISA Substrate Buffer

Diethanolamine substrate buffer (12%) for detection of alkaline phosphatase conjugates consisted of 116 mL diethanolamine; 800 mL distilled water; 100 mg $MgCl_2 \cdot 6H_2O$; 0.20 g NaN_3. Six molar HCl was added to a pH of 9.6 and the total volume brought to 1000 mL. Storage was at room temperature. The alkaline phosphatase substrate, p-nitrophenylphosphate, was added at 0.6 mg/mL immediately before use.

3.1.5 Plant Inoculation Buffer

Phosphate buffered peptone solution was used for dilution and suspension of R. meliloti strains for the inoculation of alfalfa plants in the competition study. The composition of this buffer was, per liter distilled water: peptone, 1.0 g; KH_2PO_4, 0.34 g; K_2HPO_4, 1.21 g. Autoclaved pH was 7.0 $\pm$ 0.1. This buffer was the standard diluting and inoculating buffer used with live Rhizobium in the Canadian Inoculant Testing Program.

3.2 Growth Media

3.2.1 Rhizobium Culture Media

Yeast extract mannitol broth (YEMB) used for cell culture contained per liter distilled water: yeast extract, 2.0 g; mannitol, 10.0 g; K_2HPO_4, 0.5 g; NaCl, 0.2 g; $CaSO_4 \cdot 2H_2O$, 0.2 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; H_3BO_3, 1.0 mg; $ZnSO_4 \cdot 7H_2O$, 1.0 mg; $CuSO_4 \cdot 5H_2O$, 0.5 mg; $MnCl_2 \cdot 4H_2O$, 0.5 mg; $Na_2MoO_4 \cdot 2H_2O$, 0.1 mg; Fe-EDTA, 10.0 mg. The pH was 6.8 after autoclaving. For solid media, agar was added to 1.5% (YEMA). Plate counts of commercial inoculants used YEMA supplemented with 7.5 ppm rose bengal and 20 ppm cycloheximide.

3.2.2 Plant Nutrient Solution

Nitrogen free nutrient solution for hydroponic culture of alfalfa host plants used in the competition study contained, per

liter distilled water: KCl, 0.5 g; KH_2PO_4, 0.2 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $CaSO_4 \cdot 2H_2O$, 0.2 g; H_3BO_3, 1.0 mg; $ZnSO_4 \cdot 7H_2O$, 1.0 mg; $CuSO_4 \cdot 5H_2O$, 0.5 mg; $MnCl_2 \cdot 4H_2O$, 0.5 mg; $Na_2MoO_4 2H_2O$, 0.1 mg; Fe-EDTA, 1.0 mg.

3.3 Organisms

The Balsac strain of R. meliloti was provided by Dr. Lucien Bordeleau, Agriculture Canada, Ste-Foy, Quebec. R. meliloti strains 102F34, 102F51, 102F66, 102F77 and 104A13 were provided by Dr. R.S. Smith, Nitragin Co., Milwaukee, Wisconsin. R. meliloti strains S-25 and YG-1 were provided by Dr. S.M. Lesley, Chemistry and Biology Research Institute, Agriculture Canada, Ottawa, Ontario. R. meliloti strains NRG-43, NRG-61, NRG-118, NRG-185, and NRG-259 were from the culture collection of the Northern Research Group, Agriculture Canada, Beaverlodge, Alberta.

3.4 Cell Culture

Stock cultures were stored on YEMA slants at 4°C with yearly transfers. All cultures were grown aerobically at 30°C. Individual broth cultures were grown in 75 mL YEMB media in 250 mL flasks with rotary shaking on a New Brunswick Gyrotory shaker operating at 180 rpm.

3.5 Antisera Production

Antisera were prepared against vegetative cells of R. meliloti strains NRG-43, NRG-61, NRG-185, NRG-259, and Balsac. Cells for injection were grown in YEMB for 5 days at 30°C and harvested by three fold dilution in PBS followed by centrifugation at 6,000 x g at 4°C. Cells were washed and pelleted three times in PBS, resuspended, steamed at 100°C for 30 minutes and immediately pelleted and given three additional PBS washes. Cells were resuspended to 10^{11} cells/mL and 1 mL cells emulsified with 1 mL of Freund's complete adjuvant. Primary injection was with 0.1 mL under each rear-foot toe pad and 0.25 mL intramuscularly into San Juan rabbits. Six weeks later the rabbits were intravenously administered 0.5 mL of fresh, steamed, washed cells at 10^{11}/mL followed at week 7 and week 8 by 0.25 mL injections. Sera were tested in agglutination reactions and the animals exsanguinated 7 days after the last injection. Antisera were divided and frozen with the working stocks, containing 0.04% NaN_3, kept at 4°C.

Agglutination cross-reactivity was selectively removed by repeated massive adsorptions of the antisera with steamed, washed cells. Cells for adsorption were grown in 5 L batches using sterile air for agitation and provision of O_2. Cells were harvested, washed, steamed and washed again as for injection. Cells of strains shown by agglutination to be cross-reactive with an antiserum were added at 10^{11} cells/mL antiserum and incubated at room temperature for 24 hours

before pelleting. Adsorptions were repeated until agglutination cross-reactions were negative at 1:25 dilution of the antiserum.

Antisera were prepared against alfalfa nodule contents derived from individual inoculations of aseptic plants with R. meliloti strains NRG-43, NRG-61, and NRG-185. Approximately thirty 1-2 mm nodules resultant from each strain were used for each injection. Each strain's nodules were bulked, macerated in 5 mL PBS, and allowed to settle for 1 h to separate bacterial suspensions from major plant debris. The bacteroid-containing supernate was lightly pelleted (400 x g for 20 min.), resuspended in 1 mL PBS and emulsified with 1 mL Freund's adjuvant for the primary injection. Secondary injections followed the schedule described for antisera to vegetative Rhizobium cells and were prepared from fresh nodules. Adsorbtions of antisera prepared against nodule contents were performed with vegetative cells. Bacteroid content of thirty 1-2 mm nodules macerated in 1 mL buffer was estimated by Petroff-Hausser count to be 5×10^8 cells/mL.

3.6 Micro-agglutination Test Procedure

Antisera specificity was determined by agglutination reactions in round bottom microtiter plates (Falcon 3040). Fifty uL of antiserum in serial dilution ranging from 1:100 to 1:6400 were added to each of seven wells in a row, leaving the eighth well for a non-immune serum control. Washed cells (either steamed or unsteamed) were added

(50 uL cells, O.D. 620 = 1.0; 10^9 cells/mL) to each well. Thorough mixing was obtained by gentle vibration against the vortex mixer at low speed. Results were noted after a standard incubation of 2 h at 37°C followed by overnight incubation at 4°C. Positive and negative results were readily determined visually under the magnifying lens of a colony counter. Non-agglutination resulted in a compact 1 mm diameter clump of cells in the bottom center of the well and agglutination in a uniform film of cells covering the bottom curvature of the well.

3.7 Gel Chromatography

3.7.1 Affinity Chromatography (IgG Purification)

IgG was extracted by a single passage of antiserum over DEAE Affi-Gel Blue (Bio-Rad Labs) eluting with 0.02 M TRIS-HCl buffer containing 0.028 M NaCl, pH 8.0, and monitoring effluent absorbance at 280 nm. Protein collected was concentrated to approximately 3 mg/mL (A280 nm = 1.4 = 1 mg/mL) by dialysis against polyethylene glycol followed by dialysis against PBS containing 0.02% NaN_3. Purified IgG was examined for molecular weight homogeneity by gel chromatography, then stored at 4°C.

3.7.2 Exclusion Chromatography

Non-immune sera, immune sera, adsorbed immune sera, IgG, alkaline

phosphatase, and antibody conjugates to each antiserum were examined on AcA Ultrogels (LKB) of appropriate fractionation ranges. Samples were pumped in a downward flow with a varioperpex 2120 pump, detected in a Uvicord S 2138 UV detector at 276 nm (or 206 nm using NaN_3 free eluant), and absorbances recorded on an LKB 2210 strip chart recorder. Fractions were collected on an LKB 2070 Ultrorac fraction collector.

3.8 Alkaline Phosphatase Assay

Alkaline phosphatase activity was measured by absorbance at 410 nm of the yellow color of the p-nitrophenol produced by action of alkaline phosphatase on p-nitrophenyl phosphate (PNPP). The buffer used in conjunction with PNPP to form a standard substrate has been described (sect. 3.1.4). Volumes of substrate and sample (whether alkaline phosphatase or conjugated antibody) and length of incubation were varied as required.

3.9 Antibody-Enzyme Conjugation

Conjugates were prepared by mixing 150 uL akaline phosphatase (10 mg/mL, EIA grade, Boehringer-Mannheim) with an amount of purified IgG preparation calculated to contain 1.5 mg IgG, adjusting the volume to 2.0 mL with PBS, and adding freshly thawed and diluted 12.5% aqueous glutaraldehyde (grade 1, SIGMA) to a final concentration of 0.093% (15 uL). Conjugation for 4 h at room temperature with

slight shaking was followed by immediate gel chromatography (LKB Ultrogel ACA 34, 2.5 x 80 cm) using PBS containing 0.02% NaN_3 as the elution buffer. The protein peak excluded from the gel was used in the ELISA procedure. Conjugates were not concentrated after gel passage, but bovine serum albumin was added to a concentration of 6 mg/mL for both stabilization and blockage of non-specific conjugate binding in the ELISA reaction. Conjugates were stored at $4^{o}C$.

3.10 ELISA Test Procedure

The basic ELISA procedure of Engvall and Perlmann (1971, 1972) modified by Voller et al. (1976) and Clark and Adams (1977) and as described for whole cell Rhizobium strain identification by Kishinevsky and Bar-Joseph (1978) was used with minor modifications.

Microtiter plate (Immulon M129A, Dynatech) wells were coated using purified IgG solution (10 ug/mL, 200 uL/well) in PBS for 3 h at $37^{o}C$. Plates were washed three times with PBS containing 0.05% Tween 20 (250 uL/well) on a Miniwash plate washer (Dynatech). Test antigen was added to the wells (150 uL/well) and incubated for 2 h at $37^{o}C$ followed by overnight incubation at $4^{o}C$. Plates were then washed as before and shaken dry. IgG-alkaline phosphatase conjugate was then added to each well (150 uL/well) and the plates were incubated for 4 h at $37^{o}C$. Conjugate dilution was experimentally

determined for each antigen - conjugate system to give approximately the same A410 nm values at equal cell concentrations. Plates were washed two times with PBS following conjugate incubation followed by two additional washes with phosphate-free saline to eliminate the possibility of free phosphate inhibition of the alkaline phosphatase. Freshly prepared enzyme substrate solution (0.6 mg/mL p-nitrophenylphosphate TRIS buffer salt, Eastman) in 12% aqueous diethanolamine buffer, 1 mM $MgCl_2$, pH 9.8) was added to each well (200 uL/well). Incubation was ordinarily for 1 h at 37°C, but in some cases incubations were shortened or lengthened as required and absorbance results corrected to 1 h by extrapolation. Color development was halted by the addition of 5 M NaOH (50 uL/well) with a Dynadrop SR1 (Dynatech). The absorbance in each well was measured at 410 nm with a plate reader (Micro ELISA minireader MR 590, Dynatech). Positive controls (150 uL of homologous, steamed, washed cells at 10^7 cells/mL) and negative controls (150 uL PBS) were included on every plate. The use of pressure sensitive film plate covers (Falcon 3044) during all incubations minimized erratic results in the outside rows of the microtiter plates.

3.11 Quantitative strain evaluation of commercial inoculants

Commercial peat-base inoculant samples obtained from retail outlets in Alberta and Ontario were tested for R. meliloti strain

content. Inoculants were transported to the Beaverlodge Research Station with control inoculants of known Rhizobium count to ensure that conditions of transport did not affect the microbial population of the samples. All inoculants were tested by the plant infection technique (Vincent 1970) and by plate count on YEMA supplemented with 7.5 ppm rose bengal and 20 ppm cycloheximide. After 4-5 days incubation at 30°C, representative plates from plate count tests were selected for serological evaluation and each colony evaluated by microplate agglutination and by ELISA. The ratio of strain identified colonies to total colonies was determined and used to calculate the number of rhizobia of specific strain/g inoculant. ELISA values for strain NRG-43 were not obtained because we were unable to produce a functioning ELISA conjugate specific to strain NRG-43.

For agglutination tests, colonies were individually picked and streaked on YEMA slants. After 3 days growth at 30°C, cells were suspended by gentle vortexing in 1.0 mL PBS, transferred to 1.5 mL disposable centrifuge tubes (Eppendorf) and pelleted in a micro-centrifuge (Eppendorf model 5412). Cells were steamed, washed and pelleted three times and resuspended to an approximate O.D. 620 nm = 1.0; 10^9 cells/mL. Washed cells were then tested against appropriate antisera and a non-immune serum control by adding cells (50 uL/well) derived from a single colony to a single dilution (1:150) of anti-serum (50 uL/well). Cells and antisera were thoroughly mixed prior

to incubation by gentle vibration on a vortex mixer.

For the ELISA reaction whole colonies (approximately 1-2 mm diameter) were picked as completely as possible and each suspended in 1.0 mL PBS in 1.5 mL disposable centrifuge tubes, steamed, pelleted and washed three times with PBS in the microcentrifuge. They were finally suspended in 0.75 mL PBS with 0.05% Tween 20 and used directly in the ELISA procedure.

3.12 Nodulation Competition Study Between Two *R. meliloti* Commercial Strains

3.12.1 Design

This study was designed to identify, by means of ELISA, the nodule inhabitant strains resultant from the simultaneous inoculation of aseptic alfalfa seedlings with varying numerical ratios of two commercially used *R. meliloti* strains. The possibility of dual strain inhabitation of single nodules was also under examination.

The study was established with eight treatments consisting of varying ratios of the inoculating strains Balsac and NRG-185 as follows:

Treatment	Total rhizobia/unit
T1 - Balsac alone	approx. 6×10^4
T2 - NRG-185 to Balsac, 1:100	" 6×10^4
T3 - NRG-185 to Balsac, 1:10	" 6×10^4
T4 - NRG-185 to Balsac, 1:1	" 6×10^4
T5 - NRG-185 to Balsac, 10:1	" 6×10^4
T6 - NRG-185 to Balsac, 100:1	" 6×10^4
T7 - NRG-185 alone	" 6×10^4
T8 - No inoculation	none

Each treatment was replicated four times.

3.12.2 Plant Growth Units

Medicago sativa (var. Beaver) was used exclusively as legume host. Seed was surface sterilized by twenty minute immersion in concentrated sulfuric acid followed by six washes in sterile distilled water. Seed was then germinated on water agar and sown two per unit in autoclaved, cotton plugged 21 x 3 cm glass test tubes containing 4.0 g vermiculite and 25 mL plant nutrient solution. The pH of the autoclaved growth units immediately prior to seeding was 6.4. Plants were grown for the duration of the test in a Controlled Environment (model E8H) chamber at 65-70% relative humidity with 16 h light and 8 h darkness.

3.12.3 Inoculation

Each plant growth unit (except those in the non-inoculated treatment number eight) received a total of 6×10^4 R. meliloti suspended in 1 mL phosphate peptone solution (PPS) regardless of relative ratios of the two strains. Plant units in the nil treatment eight received 1 mL sterile PPS at the time of inoculation of the other units. Plants were inoculated on the fifth day after germination. Inoculating suspensions were prepared by diluting 4 day YEMB cultures of the two strains with PBS to the same A620 nm cell density ($A_{620\ nm} = 0.065$) and verifying that Petroff-Hauser and Helber counts were equal ($A_{620\ nm} = 0.065 = 6 \times 10^7$ organisms/mL). The two cell suspensions were then diluted to 6×10^4 organisms/mL and actual inoculating doses prepared by mixing the strains at this level according to the ratios corresponding to the treatments applied.

3.12.4 Nodule Harvest

Nodules were harvested from all plant units eleven weeks after inoculation. Nodules from both plants in a growth unit were bulked and then arranged in visual order of size on PBS dampened filter paper in petri dishes. Each nodule was then placed in 0.60 mL PBS containing 0.05% Tween 20 in individual 10 x 75 mm disposable test tubes and crushed with 6 mm diameter rounded end glass rods. Two

150 uL portions of each bacteroid-plant debris suspension were then transferred to antibody coated microtiter plate wells; one in the anti-Balsac antibody ELISA format, the other in the anti-NRG-185 antibody ELISA format. No attempt was made to segregate plant debris and bacteroids although the larger bits of plant debris settled to the bottom of the tubes and were allowed to remain there. Nodules were taken in order of size to attempt a later correlation between nodule size and enzyme substrate absorbance results. All nodules were tested against both antisera.

4. RESULTS

4.1 Agglutination results for antisera prepared against vegetative, pure culture, R. meliloti cells

The non-immune sera of rabbits subsequently used to produce anti-Rhizobium antibodies showed little tendency to agglutinate six R. meliloti strains (Table 2). The slight tendency of strain NRG-185 to agglutinate in weakly diluted non-immune sera (Table 2) required that a higher dilution of non-immune sera be used in negative control suspensions. A final dilution of 1:200 non-immune sera from a single rabbit was arbitrarily selected and subsequently used for negative control in all agglutination testing.

Antisera raised against each of the six strains showed cross-reactivity in agglutination testing (Table 3). The fact that each of the six antisera agglutinated strains NRG-43 and NRG-185 indicated that a common antigen(s) exists among the strains. Antisera raised against strains NRG-259 and NRG-260 produced identical agglutination patterns when tested against the six strains, and only these two antisera agglutinated either NRG-259 or NRG-260 (Table 3). The apparent identity between strains NRG-259 and NRG-260 was confirmed when cross-adsorption of either antiserum with cells used to raise the other completely removed agglutinability (Table 4). Strains NRG-259 and NRG-260 were both ineffective (Nod^+, Nif^-) and were collected from alfalfa nodules taken from the

Table 2. Agglutination reactivity of pre-immune sera .

Antigen	Reciprocal titer of sera					
	NRG-43	NRG-61	NRG-185	NRG-259	NRG-260	Balsac
NRG-43	nr*	nr	nr	nr	nr	nr
NRG-61	nr	nr	nr	nr	nr	nr
NRG-185	nr	nr	10	nr	nr	10
NRG-259	nr	nr	nr	nr	nr	nr
NRG-260	nr	nr	nr	nr	nr	nr
Balsac	nr	nr	nr	nr	nr	nr

*nr - No reaction at 1:10 dilution of sera.

Table 3. Agglutination cross-reactivity of unadsorbed antisera

Antigen	Reciprocal titer of antisera					
	NRG-43	NRG-61	NRG-185	NRG-259	NRG-260	Balsac
NRG-43	1600	1600	1600	400	400	3200
NRG-61	50	1600	50	nr	nr	nr
NRG-185	1600	50	1600	50	50	1600
NRG-259	nr*	nr	nr	1600	1600	nr
NRG-260	nr	nr	nr	800	800	nr
Balsac	nr	nr	50	nr	nr	1600

*nr - No reaction at 1:25 dilution of antisera.

Table 4. Agglutination reactivity of antisera adsorbed against all cross-reacting strains

Antigen	Reciprocal titer of antisera					
	NRG-43	NRG-61	NRG-185	NRG-259	NRG-260	Balsac
NRG-43	1600	nr	nr	nr	nr	nr
NRG-61	nr*	1600	nr	nr	nr	nr
NRG-185	nr	nr	1600	nr	nr	nr
NRG-259	nr	nr	nr	nr**	nr	nr
NRG-260	nr	nr	nr	nr	nr**	nr
Balsac	nr	nr	nr	nr	nr	1600

*nr - No reaction at 1:25 dilution of antiserum.

** Antisera to strains NRG-259 and NRG-260 retained agglutinability to both strains at 1:1600 dilution until adsorbed with cells of either NRG-259 or NRG-260.

same field on the same day. For further work, it was assumed that NRG-259 and NRG-260 were the same strain and experiments were carried out with strain NRG-259 only.

Cross-reactivity was selectively removed from each antiserum by repeated massive adsorbtions with cells of cross-reacting strains. This resulted in strain specificity without loss in specific titer (Table 4). Adsorbtion of any of the antisera with homologous cells resulted in complete loss of agglutinability. Each of R. meliloti strains NRG-43, NRG-61, NRG-185, NRG-259-260; and Balsac possessed sufficient individual antigenic character for serological strain identification provided that the antisera used were suitably adsorbed against heterologous cells.

Addition of mercaptoethanol (to 0.1M) to the five antisera had no effect on homologous agglutination titer suggesting that the agglutination antibody present in the antisera is of the IgG class. This was confirmed by demonstrating retention of specific agglutinability in the IgG fractions purified from the respective antisera by $(NH_4)_2SO_4$ precipitation, affinity chromatography, and gel chromatography.

4.2 Agglutination results for antisera prepared against bulked mascerated alfalfa nodules of single strain origin

Antisera raised by injection of bulked single strain nodule contents

were capable of agglutinating homologous vegetatively grown R. meliloti cells and showed patterns of cross-reactivity similar to those found for antisera raised against vegetative cells. Adsorbtion of these nodule-produced antisera with cross-reacting vegetative cells produced strain specific antisera (Table 5). The low titers of these antisera were thought to reflect the small quantity of antigen available for injection. No attempt was made to separate plant material, bacteroids, and vegetative type cells from within the nodules prior to injection which precludes the assumption that antibody raised to nodule contents was due to bacteroid antigen alone.

4.3 Chromatography of IgG-alkaline phosphatase conjugates

Conjugate reaction mixes were preparatively chromatographed on Ultrogel AcA 34 (2.5 x 80 cm column) following the conjugation procedures in order to removed unbound IgG, free alkaline phosphatase, and glutaraldehyde.

4.3.1 Calibration of the column

The exclusion volume of the column was determined using blue dextran (M.W. = 2×10^6 daltons) monitoring effluent fractions at 280 nm. Catalase (M.W. = 240,000 daltons) and aldolase (M.W. = 158,000 daltons)

Table 5. Agglutination reactivity of antisera raised against single strain nodule contents before and after adsorbtion with cross-reacting strains

Antigen	Reciprocal titer of antisera					
	Before adsorbtion			After adsorbtion		
	NRG-43	NRG-61	NRG-185	NRG-43	NRG-61	NRG-185
NRG-43	400	nr	200	400	nr	nr
NRG-61	200	200	50	nr	200	nr
NRG-185	400	25	400	nr	nr	400

standards were run to define the position at which IgG and alkaline phosphatase should elute (Fig. 1). Purified IgG and EIA grade alkaline phosphatase eluted in the same position as the aldolase standard.

4.3.2 Chromatography of conjugate reaction products

The entire reaction mixture for each antibody-enzyme conjugate was chromatographed on the Ultrogel AcA 34 column. The molecular weight distribution pattern shown in Fig. 2 for the Balsac antibody-enzyme conjugate mixture was typical of the others. In each case unreacted protein showing alkaline phosphatase activity was found (fractions 150-160). The excluded peak (nominally + 350,000 M.W. material) was pooled for use in the ELISA procedure. Chromatography of a sample of the pooled excluded peak from Fig. 2 on Bio-gel A 50 M (nominal fractionation range = $1 \times 10^5 - 50 \times 10^6$ daltons) showed a range of molecular weights ranging from 2×10^6 to approximately 350,000 daltons.

Under the conditions of conjugation used in these experiments only a relatively small proportion of the total alkaline phosphatase activity is found in the peak excluded from the gel. For the Balsac conjugate shown in Fig. 2 the area under the alkaline phosphatase activity curve for fractions 84-94 equals 20.1% of the total activity and this was typical of the other conjugate reaction mixes.

FIGURE 1

ELUTION PROFILE OF STANDARDS ON AN
LKB AcA 34 GEL FILTRATION COLUMN

Three standards were individually applied to an AcA 34 column (2.5 x 80 cm) of nominal M.W. fractionation range 20,000 to 350,000 daltons. The column was equilbrated and eluted with PBS and eluate monitored at 280 nm.

I = blue dextran M.W. = 2,000,000 daltons

II = catalase M.W. = 240,000 daltons

III = aldolase M.W. = 158,000 daltons

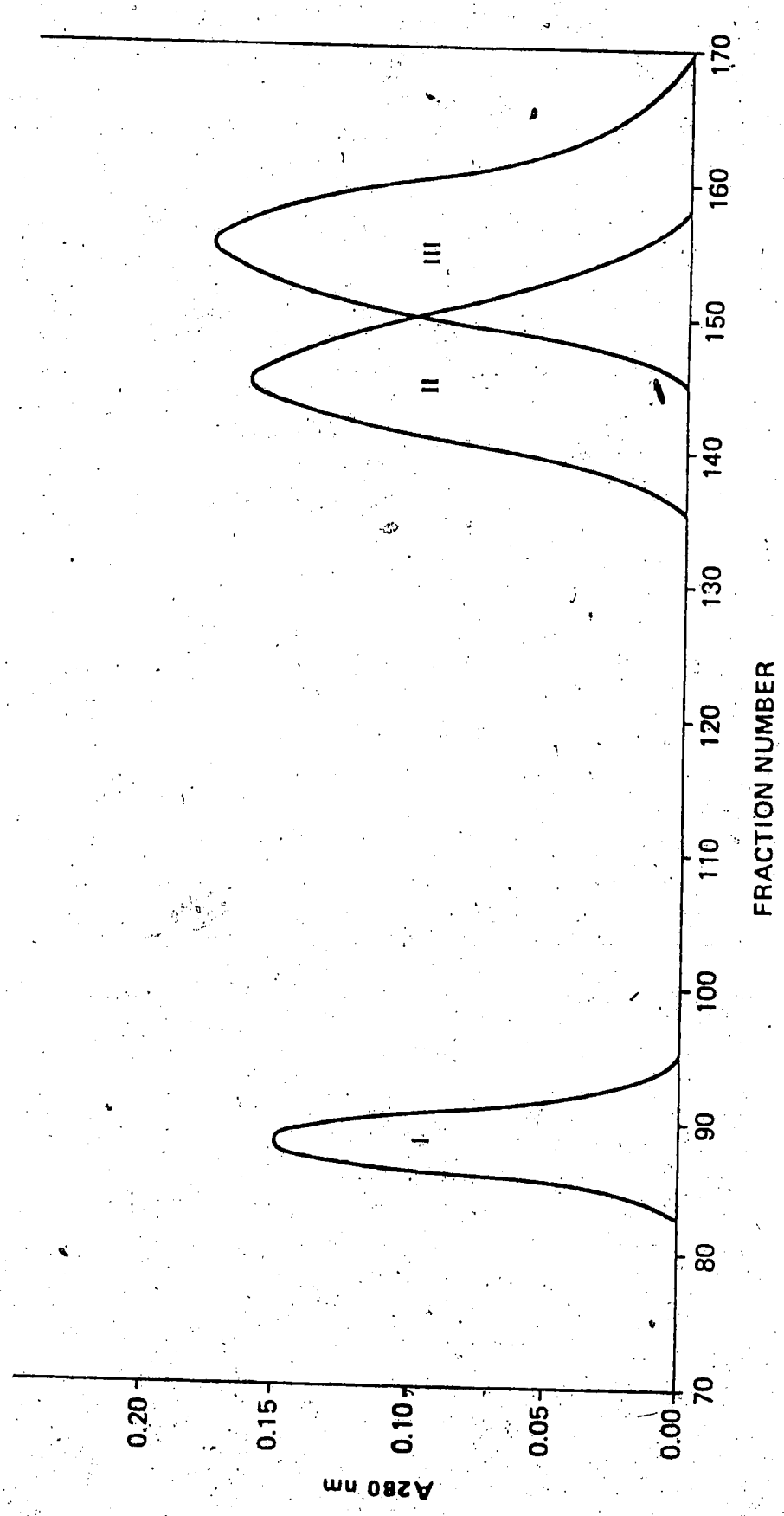

A280 nm
0.00
0.05
0.10
0.15
0.20
70
80
90
100
110
120
130
140
150
160
170
FRACTION NUMBER
I
II
III

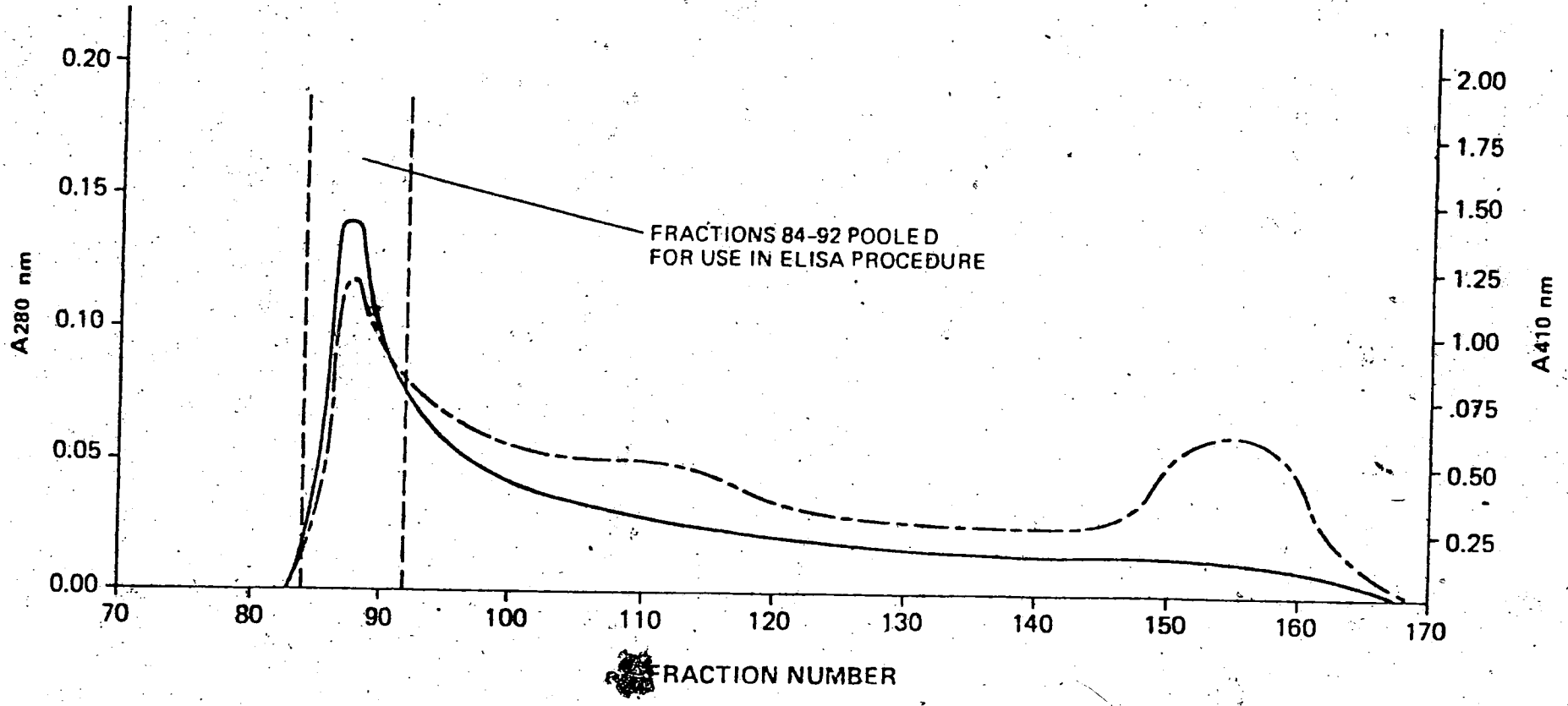
FIGURE 2

ELUTION PROFILE OF ANTIBODY-ENZYME CONJUGATION REACTION MIX ON AN AcA 34 GEL

Anti-Balsac IgG and alkaline phosphatase were mixed and allowed to react as described in Materials and Methods. The entire reaction mix was then chromatographed on the AcA 34 gel filtration column described by Fig. 1. Eluate was monitored at 280 nm. Fractions were collected (1.5 mL) and measured for alkaline phosphatase activity (A_{410}nm).

——— = A_{280}^{nm}

— - — = A_{410}^{nm}


0.20
0.15
0.10
0.05
0.00
A280 nm
2.00
1.75
1.50
1.25
1.00
.075
0.50
0.25
A410 nm
FRACTIONS 84-92 POOLED
FOR USE IN ELISA PROCEDURE
70
80
90
100
110
120
130
140
150
160
170
FRACTION NUMBER

4.4 ELISA reactivity of five R. meliloti antibody-enzyme conjugates

4.4.1 Determination of sensitizing antibody concentration

The effect of varying the sensitizing antibody concentration on the A_{410}nm value generated in the final step of the ELISA reaction is shown in Fig. 3. Enzyme activities of the conjugates were equalized before use. The differences in overall A_{410}nm values generated by the various ELISA conjugates therefore reflects differences in antibody affinity and/or antibody-antigen concentrations rather than in amounts of conjugated enzyme available. It can be seen from Fig. 3 that sensitizing antibody concentrations greater than 10 ug/mL were wasteful of the sensitizing antibody preparation. Periods of sensitizing antibody incubations in excess of 4h did not increase the final A_{410}nm values seen, in fact, overnight sensitizing incubations appeared to lead to widely erratic final A_{410}nm values among replicate samples. To minimize variation, a sensitizing antibody concentration of 10 ug/mL and an incubation period of 3h was subsequently used in all ELISA experiments.

4.4.2 Effect of antigen concentration (cells/mL) on ELISA response of five antibody-enzyme conjugates

The specificity of each conjugate with homologous and heterologous

FIGURE 3

EFFECT OF INCREASING SENSITIZING ANTIBODY CONCENTRATION ON THE ELISA A_{410}^{nm} RESULT FOR FIVE HOMOLOGOUS SENSITIZING ANTIBODY-ANTIGEN-CONJUGATE SYSTEMS

I = anti NRG-259 system

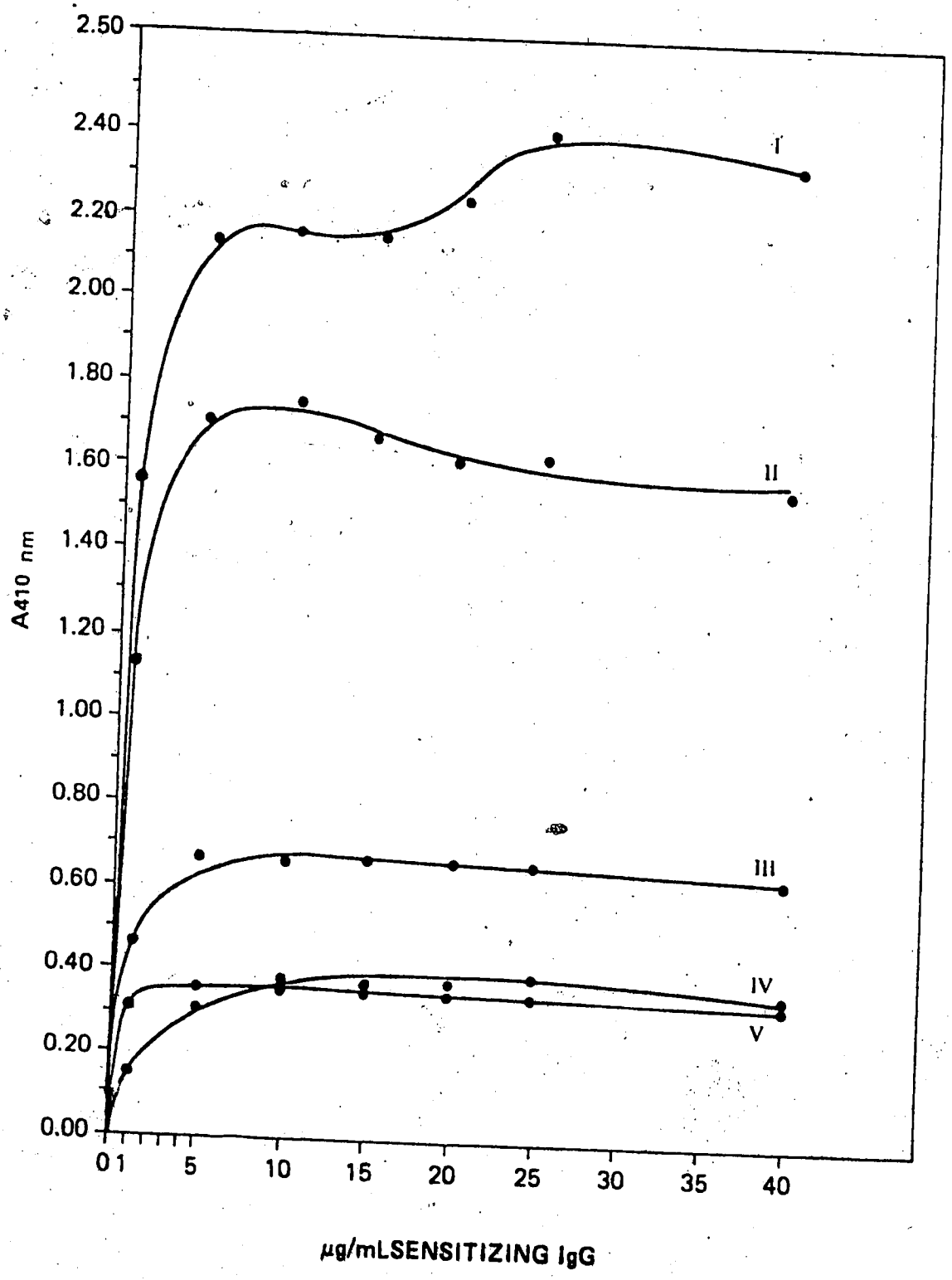
II = anti NRG-61 system

III = anti Balsac system

IV = anti NRG-185 system

V = anti NRG-43 system

Conjugates were adjusted to equal alkaline phosphatase activities before use. The antigens were homologous whole cells at 10^8 cells per ml.


2.50
2.40
2.20
2.00
1.80
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
A410 nm
I
II
III
IV
V
0 1 5 10 15 20 25 30 35 40
µg/mLSENSITIZING IgG

cells at five concentrations is shown in Fig. 4 A-E. The conjugate prepared against strain NRG-43 did not give a significant A_{410} nm response even at 10^8 cells/mL (Fig. 4, E). Variation in sensitizing antibody and/or conjugate concentration failed to provide a specific ELISA response to strain NRG-43 as did antibody and conjugates prepared with adsorbed anti-NRG-43 IgG from two other rabbits.

Anti-NRG-61 conjugate bound to the plate wells in a uniform, but non-specific manner as shown by the high background A_{410} nm values seen in the control wells receiving no antigen (Fig. 4). Despite this non-specific binding, a high degree of specific binding was seen when this conjugate was used against strain NRG-61 (Fig. 4, B). The non-specific binding of NRG-61 conjugate was subsequently reduced to a low level by adding additional bovine serum albumin to the conjugate (10 mg/mL). This level of BSA apparently provided a high BSA to conjugate ratio allowing the BSA to effectively out-compete for non-specific protein binding sites on the polystyrene.

Despite low homologous levels of Balsac and NRG-185 ELISA response (relative to the homologous responses of conjugates to NRG-259 and NRG-61), very little cross-reactivity was seen between strains Balsac and NRG-185 with either conjugate (Fig. 4, C & D). This allowed for the ELISA distinction between these two strains in a microbiologically defined study of competition for nodule formation between them (see section 4.5).

FIGURE 4

EFFECT OF ANTIGEN CONCENTRATION ON ELISA RESPONSE OF HOMOLOGOUS AND HETEROLOGOUS ANTIBODY SYSTEMS

The A_{410}^{nm} ELISA response of each of the five antibody systems is shown in relation to four antigen concentrations and a no antigen control (NC) for:

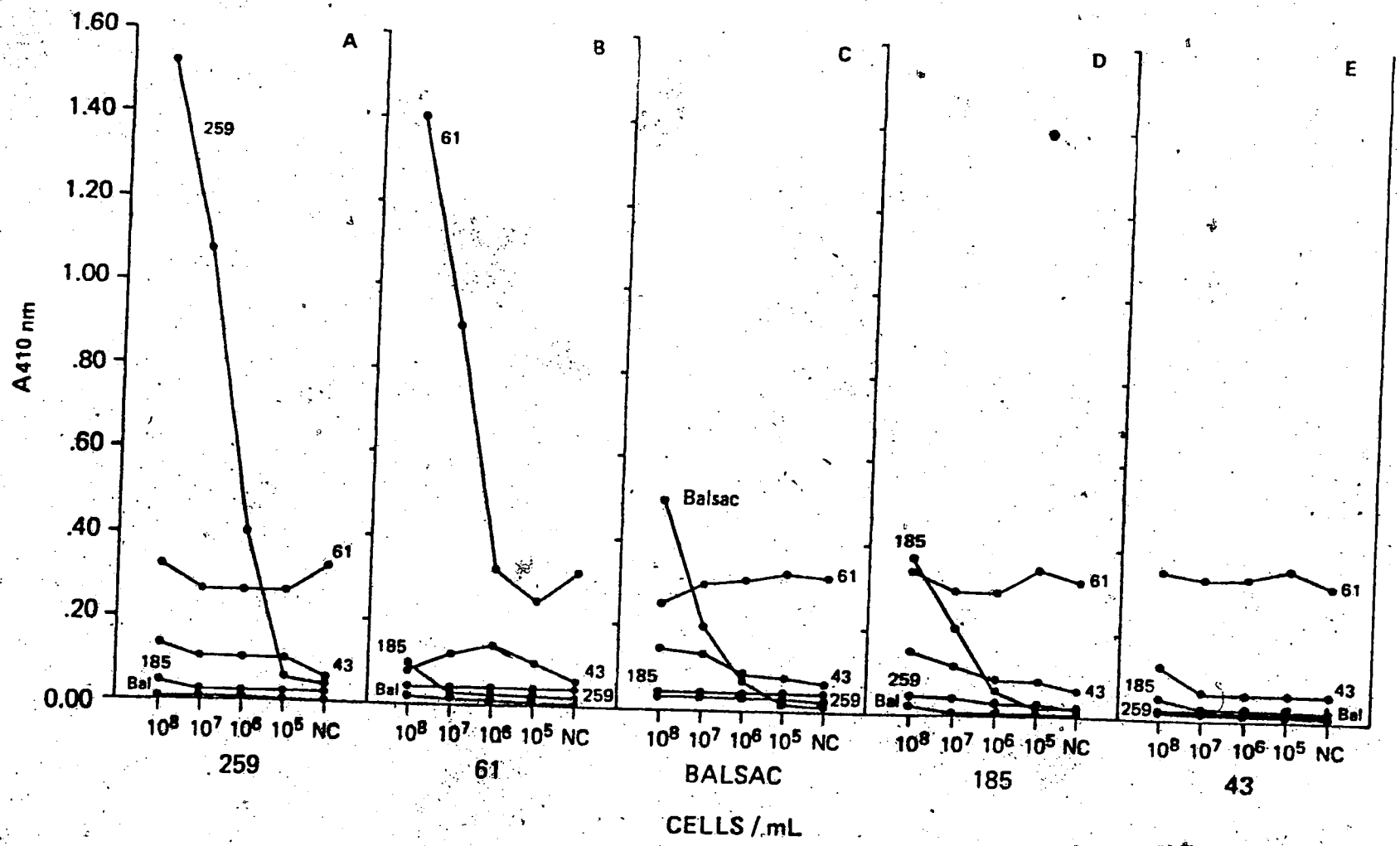
A = NRG-259 cells

B = NRG-61 cells

C = Balsac cells

D = NRG-185 cells

E = NRG-43 cells


A410 nm
1.60
1.40
1.20
1.00
.80
.60
.40
.20
0.00
A
B
C
D
E
259
61
Balsac
185
43
Bal
10^8 10^7 10^6 10^5 NC
259
61
BALSAC
185
43
CELLS / mL

An experiment was performed to determine the degree of ELISA specifity exhibited by the five conjugates when tested against R. meliloti strains not used for adsorbtion of antisera prior to conjugate production. No cross-reactivity was found, indicating that the specific identifying antigens of strains NRG-61, NRG-185, NRG-259 and Balsac were not present in any of the other strains (Table 6).

4.5 Nodulation competition study between two commercial Canadian R. meliloti strains

Aseptic alfalfa (var. Beaver) seedlings grown in N free nutrient solution in large glass cotton-plugged tubes (two plants per tube = one unit) were inoculated with seven varying numerical ratios of two R. meliloti strains. Eight treatments were applied in four replicates as follows:

	Treatment	Total rhizobia/unit
T 1	Balsac alone	6×10^4
T 2	NRG-185:Balsac 1:100	6×10^4
T 3	NRG-185:Balsac 1:10	6×10^4
T 4	NRG-185:Balsac 1:1	6×10^4
T 5	NRG-185:Balsac 10:1	6×10^4
T 6	NRG-185:Balsac 100:1	6×10^4
T 7	NRG-185 alone	6×10^4
T 8	No inoculation	none

Table 6. Specificity of antibody-enzyme conjugates tested against *R. meliloti* strains with the ELISA procedure

Strain	ELISA antibody-enzyme conjugate				
	NRG-43	NRG-61	NRG-185	NRG-259	Balsac
NRG-43	.05*	.05	.02	.03	.06
NRG-61	.04	1.41	.07	.06	.05
NRG-185	.03	.03	2.19	.06	.04
NRG-259	.03	.03	.07	3.87	.03
Balsac	.06	.04	.07	.06	2.06
NRG-118	.06	.02	.01	.02	.01
102F34	.05	.01	.01	.02	.02
102F51	.07	.00	.01	.01	.02
102F66	.04	.01	.01	.00	.00
102F77	.04	.01	.01	.02	.01
104A13	.05	.02	.01	.01	.01
S-25	.07	.00	.02	.02	.01
YG-1	.07	.01	.02	.02	.01

* All values are A_{410}nm absorbances obtained with 10^8 vegetative cells and corrected to a substrate incubation period of 1.0 h.

Nodules were harvested from all plant units eleven weeks after inoculation and each nodule individually tested for strain identity against both anti-Balsac ELISA conjugate and anti-NRG-185 ELISA conjugate. Uninoculated control plants (T 8) had no nodules.

The average number of nodules per plant (excluding uninoculated control plants) was 5.75, with a total of 322 nodules formed. These nodules were numerically distributed among treatments and replicates as shown in Fig. 5. While the average number of nodules in replicates appeared to decrease slightly as the numberically dominant inoculating population shifted from the Balsac strain to NRG-185 (T 1-7), the large variability in nodule number between replicate treatments precluded meaningful conclusions.

All nodules were individually macerated in PBS and tested for Rhizobium strain inhabitant identity in the double antibody sandwich ELISA format. Each nodule's content was tested separately against antibody specific to strain NRG-185 and antibody specific to strain Balsac. The resultant A_{410}nm values were determined after 1h substrate incubation and are represented graphically in Fig. 6. Fig. 6 represents the average A_{410}nm value of the four replicates in a treatment (with the average of the extreme replicate in either direction also shown) for both ELISA antibody systems (anti-NRG-185 and anti-Balsac). The average value of the 54 negative controls (receiving PBS in place of nodule antigen) dispersed throughout the eight microtiter plates used in this test was A_{410}nm = 0.037. There were minimal differences

FIGURE 5

NUMBER OF ALFALFA NODULES PRODUCED BY SEVEN VARYING INOCULATION TREATMENT RATIOS IN A COMPETITION STUDY BETWEEN TWO COMMERCIAL INOCULANT STRAINS

Treatments

T 1 Balsac alone

T 2 NRG-185:Balsac, 1:100

T 3 NRG-185:Balsac 1:10

T 4 NRG-185:Balsac 1:1

T 5 NRG-185:Balsac 10:1

T 6 NRG-185:Balsac 100:1

T 7 NRG-185 alone

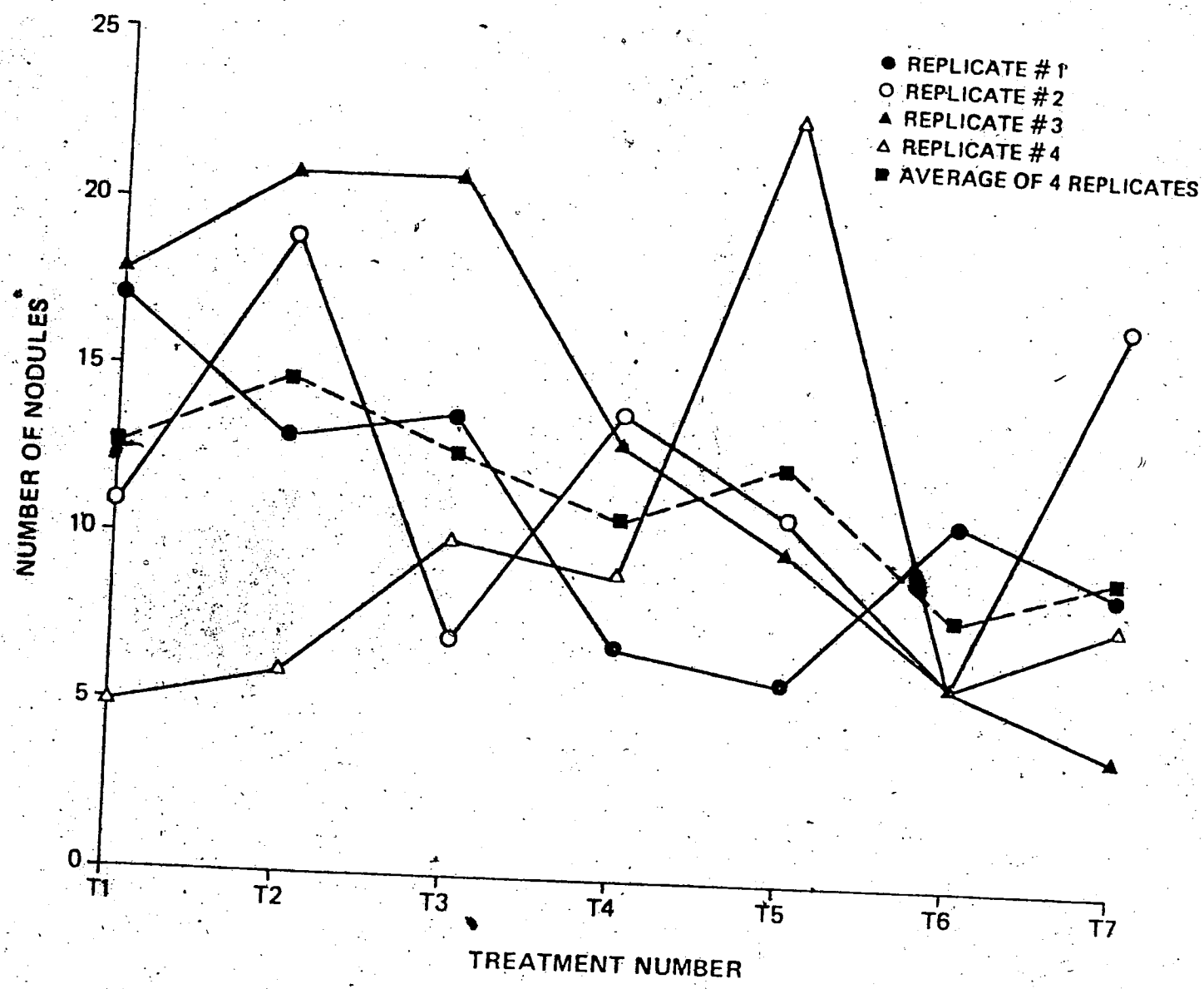

25
20
15
10
5
0
NUMBER OF NODULES
REPLICATE # 1
REPLICATE # 2
REPLICATE # 3
REPLICATE # 4
AVERAGE OF 4 REPLICATES
T1
T2
T3
T4
T5
T6
T7
TREATMENT NUMBER

FIGURE 6

ELISA RESPONSE (A_{410}nm) OF COMPETITION STUDY NODULES TO ANTIBODY CONJUGATES PREPARED AGAINST INOCULATING STRAINS

All nodules (322) were tested against both antibody systems and the average A_{410}nm value generated against each system is shown. The range lines indicate the average A_{410}nm values of the extreme replicates.

	Treatment
T 1	Balsac alone
T 2	NRG-185:Balsac, 1:100
T 3	NRG-185:Balsac 1:10
T 4	NRG-185:Balsac 1:1
T 5	NRG-185:Balsac 10:1
T 6	NRG-185:Balsac 100:1
T 7	NRG-185 alone

between negative control values for the two separate antibody-enzyme conjugate systems.

Expression of A_{410}^{nm} values in terms of strain identity required that a particular minimum A_{410}^{nm} value be selected as indicative of a particular strain's presence. The selection of such a value, while critical to interpretation of results, was somewhat arbitrary due to the fact of great variation in nodule size and thus antigen concentration in the ELISA. Since variation in concentration between 10^5 cells/mL and 10^9 cells/mL has large effects on A_{410}^{nm} value generated (Fig. 4), the determinative value selected had to be small enough to allow identification of the smaller nodules and large enough to avoid false positive identifications. For this study an A_{410}^{nm} value of 0.15 was selected as a reasonable minimum value for allocation of nodule inhabitants to strain categories. This value is four times the background value (negative control = 0.037) and high enough that no nodule in the single strain inoculation treatments (T 1 and T 7) is seen as cross-reactive to the heterologous conjugate. The use of A_{410}^{nm} = 0.15 as the value for lower limit for identification defined eleven nodules of the 322 tested, or 3.4% as unidentifiable (A_{410}^{nm} less than 0.15 with both antisera). The effect of selecting a higher A_{410}^{nm} value as indicative of strain identity is to decrease the number of nodules identified as inhabited by both strains while increasing the number unidentifiable. Selecting a lower value has the opposite effect (Table 7). The relationships between the number of

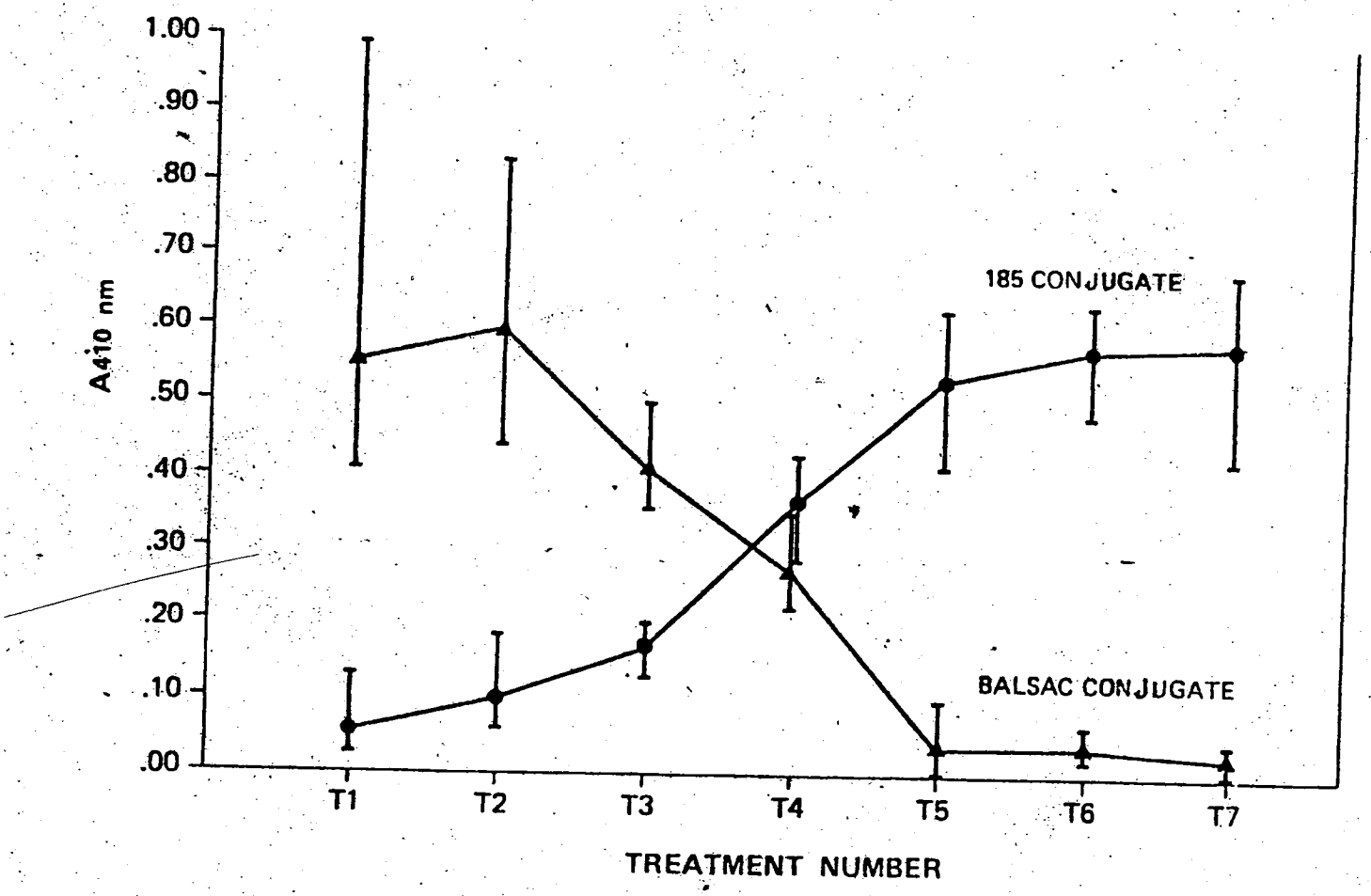

1.00
.90
.80
.70
.60
.50
.40
.30
.20
.10
.00
A410 nm
185 CONJUGATE
BALSAC CONJUGATE
T1
T2
T3
T4
T5
T6
T7
TREATMENT NUMBER

Table 7. Effect of change in lower limit A410nm value taken as indicative of a positive ELISA strain identification on nodule numbers allocated to identification categories

Inoculation Treatment Number	$A_{410}^{nm} > 0.10$				$A_{410}^{nm} > 0.15$			
	Bal[1]	185[2]	DI[3]	U[4]	Bal	185	DI	U
1	48	0	3	0	51	0	0	0
2	45	2	11	1	48	2	8	1
3	25	5	21	1	27	5	19	1
4	13	18	10	2	14	18	9	2
5	6	34	5	5	3	39	2	6
6	1	27	1	0	1	27	1	0
7	0	35	2	1	0	37	0	1
Totals	138	121	53	10	144	128	39	11

	$A_{410}^{nm} > 0.20$				$A_{410}^{nm} > 0.25$			
	Bal	185	DI	U	Bal	185	DI	U
1	49	0	0	2	43	0	0	6
2	52	2	4	1	54	2	1	2
3	30	6	13	3	31	6	12	3
4	16	19	6	2	16	20	5	2
5	1	42	0	7	1	41	0	8
6	1	28	0	0	1	27	0	3
7	0	37	0	1	0	36	0	2
Totals	149	134	23	16	146	132	18	26

[1] Balsac strain.

[2] NRG-185 strain.

[3] Doubly inhabited by Balsac and NRG-185 strains.

[4] Unidentified.

nodules identified as single strain inhabited, double strain inhabited, and strain unidentified, using A_{410}nm = 0.15 as the indicative limit, is shown in Fig. 7. The preponderance of nodules identified as doubly inhabited occurred in treatment 3 where the inoculant ratio was 10:1 in favor of the Balsac strain over strain NRG-185. Comparison of Fig. 7 with Table 7 shows than an increase in the determinative A_{410}nm value from 0.15 to 0.25 would decrease the size, but not the occurrence of the doubly inhabited nodule population peak in treatment 3.

Conversion of the data presented in Fig. 7 to percent of total nodules in a treatment identified by strain reveals a nearly symmetrical relationship between single strain inhabited nodules and inoculating ratios (Fig. 8). Apparently neither inoculating strain had any marked advantage in forming single strain inhabited nodules under the conditions of this test. Single strain inhabited nodules comprised 83.5% of the total. Doubly inhabited nodules, however, were not distributed evenly around the point of equal inoculation (treatment 4). Altogether, 13.0% of the nodules in the experiment were classified as doubly inhabited (using the 0.15 A_{410}nm value as the indicative value). The fact that only treatments 2-6 were inoculated with both strains limits the number of nodules where dual strain inhabitation could occur. Treatments 2-6 had a total of 234 nodules of which 42 or 17.9% were classed as inhabited by both strains. Of the 42 doubly occupied nodules nearly one-half (45.2%) were found in treatment

FIGURE 7

NUMBER OF NODULES STRAIN IDENTIFIED IN EACH COMPETITION STUDY TREATMENT

Treatment	
T 1	Balsac alone
T 2	NRG-185:Balsac, 1:100
T 3	NRG-185:Balsac 1:10
T 4	NRG-185:Balsac 1:1
T 5	NRG-185:Balsac 10:1
T 6	NRG-185:Balsac 100:1
T 7	NRG-185 alone

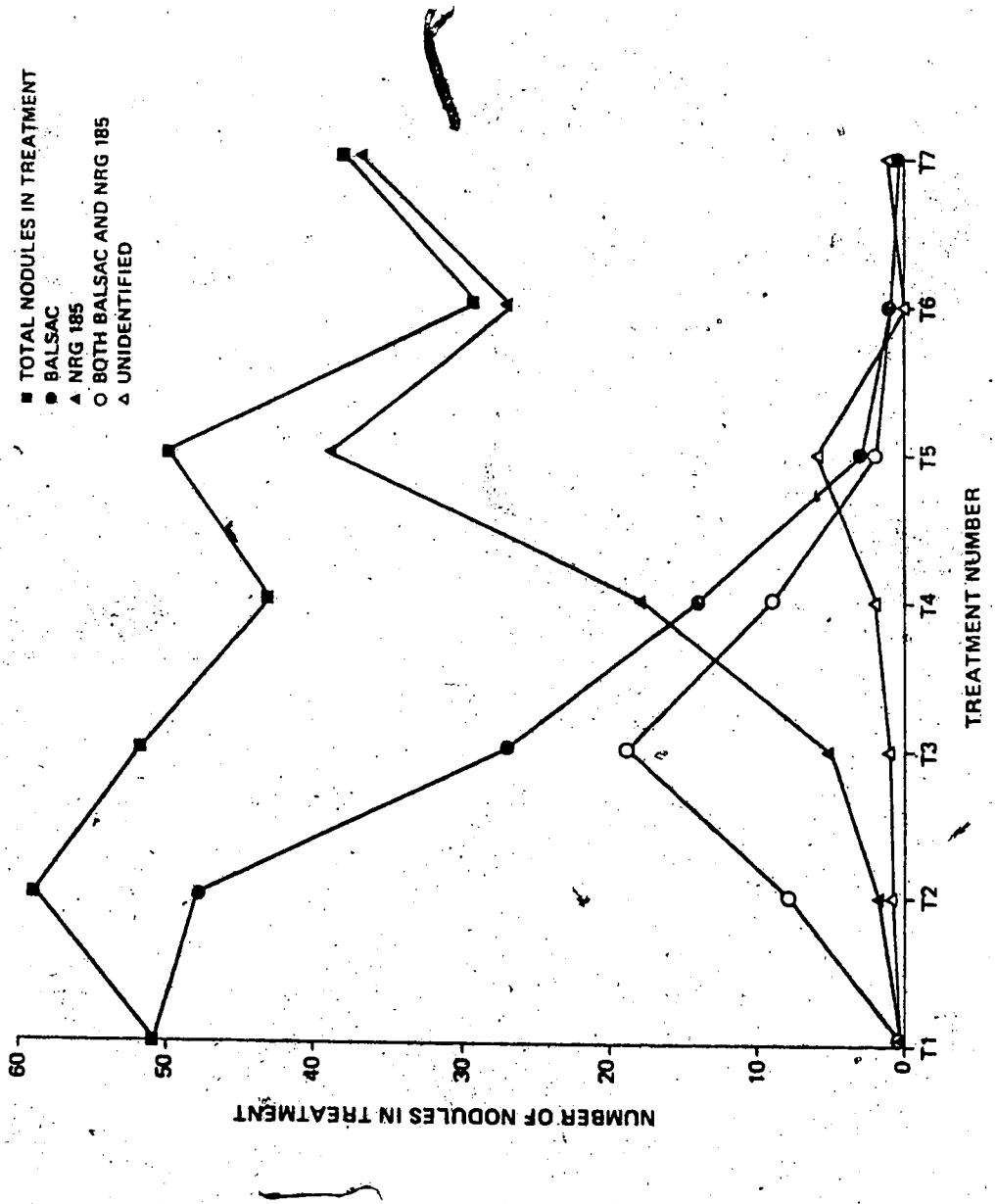

■ TOTAL NODULES IN TREATMENT
● BALSAC
▲ NRG 185
○ BOTH BALSAC AND NRG 185
△ UNIDENTIFIED
NUMBER OF NODULES IN TREATMENT
0
10
20
30
40
50
60
T1
T2
T3
T4
T5
T6
T7
TREATMENT NUMBER

FIGURE 8

PERCENT OF TOTAL NODULES IN

COMPETITION STUDY

TREATMENTS IDENTIFIED BY STRAIN

T 1 Balsac alone

T 2 NRG-185:Balsac, 1:100

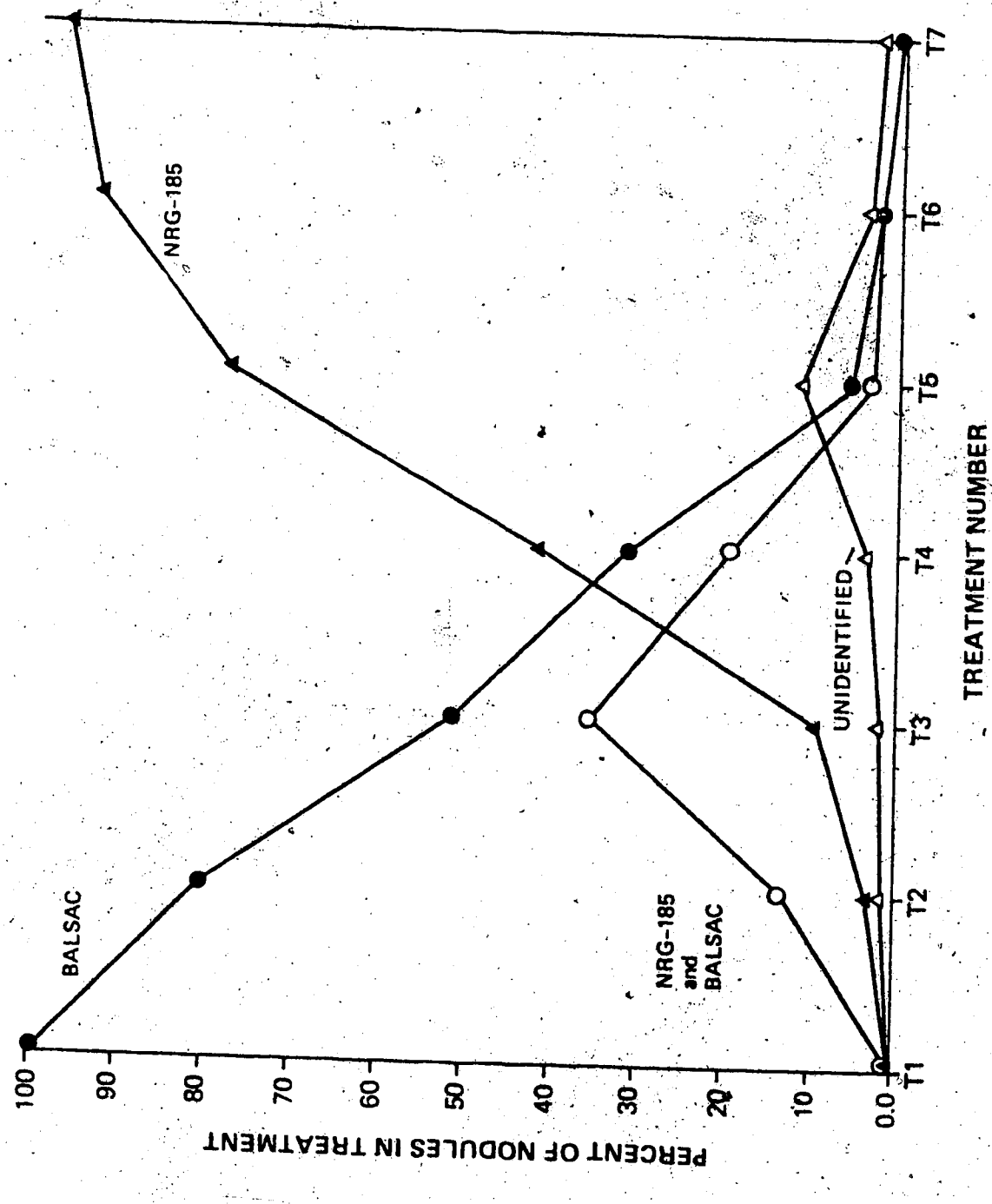
T 3 NRG-185:Balsac 1:10

T 4 NRG-185:Balsac 1:1

T 5 NRG-185:Balsac 10:1

T 6 NRG-185:Balsac 100:1

T 7 NRG-185 alone


PERCENT OF NODULES IN TREATMENT
100
90
80
70
60
50
40
30
20
10
0.0
T1
T2
T3
T4
T5
T6
T7
TREATMENT NUMBER
BALSAC
NRG-185
NRG-185 and BALSAC
UNIDENTIFIED

3 in which the Balsac inoculating organism was applied at 10 times the rate of strain NRG-185. A further 19% of the total number of doubly infected nodules were found in treatment 2, in which the Balsac inoculating strain was applied at a rate 100 times the rate of Strain NRG-185. Thus, 64.2% of the doubly infected nodules resulted from dual strain inoculation in which the Balsac strain had a numerical advantage of 10:1 or better.

4.6 Strain analysis of commercial alfalfa inoculants

Four separate lots of commercial alfalfa inoculant were sampled at various Alberta and Ontario retail outlets and transported to the Beaverlodge Research Station with control inoculants of known Rhizobium count. The control inoculants were tested for number of rhizobia of proper species by the plant infection technique. Control inoculant Rhizobium populations were determined by plate count and had not been affected by conditions of transport. All of the commercial inoculants tested had been manufactured in the United States by the Nitragin Company (specifically for Canadian distribution) and the package labels indicated that the inoculants contained either the Balsac strain or a combination of strains NRG-43 and NRG-185. The inoculants contained 4.8 - 15.3 x 10^8 rhizobia/g as determined by the plant infection technique (Table 8). The Canada Fertilizers Act and Regulations requires that an alfalfa inoculant, when applied to

Table 8. Quality evaluation of 4 samples of commercial alfalfa inoculants determined by the plant infection count, plate count, agglutination test and ELISA test

Sample No.	R. meliloti strain	rhizobia/g inoculant ($x10^8$)			
		plant infection	plate count	agglutination	ELISA
1.	NRG-43	11.1(4.0-30.6)*	18.4 ± 7.9**	0.5	nd***
	NRG-185			12.5	11.5
2.	NRG-43	4.8(1.8-12.7)	23.4 ± 4.6	0.6	nd
	NRG-185			17.3	17.3
3.	NRG-43	8.6(3.2-44.2)	12.3 ± 0.8	0.3	nd
	NRG-185			8.0	7.4
4.	Balsac	15.3(8.6-74.0)	25.4 ± 4.2	13.8	13.1

* 95% confidence limits
** ± SE
*** nd - not determined

seed at the manufacturer's recommended rate must provide 10^3 viable rhizobia of designated species per seed (Anon. 1979). The minimum requirement for these products is therefore 7.8×10^7 rhizobia/g inoculant, calculated as follows:

manufacturer's recommended rate of application	- 142 g inoculant/25 kg seed
alfalfa	- 4.41×10^5 seeds/kg
required rhizobia/seed	- 1000

$$\frac{4.41 \times 10^5 \times 25 \times 10^3}{142} = 7.76 \times 10^7 \frac{\text{rhizobia required}}{\text{g inoculant}}$$

All samples tested met this requirement (Table 8).

Plate counts of the inoculant samples were somewhat higher than plant infection counts because the inoculants were prepared using non-sterile peat carrier and contaminants visually indistinguishable from rhizobia were common on the semi-selective media used. Agglutination results (Table 8) indicated that the ratio of strain NRG-185 to NRG-43 in the mixed strain inoculants was about 25:1 instead of the desired 1:1 ratio. The inoculant manufacturer produced these inoculants by simultaneous inoculation with starter cultures of the two organisms into the final mass production growth medium. The imbalance of strain numbers in the mixed inoculant could be due to differential growth rates in either broth or carrier, or to differential survival abilities in the carrier.

The agglutination and ELISA strain analysis results were in close agreement, and gave values similar to those obtained by plant infection counts (Table 8). Based on the serological results, all inoculants

tested exceeded the Canadian standard of 10^3 rhizobia/seed (7.8×10^7 rhizobia/g) if applied at the recommended rate. The Balsac and NRG-185 specific ELISA antibodies were tested against the five R. meliloti strains (102F34, 102F51, 102F66, 102F77 and 104A13) ordinarily used by the Nitragin Company in its production of alfalfa inoculants and found to be non cross-reactive (Table 6). ELISA determinations of strain NRG-43 in the inoculants were not attempted due to the lack of a functioning conjugate against this strain (see section 4.4.2).

4.7 Optimization of ELISA response of R. meliloti antibody-enzyme conjugates

Although prepared in identical procedures, conjugates specific to strains NRG-259 and NRG-61 showed much greater sensitivity than those specific to NRG-185 and Balsac (Fig. 4). Gel chromatographic examination of all of the conjugates showed no significant differences in molecular weight distribution patterns among them. Chromatographic examination of the IgG fractions used to prepare the conjugates, however, revealed that the Balsac, 185, and 43 IgG preparations were contaminated with protein eluting at the same position as calibration standard bovine serum albumin. The IgG fractions used to prepare the 61 and 259 conjugates appeared homogenous in molecular weight eluting in the same position as highly purified commercial alkaline phosphatase. Repurification of the Balsac, 185 and 43 IgG fractions

by affinity column chromatography (DEAE Affi-Gel Blue) and repeated $(NH_4)SO_4$ precipitations purified the Balsac, 185, and 43 IgG preparations of the suspected serum albumin leaving solutions of homogenous molecular weight eluting in the proper column position. Conjugation as before, but using the repurified IgG, significantly improved the ELISA response for conjugates against strains NRG-185 and Balsac (Fig. 9). Non-specific binding of conjugate 61 (Fig. 4) was effectively reduced by increasing the BSA content of that conjugate to 10 mg/mL. Repurification of the anti NRG-43 IgG followed by reconjugation again failed to produce a functional ELISA conjugate against that strain.

FIGURE 9

OPTIMIZATION OF ELISA RESPONSE OF ANTIBODY-ENZYME CONJUGATES THROUGH REPEATED PURIFICATION OF SPECIFIC IgG PRIOR TO CONJUGATION

The A_{410}^{nm} ELISA response of each of the five antibody systems is shown in relation to four antigen concentrations and a no antigen control (NC) for:

A = NRG-259 cells

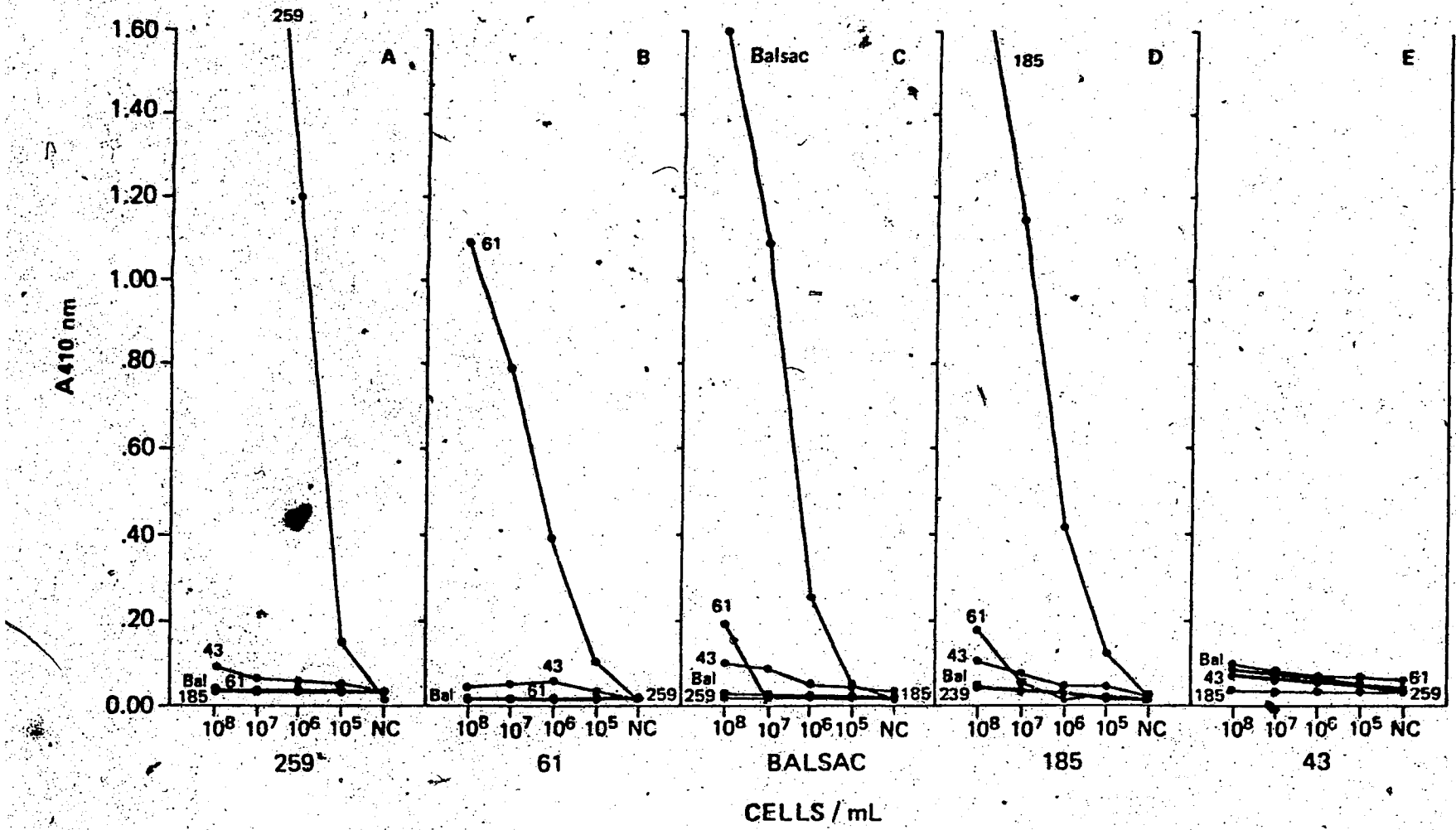
B = NRG-61 cells

C = Balsac cells

D = NRG-185 cells

E = NRG-43 cells

The increase in ELISA response of anti-Balsac and anti-185 conjugates through extra attention to IgG purification can be seen by comparison of Fig. 9 with Fig. 4.


A410 nm
1.60
1.40
1.20
1.00
.80
.60
.40
.20
0.00
A
B
C
D
E
259
61
Balsac
185
43
Bal
10^8 10^7 10^6 10^5 NC
259
61
BALSAC
185
43
CELLS / mL

5. DISCUSSION

5.1 General

The results presented have demonstrated that five of six R. meliloti strains examined are serologically distinct; that designated Canadian selected strains were in fact present in alfalfa inoculants sold by cooperating manufacturers marketing in Canada; and that ELISA techniques for the identification of small antigen quantities, such as from plate colonies or root nodules, are feasible. The basis for demonstration of specific serological reactions among the strains was the use of heavily adsorbed antibody to detect heat stable, cell-bound, antigen.

5.2 Serological quality control evaluation of commercial inoculants

Prior to this work, strains of R. meliloti adapted to regional conditions in Canada had been selected, field tested, and released for commercial production of alfalfa (Medicago spp.) inoculants. These inoculants are manufactured in the U.S.A. and marketed in Canada on a regional basis. Inoculants containing the Balsac strain from the Ste-Foy Research Station (Bordedeau et al. 1977) are

marketed in eastern Canada and inoculants containing two strains (NRG-43 and NRG-185) from the Beaverlodge Research Station (Rice and Olsen 1981) are marketed in western Canada. During the 1981 and 1982 seasons, approximately 95% of the alfalfa inoculants tested in Canada under the national inoculant quality control program contained one or the other of the Canadian developed R. meliloti strains.

Inoculant quality control in Canada is presently based on Rhizobium enumeration as determined by plant infection counts which give estimates of the number of viable Rhizobium of proper species per unit weight of inoculant. The introduction of specified Rhizobium strains in inoculants particularly prepared for Canadian distribution requires that inoculant quality control techniques be modified to enumerate Rhizobium of specific strain per-unit weight inoculant. The introduction of antibiotic resistance markers into these strains is appealing in terms of ease of marker introduction and recognition, but poses possibilities of unseen modifications to the carefully selected strains (Jones and Bromfield 1978; Josey et al. 1979; Zelazna-Kowalska 1971). Serological techniques are the most specific methods for identifying micro-organisms based on natural marker characteristics (Dudman 1977; Schwinghamer and Dudman 1980). Other workers have concluded that serological techniques are of severely limited use in the identification of R. meliloti strains

(Sinha and Peterson 1980; Humphrey and Vincent 1975). Here, adsorbed antisera were used to detect unshared antigen(s) attached to the cell (non-diffusable) permitting identification of R. meliloti strains. The micro-agglutination and enzyme-linked immunosorbent assay (ELISA) techniques described confirmed the presence of the Canadian-developed strains in alfalfa inoculants sold in Canada during 1981 (Olsen et al. 1983). These techniques could be used to form the basis of an inoculant quality control program based on serological determinations as opposed to plant infection tests. The advantage of the serological determination is the ability to identify specific strains while shortening the time period required for testing. The ELISA determination is rapid with results available within five days (including the plate count grow-out period) whereas three to four weeks are required to evaluate an inoculant by the plant infection technique. The agglutination analysis of inoculants is accurate and simple, but is cumbersome in that each colony isolate requires regrowth to generate sufficient cell numbers and each cell suspension further requires optical adjustment to 10^9 cells per mL (O.D. 620 nm = 1.0).

The serological identification of an unknown such as a Rhizobium strain in a commercial inoculant requires the assumption that a positive immunological response equates the unknown with the strain to which the antiserum was raised. This assumption is valid only

when it is possible to fully document the specificity of the reaction. Even where extensive experience suggests that a given antisera has a high degree of specificity, possibilities exist that different strains may be serologically identical. It is therefore difficult to be certain that a strain obtained from a commercial inoculant is the same strain with which the manufacturer was provided. However, in the study described, it has been demonstrated that the adsorbed antisera used did not serologically react with other R. meliloti strains ordinarily used by the manufacturer in the production alfalfa inoculants (Table 6). Similarly, there was no cross-reactivity between any inoculant isolate and antisera heterologous to the strain or strains identified on the inoculant package label. This provided a high degree of confidence in serological techniques for evaluating alfalfa inoculants containing the R. meliloti strains selected for Canadian conditions.

5.3 The ELISA

The use of (and literature describing) ELISA techniques has grown exponentially during the last decade. Originally employed for the detection of antibody protein (Engvall and Perlmann 1971), the technique was rapidly expanded to the detection of soluble antigen of many types and to the detection of viral antigens. The

use of the ELISA for detection and identification of surface antigens on bacterial whole cells has been a relatively late development and was first reported for Rhizobium strain identifications in 1978 (Kishinevsky and Bar-Joseph 1978).

The relative mass of a bacterial cell to that of a soluble protein or to a virus particle is enormous. The successful application of double antibody sandwich microtiter plate ELISA techniques to bacterial whole cell strain identifications depends on the establishment of sufficient bridges between antibody, non-covalently bound to polystyrene well surfaces, and the specific antigen which is bound to the relatively massive cell. These two key bindings (antibody to polystyrene and antibody to cell) are essential to the capture of the cell, thereby allowing detection of specific antigen on the cell through the medium of an additional, enzyme-tagged, specific antibody. The cell capture must be of sufficient binding strength to hold the cells over the two day period of testing while being subjected to rigorous triple washings before cell binding, after cell binding, and following antibody-enzyme conjugate binding. The work reported here made use of antisera massively adsorbed against heterologous cross-reacting cells. Where the cross-reacting antigen on the cell surface was relatively massive, the adsorbtive removal of antibodies to these sites would be expected to further reduce the overall binding of the cells to the plate wells via the use of adsorbed first antibody as binding agent. Adsorbed second antibody carrying the enzyme tag would similarly have fewer

sites for binding onto the cell; the net effect of both reductions in binding is the final lessening of bound enzyme which amounts to reduced sensitivity for the assay. Speculatively, it is felt that one or both of these binding reductions are sufficient to account for the differences in sensitivity of the ELISA identity determinations of Rhizobium meliloti strains described in this work.

The failure of the ELISA to identify strain NRG-43 may also be related to these phenomena. Strain NRG-259, for example, was clearly the least cross-reactive of the strains examined and antiserum to strain NRG-259 required much less adsorption to achieve agglutination specificity than did any of the others examined. Strain NRG-259 shows the greatest ELISA sensitivity by far, yet these results are achieved with antibody-enzyme conjugate concentrations twenty times as dilute as those required for similar color development, in a given time, with strains Balsac or NRG-185. Of the antisera produced against each of the five strains, that raised against strain NRG-43 required the most extensive adsorptions against all heterologous cell types to yield agglutination specificity.

It is possible that the purified IgG antibodies used in the production of enzyme-antibody conjugates against strain NRG-43 had been adsorbed to extinction if each of the differing NRG-43 antigens are shared by one or more of the adsorbing strains. If this is so, it is necessary to assume that the 160,000 dalton protein (presumably

IgG) used in the preparation of anti NRG-43 conjugates was either not antibody or was antibody which had no reactivity with strain NRG-43. It should be pointed out that within a microbiologically defined system (in which all strains present are known, though not individually) it has been possible to identify strain NRG-43 through the use of antibody conjugates prepared against strain NRG-185, but not adsorbed against strain NRG-43. These conjugates react with both strains NRG-185 and NRG-43 whereas fully adsorbed conjugates prepared against NRG-185 react only with NRG-185, thus NRG-43 may be differentiated from NRG-185. This latter approach is not suitable, however, to strain identification in nodules or inoculants as the possibility of dual strain inhabitation is real in nodules and colonies isolated from commercial inoculants cannot be considered as a part of a microbiologically defined system.

Work to define the relative importance of the amount of binding available to hold cells to microtiter plate wells versus the number of bindings of enzyme-tagged specific antibody remains to be done. One approach will be to use [illegible] adsorbed generalized anti-Rhizobium antibody for the cell plate binding followed with adsorbed, highly specific, enzyme [illegible] antibody for strain detection. If such an approach yields greater sensitivity without significant loss of specificity, then cell binding and capture is a significant problem; a problem only exacerbated when bacteroids, with cell sizes much larger than vegetative cells, are to be identified. In actual

practice for the applied Rhizobium lab, however, the whole binding issue is somewhat moot as the demonstrated ability to identify strains at 10^6-10^7 cells per mL allows the ready identification of nodules weighing as little as 0.1 mg; as small as is practical to isolate. In any case, we have recently found (work not yet reported) that it is possible to dispense altogether with the "double antibody sandwich" ELISA through the simple expedient of baking the Rhizobium cells into the microtiter plate wells at 94°C (temperatures above this warp the plates). In our Rhizobium serology, the decision was taken to produce antibody to lipopolysaccharide of the cell walls, antigen which is not notably heat sensitive. Antibody was therefore produced by the injection of washed Rhizobium whole cells which had been steamed for 30 minutes at at least 100°C. This antibody binds well to Rhizobium cells which have been essentially heat fixed to the polystyrene of microtiter plate wells. Unconjugated specific (adsorbed) rabbit antibody in PBS-Tween 20 buffer is added directly to the dried Rhizobium in plate wells and after a two h incubation is followed with enzyme conjugated swine anti-rabbit IgG antibody. This approach has allowed the ready detection of Rhizobium strains at 10^5-10^6 cells per mL and eliminates the overnight incubation of cells for attachment to sensitizing antibody. It has also proved feasible to use directly the specific rabbit antibody-enzyme conjugates prepared for the double antibody sandwich procedure on dried Rhizobium bound in the plate wells. This direct form of

the ELISA further shortens the period required for testing, but is apparently less sensitive with detection limits in the 10^6-10^7 cells per mL range. Both techniques are readily performed in one 8 h work period.

Regardless of the particular technique applied, the ELISA requires antibody of high purity and specificity. In ELISA applications involving the "double antibody sandwich", such as the work described in this report, nanogram quantities of protein are bound in the sensitizing antibody coat (Pesce et al. 1977). If a significant proportion of this coating protein is other than specific antibody the quantity of antigen bound is concommitantly reduced. In practice, antibody-enzyme conjugates are generally prepared from an aliquot of the antibody stock from which sensitizing antibody coating solution is prepared. If the antibody stock is less than pure, not only in terms of being biologically active IgG, but in terms of being active against a specific desired antigen(s), then conjugate prepared from it cannot reach it's full potential. These factors, arising from the heterogeneity of animal antisera, form the basis of the numerous advantages to be gained from the use of monoclonal antibody systems.

The coating buffer used for sensitizing antibody deposition onto polystyrene for microtiter plate ELISAs is almost universally a carbonate-bicarbonate buffer of pH 9.6. This buffer is unstable and requires room temperature storage with fresh recomposition every

two weeks (Voller et al. 1979). Experiments in our lab demonstrated no effect on ELISA results through the use of our normal carrier buffer of PBS, pH 7.2, as sensitizing antibody coating buffer. The use of PBS for coating buffer allowed the refrigerated storage and multiple reuse of stock dilutions of sensitizing antibody at 10 ug protein per mL. Such reuse of the sensitizing antibody showed no decrease in ELISA sensitivity over a period of one year and allowed for considerable conservation of purified adsorbed antibody which was in short supply.

5.4 Nodulation competition study

Competition between Rhizobium strains for legume nodulation involves such a complex set of interactions that there is imposed a requirement for cautious and conservative interpretation of results. The results of the nodulation competition study described in this report apply to the sudden and simultaneous inoculation of one alfalfa cultivar, rooted aseptically in vermiculite, under glass, under a given moisture, temperature, nutrient and pH status, with artificially grown, early stationary phase R. meliloti cells. Such results simply cannot be extrapolated to field conditions.

As described earlier, some 95% of the alfalfa inoculant sold in Canada in 1981 and 1982 contained Canadian developed R. meliloti strains distributed differentially on a regional basis. Considerable logistical inoculant supply simplification could be gained were it

considered feasible and wise to mix these strains into a single inoculant which could then be distributed nationwide. This desirability of a mixed strain Canadian alfalfa inoculant provides the impetus for studies of interactions and relative competitive nodulation abilities between the strains involved.

The results of the nodulation study described in this report indicate an essentially equal status of competitive nodulation ability between the Balsac (eastern) and NRG-185 (western) strains. No evidence of antagonism (or synergism) was noted. It appears (on the basis of the single study, under the conditions described) that strain NRG-185 may be more able to share nodule inhabitancy, in the face of superior opposing numbers, than the Balsac strain. That this phenomenon offers any advantage to strain NRG-185 is unlikely, given the relatively small percentage of nodules found to be inhabited by both strains. While the results are encouraging to the possibility of establishing a single mixed strain Canadian alfalfa inoculant, considerable work remains. Work is required to investigate the abilities of the strains to nodulate other cultivars under varying environmental parameters and, in particular, under field conditions. The relative multiplication and survival characteristics of the strains while mixed in peat carrier will also require examination. Probably the most significant aspect of the competition study results described is that reasonable, believable results were obtained which lends credence to the Rhizobium-ELISA protocols used, thereby creating an avenue by which the necessary further studies may be conducted.

BIBLIOGRAPHY

ANONYMOUS. 1979. Fertilizers Act and Fertilizers Regulations, Office Consolidation. C.R.C., c. 666 amended by P.C. 1978-3455, P.C. 1979-1271. Government of Canada.

BAL, A.K., and P.P. WONG. 1982. Infection process and sloughing off of rhizobial outer membrane in effective nodules of lima bean. Can. J. Microbiol. 28:890-896.

BALDWIN, I.L., and E.B. FRED. 1929. Nomenclature of the root nodule bacteria of the Leguminosae. J. Bacteriol. 17:141-150.

BARNET, Y.M. 1972. Bacteriophages of Rhizobium trifolii. I. Morphology and host range. J. Gen. Virol. 15:1-15.

——— 1979. Properties of Rhizobium trifolii isolates surviving exposure to specific bacteriophages. Can. J. Microbiol. 25:979-986.

BERGER, J.A., S.N. MAY, L.R. BERGER, and B.B. BOHLOOL. 1979. Colorimetric enzyme-linked immunosorbent assay for the identification of strains of Rhizobium in culture and in the nodules of lentils. Appl. Environ. Microbiol. 33:642-646.

BOHLOOL, B.B., and T.D. BROCK. 1974. Population ecology of Sulfolobus acidocoldarius. ii. Immunoecological studies. Arch. Microbiol. 97:181-194.

BORDELEAU, L.M., H. ANTOUN, and R.A. LACHANCE. 1977. Effets des souches de Rhizobium meliloti et des coupes successives de la luzerne (Medicago sativa) sur la fixation symbiotique d'azote. Can. J. Plant Sci. 57:433-439.

BROCKWELL, J. 1980. Experiments with crop and pasture legumes - principles and practice. In Methods for evaluating biological nitrogen fixation. Edited by F.J. Bergerson. John Wiley and Sons, New York. pp. 417-480.

BROCKWELL, J., E.A. SCHWINGHAMER, and R.R. GAULT. 1977. Ecological studies of root-nodule bacteria introduced into field environments - V. 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.

CARLSON, R.W., R.E. SANDERS, C. NAPOLI, and P. ALBERSHEIM. 1978. Host-symbiont interactions. III. Purification and partial characterization of Rhizobium lipopolysaccharides. Plant Physiol. 62:912-917.

CLARK, M.F., and A.N. ADAMS. 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. Sci. 26:121.

CLOONAN, M.J., and B. HUMPHREY. 1976. A new method for strain identification of Rhizobium trifolii in nodules. J. Appl. Bact. 40:101-107.

COOPER, J.E. 1979. Rapid method for counting antibiotic-resistant rhizobia in soils. Soil Biol. Biochem. 11:433-435.

DAZZO, F.B., and W.J. BRILL. 1979. Bacterial polysaccharide which binds Rhizobium trifolii to clover root hairs. J. Bacteriol. 137:1362-1373.

DEMELON, A., and A. DUNEZ. 1936. Nouvelles observation sur le bacteriophage et la fatique des sols cultives en luzerne. Ann. Agron. 6:1-21.

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. Immunodiffusion analysis of the extracellular soluble antigens of two strains of Rhizobium meliloti. J. Bacteriol. 88:782-794.

——— 1971. Antigenic analysis of Rhizobium japonicum by immunodiffusion. Appl. Microbiol. 21:973-985.

——— 1977. Serological methods and their application to dinitrogen-fixing organisms. In A treatise on dinitrogen fixation. Section IV. Edited by R.W.F. Hardy and A.H. Gibson. John Wiley and Sons, New York. pp. 487-508.

DUDMAN, W.F., and J. BROCKWELL. 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.

ECKHARDT, M.M., I.L. BALDWIN, and E.B. FRED. 1931. Studies on the root nodule organism of Lupinus. J. Bacteriol. 21:273-285.

ENGVALL, E., and P. PERLMANN. 1971. Enzyme-linked immunosorbent assay (ELISA). Quantitiative assay of immunoglobulin G. Immunochemistry, 8:871-874.

——— 1972. Enzyme-linked immunosorbent assay (ELISA). III. Quantitation of specific antibodies by enzyme-labelled anti-immunoglobulin in antigen-coated tubes. J. Immunol. 109:129-135.

FLIERMANS, C.B., and T.C. HAZEN. 1980. Immunofluorescence of Aeromonas hydrophila as measured by fluorescence photometric microscopy. Can. J. Microbiol. 26:161-168.

FRED, E.B., I.L. BALDWIN, and E. McCOY. 1932. Root nodule bacteria and leguminous plants. University of Wisconsin Studies in Science, No. 5. University of Wisconsin Press, Madison.

GRAHAM, P.H. 1976. Identification and classification of root nodule bacteria. In Symbiotic nitrogen fixation in plants. Edited by P.S. Nutman. Cambridge University Press, Cambridge. pp. 99-112.

HILDEBRAND, R.L. 1979. ELISA (enzyme-linked immunosorbent assay) In Rapid diagnosis in infectious disease. Edited by M.W. Rytel. CRC Press, Inc. pp. 71-88.

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

HUMPHREY, B.A., and J.M. VINCENT. 1969. The somatic antigens of two strains of Rhizobium trifolii. J. Gen. Microbiol. 59:411-425.

——— 1975. Specific and shared antigens in strains of Rhizobium meliloti. Microbios, 13:71-76.

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

JONES, D.G., and S.J. MORLEY. 1981. The effect of pH on host plant 'preference' for strains of Rhizobium trifolii using fluorescent ELISA for strain identification. Ann. Appl. Biol. 97:183-190.

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

JOHNSTON, A.W.B. and J.E. BERINGER. 1975. Identification of the Rhizobium strains in pea root nodules using genetic markers. J. Gen. Microbiol. 87:343-350.

JORDAN, D.C., and O.N. ALLAN. 1974. Family Rhizobiaceae. In Bergey's manual of determinative bacteriology, 8th ed. Edited by R.E. Buchanan and N.E. Gibbons. The Williams and Wilkans Co., Baltimore. pp. 261-267.

JOSEY, D.P., J.L. BEYNON, A.W.B. JOHNSTON, and J.E. BERINGER. 1979. Strain identification in Rhizobium using intrinsic antibiotic resistance. J. Appl. Bacteriol. 46:343-350.

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

KISHINEVSKY, B., and D. GURFEL. 1980. Evaluation of enzyme-linked immunosorbent assay (ELISA) for serological identification of different Rhizobium strains. J. Appl. Bacteriol. 49:517-526.

KONDEROSI, A., G.B. KISS, T. FORRAI, E. VINCZE, and Z. BANFALVI. 1977. Circular linkage map of Rhizobium meliloti chromosome. Nature (London), 268:525-527.

LESLEY, S.M. 1982. A bacteriophage typing system for Rhizobium meliloti. Can. J. Microbiol. 28:180-189.

LINDEMAN, W.C., E.L. SCHMIDT, and G.E. HAM. 1974. Evidence for double infection within soybean nodules. Soil Sci. 118:274-279.

MEANS, M., H.W. JOHNSON, and R.A. DATE. 1964. Quick serological method of classifying strains of Rhizobium japonicum in nodules. J. Bacteriol. 87:547-553.

MEADE, H.M., and E.R. SIGNER. 1977. Genetic mapping of Rhizobium meliloti. Proc. Natl. Acad. Sci. U.S.A. 74:2076-2078.

MORLEY, S.J., and D.G. JONES. 1980. A note on a highly sensitive modified ELISA (enzyme-linked immunosorbent assay) technique for Rhizobium strain identification. J. Appl. Bacteriol. 49:103-109.

NAKANE, P.K., and G.B. PIERCE JR. 1967. Enzyme-labeled antibodies: preparation and application for the localization of antigens. J. Histochem. Cytochem. 14:929.

NORRIS, D.O. 1958. A red strain of Rhizobium from Lotononis bainesii Baker. Aust. J. Agric. Res. 9:629-632.

OBATON, M. 1973. The use of spontaneous antibiotic resistant mutants for the ecological study of Rhizobium. Bull. Ecol. Res. Comm. (Stockholm) 17:170-171.

OLSEN, P.E., W.A. RICE, G.W. STEMKE, and W.J. PAGE. 1981. The identification of Canadian-developed strains of Rhizobium meliloti in commercial alfalfa inoculants. Program and Abstracts, Eighth North American Rhizobium Conference, Winnipeg. p. 94.

———1983. Strain specific serological techniques for the identification of Rhizobium meliloti in commercial alfalfa inoculants. Can. J. Microbiol. 29:225-230.

PARKER, C.A., and P.L. GROVE. 1970. Rapid serological identification of rhizobia in small nodules. J. Appl. Bacteriol. 33:248-252.

PESCE, A.J., D.J. FORD, M. GAIZUTIS, and V.E. POLLAK. 1977. Binding of protein to polystyrene in solid-phase immunoassays. Biochem. Biophys. Acta, 492:399-407.

PINTO, C.M., P.Y. YAO, and J.M. VINCENT. 1974. Nodulating competitiveness amongst strains of Rhizobium meliloti and R. trifolii. Aust. J. Agric. Res. 25:317-329.

RAM, J.S., P.K. NAKANE, D.G. RAWLINSON, and G.B. PIERCE. 1966. Enzyme-labeled antibodies for ultrastructural studies. Fed. Proc. Abstr. 25:732.

RICE, W.A., and P.E. OLSEN. 1981. Synergism between effective and ineffective strains of Rhizobium meliloti. In Current perspectives in nitrogen fixation. Edited by A.H. Gibson and W.E. Newton. Australian Academy of Science, Canberra. p. 440.

ROBERTS, G.P., W.T. LEPS, L.E. SILVER, and W.J. BRILL. 1980. Use of two-dimensional polyacrylamide gel electrophoresis to identify and classify Rhizobium strains. Appl. Environ. Microbiol. 39:414-422.

RUSSA, R., URBANIK, T., ZURKOWSKI, W., and LORKIEWICZ, Z. 1981. Neutral sugars in lipopolysaccharides of Rhizobium trifolii and it's non-nodulating mutant. Plant and Soil, 61:81-85.

SCHMIDT, E.L. 1973. Fluorescent antibody technique for the study of microbial ecology. In Modern methods in the study of microbial ecology. Edited by T. Roswall. Bull. Ecol. Res. Comm. (Stockholm), 17:67-76.

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

——— 1980. Methods for identifying strains of diazotrophs. In Methods for evaluating biological nitrogen fixation. Edited by F.J. Bergerson. John Wiley and Sons, New York. pp. 337-365.

SINHA, R.C., and E.A. PE[redacted]ON. 1980. Homologous serological analysis of Rhizobium meliloti strains by immunodiffusion. Can. J. Microbiol. 26:1157-1161.

STANIEWSKI, R. 1970a. Relationship among different Rhizobium strains determined by phage lysis. Acta Microbiol. Pol. Ser. B, 2:3-12.

——— 1970b. Typing of Rhizobium by phages. Can. J. Microbiol. 16:1003-1009.

TRINICK, M.J. 1969. Identification of legume nodule bacteria by the fluorescent antibody technique. J. Appl. Bacteriol. 32:181-186.

VAN WEEMEN, B.K., and A.H.W.M. SCHUURS. 1971. Immunoassay using antigen-enzyme conjugates. FEBS Lett. 15:232-236.