RELEASE AND RECOVERY OF *RHIZOBIUM* FROM TROPICAL SOILS FOR ENUMERATION BY IMMUNOFLUORESCENCE

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ABSTRACT

Immunofluorescence (IF) provides a direct method for in situ autecological studies of microorganisms; it allows for the simultaneous detection and identification of the desired organism in its natural habitat. With the development of a quantitative membrane filter - immunofluorescence technique, the range of applications of IF were extended to include quantitative studies of microorganisms directly from soil.

The overall objective was to study the ecology of chickpea Rhizobium in tropical soils. To accomplish this, the research described in this thesis was concerned with: (1) determining the serological characteristics of 27 strains of chickpea (Cicer arietinum L.) rhizobia, by immunofluorescence and immunodiffusion, for use in ecological studies; (2) evaluation of the quantitative membrane filter - immunofluorescence technique for studies of Rhizobium in tropical soils; (3) the development of successful modifications of the quantitative method to optimize release and recovery of Rhizobium from tropical soils.

To employ the quantitative technique for the study of chickpea Rhizobium in tropical soils, fluorescent antibodies (FA's) were prepared from the somatic antigens of the following strains: Nitragin strains 27A3, 27A8, 27A11, USDA strain 3HOa1; and NifTAL strains TAL-480, TAL-619, and TAL-620. Twenty-seven, strains of chickpea rhizobia were screened with these seven FA's; the immunofluorescent reactions defined five groups. Group I, corresponding to serogroup Nitragin 27A3, contained only the homologous strain. Group II, serogroup Nitragin 27A8, Nitragin 27A11, TAL-619, and TAL-620, contained 15 cross-reacting strains. The four strains, Nitragin 27A8, Nitragin 27A11, TAL-619, and TAL-620 were shown to have identical antigens by FA-cross adsorption, and by immunodiffusion with whole cell antiserum. These four strains constituted one serotype. Group III, serogroup TAL-480, contained two reactive

strains TAL-480 and TAL-622. Group IV, serogroup 3HOa9, was specific for the homologous FA. Eight strains failed to react with any FA (Group V). No cross-reactions were detected among 19 other strains of fast- and slow-growing rhizobia.

FA and immunodiffusion were used to compare the antigens of two strains of chickpea rhizobia obtained from both pure cultures and from nodules. The immunofluorescent reactions of the nodules containing these strains paralleled the reactions of their parent cultures. A difference was detected in the quality of fluorescence between the nodule bacteria and their parent cultures. The fluorescent outline of cells from culture was sharp and well defined, while that of the nodule-bacteria was diffuse and thick. In immunodiffusion agar gels, nodule antigens were freely diffusable while culture antigens required heat-treatment.

The efficiency of the quantitative membrane filter technique for recovering fast- and slow-growing rhizobia from tropical soils was evaluated with eight soils, from three of the major soil orders (Oxisols, Inceptisols, Vertisols). Recovery of added rhizobia from seven soils was less than or equal to 13%. A recovery of 100% of the added cells was obtained with one Inceptisol.

In a sand:soil (Oxisol) mixture, increasing the soil content from 0% (i.e. 10 g sand) to 100% soil (10 g soil) caused a decrease in recovery of two fast-growing strains of Rhizobium from 100% to less than 1%.

Modifications to the usual quantitative membrane filter--immunofluorescence technique yielded consistently high and reproducible recoveries of both fast- and slow-growing rhizobia from tropical soils. The modified procedure involved suspending the soil by shaking with glass beads on a wrist-action shaker. The diluent consisted of partially hydrolyzed gelatin (0.1%)-0.1M $(NH_4)_2HPO_4$. Growth of fast and slow growing strains of Rhizobium in a sterile Hawaiian Oxisol was followed by plate counts,

the quantitative procedure and the modified quantitative procedure. Parallel growth curves obtained with plate counts and the modified quantitative procedure indicated close agreement, while counts with the original procedure were 1000 times lower.

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LIST OF ABBREVIATIONS AND SYMBOLS

Å	angstroms
CEC	cation exchange capacity
FA	fluorescent antibody
g	gram
IF	immunofluorescence
M	molar
ml	millilite
mm	millimeter
MSRP	Modified Soil Release Procedure
NaHMP	Sodium Hexa-Meta Phosphate
O.M.	organic matter
PBS	Phosphate Buffered Saline
PC	Plate Counts
PHG	Partially Hydrolyzed Gelatin
SRP	Soil Release Procedure
µl	micrometer
µm	micrometer

CHAPTER 1

GENERAL INTRODUCTION

The small size of bacteria dictates that they be viewed directly in nature with the aid of a microscope. This is easily done in aquatic habitats. Unfortunately the particulate nature of soil prevents easy viewing and enumeration of microorganisms by conventional light microscopy. Cells may attach to opaque soil particles and when stained by typical bacteriologic dyes remain obstructed from view. Additionally, small pieces of organic and mineral matter may be mistaken for bacteria.

To overcome some of these difficulties a number of specialized techniques have been adopted to observe, study and enumerate bacteria microscopically in soil. Several of these techniques take advantage of the smooth, artificial surface of glass. Some examples are: the Perfil'ev capillary technique (Perfil'ev and Gabe, 1969), the Cholodny buried slide (see Johnson and Curl, 1972) and the Breed slide (framer and Schmidt, 1964). Although useful, none of these techniques offer the potential applications of fluorescent antibody (FA) methodology. The application of immunofluorescence (IF) to the Rhizobium model system (Schmidt et al., 1968) allowed investigators for the first time to simultaneously observe and identify a microorganism of interest directly from the soil amidst a plethora of other organisms.

Specific quantitative techniques necessary to measure biomass, growth rate in soil, and growth responses to environmental variables are important to the soil microbial ecologist. When a conventional Breed slide is stained with FA a density of approximately 10^6 cells/gram of soil is necessary to encounter one cell in ten microscope fields (100 X objective) (Bohlool, 1971; Schmidt, 1978). In order to enumerate natural populations, usually less than 10^6 cells/gram of soil, it becomes necessary to separate the bacteria from interfering soil particles and concentrate them for enumeration.

In 1973 Bohlool and Schmidt (1973a) described a technique in which cells recovered from soil on non-fluorescent membrane-filters, and stained with the appropriate FA, were enumerated by immunofluorescence. Although applied in several studies of rhizobia in soils and rhizospheres (Bohlool and Schmidt, 1973a; Reyes and Schmidt, 1979; Vidor and Miller, 1979) difficulties in the efficiency of recovery of rhizobia were noted (Schmidt, 1974; Reyes and Schmidt, 1979; Vidor and Miller, 1979; Wollum and Miller, 1980). In addition, May (1978, Personal Communication) and Kingsley and Bohlool (unpublished) obtained very poor recoveries of lentil and chickpea Rhizobium respectively from a Hawaiian Oxisol.

This research was concerned with: (1) the preparation of fluorescent antibodies for, and determining the serological characteristics of strains of Cicer rhizobia for use in ecological studies; (2) assessment of the sorptive nature of several temperate and tropical soils for Rhizobium when assayed by the quantitative membrane-filter technique (Bohlool and Schmidt, 1973a); and (3) the development of successful modifications of the quantitative method so that bacteria can be easily enumerated in tropical soils.

CHAPTER 2

LITERATURE REVIEW

Bacillus radicicola, the root-nodule bacteria of legumes, were first isolated, described, and named by Beijerinck, the father of microbial ecology. These organisms now constitute the genus Rhizobium--the name proposed by Frank in 1889 (Fred et al., 1932). From Beijerinck's report in 1888 to the present, the Rhizobiaceae have been the object of intense investigation and are probably among the most widely studied of the soil microorganisms.

Interest in these bacteria stems from the unique nitrogen-fixing symbiotic association they have with their legume hosts. Legumes are among the world's most important crop plants, second only to grains (Advisory Committee on Technology and Innovation 1979). Thus it seems only natural that the symbiont be intensely studied. While the actual mechanisms of host specificity remain elusive, questions concerning the life of these bacteria in the soil and the soil properties which influence their growth, persistance, and success or failure in nodulation can be answered. These answers can be readily applied to increasing legume yields through enhanced symbioses.

I. Use of Serological Techniques in Studies of Rhizobium

Serological techniques have been in use for many years to investigate the Rhizobiaceae. The discovery by Klimmer and Kruger (in Fred et al., 1932) that bacteria isolated from different species of legumes could be distinguished serologically, made serological methods extremely attractive for strain identification. Stevens (1923) and later Wright (1925) found that different strains isolated from the same species of plant, and therefore belonging to the same inoculation group, were serologically unrelated. In fact, Hughes and Vincent (1942) found strains isolated from different nodules on the same plant which were serologically unique. The results of these early

investigations pointed to the great serological diversity now known to exist in the Rhizobiaceae.

A. Agglutination

Agglutination was one of the first methods to be applied to serological investigations of rhizobia. It is among the simplest of serological techniques to use and it has been widely exploited in many taxonomic and ecologic investigations. Bushnell and Sarles (1939) used the technique to define three types of antigens on rhizobia. They reported on the antigenic specificity between and within rhizobia from soybean, cowpea, and lupin cross-inoculation groups. They found no correlation between the ability of rhizobia from the three legumes to cross-inoculate and cross-agglutinate. This important observation was recently restated by Vincent (1977): strains which are related or apparently related serologically can be entirely unrelated in other characteristics. Bushnell and Sarles (1939) also confirmed the results of Stevens (1923) who found that due to the serological diversity within a species of Rhizobium all strains cannot be identified by the agglutination test.

Kleczkowski and Thornton (1944) used agglutination to study the serological relatedness between and within pea and clover strains of rhizobia. They tested six antisera (four clover, two pea) against 161 strains of R. trifolii, 29 R. leguminosarum, 5 each of R. meliloti and R. lupini, and 13 non-Rhizobium soil isolates. Partial cross-reactions occurred in the clover and pea groups which were removed after adsorption of antisera with the cross-reacting antigens. No cross-reactions were detected outside of the clover and pea groups; and none of the 13 soil isolates agglutinated. No "group" antigen common to all the strains was found and attempts to link effectiveness or ineffectiveness to any serological property failed.

Koontz and Faber (1961) used agglutination-adsorption (Edwards and Ewing, 1955) to characterize the somatic antigens of 25 strains of Rhizobium resulting in six distinct serogroups. Antigenic similarities and physiological characteristics could not be related.

Graham (1963), in a similar study to that of Kleczkowski and Thornton (1944), prepared antisera against somatic antigens and whole cell/flagellar antigens of 58 strains of root-nodule bacteria and 16 strains of agrobacteria. He tested the antisera by tube agglutination against 113 strains of Rhizobium, 20 strains of Agrobacterium and 20 strains of other, possibly related bacteria. On the basis of the agglutination reactions he categorized the rhizobia into three serologically distinct groups. Cross-reactions were more common when whole cell antisera were used than when agglutinations were run with somatic antisera.

Tube agglutination techniques utilize antigens obtained from pure cultures. Means et al. (1964) adapted the methods to type bacteria directly from root-nodules. They observed that the agglutination reaction of pure cultures and of root-nodule homogenates were identical for 15 of 17 strains tested, and recommended that this technique be used for a quick classification of nodules. This method was further modified into a micro-agglutination test (Damirgi et al., 1967). In micro-agglutination a drop of dilute nodule homogenate is mixed with a drop of dilute antiserum in a depression plate and allowed to react. In this way the number of serologic tests of even small nodules can be increased greatly.

B. Immunodiffusion

The technique of Ouchterlony double-diffusion has been widely used to study the antigens of root-nodule bacteria for both taxonomic and ecologic purposes (Dudman, 1964, 1971; Dudman and Brockwell, 1968; Gibbins, 1967; Humphrey and Vincent, 1965, 1969, 1975; Vincent and Humphrey, 1968, 1970, 1973). Strains

which cross-react in agglutination tests because of minor similarities in their surface, particulate antigens (such as flagella) do not necessarily share other antigens as shown by immunodiffusion--a method which uses soluble antigens diffusing through gels (Eisen, 1974; Dudman, 1977).

The gel diffusion method permits the enumeration and comparison of antigens with minimal effort; but the confidence with which strains can be identified will increase in proportion to the number of antigens detected (Dudman, 1964, 1977; Eisen, 1974). Relationships between various antigens are established by observing the nature of the interaction at the junction of precipitin bands from the various wells, the number of precipitin bands being equal to the minimal number of separately diffusable soluble antigens present in the antigen well.

The somatic antigens of many Rhizobium strains diffuse slowly in the agar gels; they yield either no precipitin bands or only weak bands close to the antigen well since the location of bands is dependent upon the relative concentrations of diffusable antigens and antibodies (Eisen, 1974). Heating for various periods of time dissociates the poorly diffusable somatic antigen molecules and makes them more soluble; thus it is one of the easiest methods of antigen preparation for gel diffusion (Skrdleta, 1969; Dudman, 1971; Humphrey and Vincent, 1975). Gibbins (1967) found ultrasonic disruption prevented precipitin band formation; however, band formation was restored by heating the sonicated antigen preparation. Sonication is a useful method to liberate internal antigens which generally are not strain specific (Humphrey and Vincent, 1965; Vincent and Humphrey, 1970).

Dudman (1964) was the first investigator to adapt gel diffusion to studies of Rhizobium. His investigation of the extracellular soluble antigens of two strains of R. meliloti indicated that the two strains shared all extracellular antigens except for those strain-specific fast-diffusing polysaccharides, which were useful for identification purposes. Since the two

strains did not cross-agglutinate he proposed that the strain-specific polysaccharides dominated their surfaces.

Humphrey and Vincent (1965) used gel-diffusion to show that whole-cells of R. trifolii strains grown on calcium-deficient medium yielded identical immunodiffusion patterns with mechanically disintegrated calcium-adequate bacteria. Earlier work by Vincent (1962) had shown that these strains required calcium for normal growth. The identical immunodiffusion patterns indicated that the walls of the untreated calcium-deficient bacteria were more fragile and the cells underwent autolysis with the release of their internal antigens.

In a later publication Humphrey and Vincent (1969) indicated that the somatic antigens of two strains of R. trifolii were strain specific. However, the internal antigens obtained by mechanically disrupting the cells were identical and could not be used to differentiate between strains.

Skrdleta (1969) utilizing gel-diffusion to study the serological relatedness of strains of R. japonicum divided the 11 strains into two somatic serogroups. The somatic antigens were more specific to differentiate between individual strains than those of flagella. Dazzo and Hubbel (1975) in contrast to the results obtained by Bushnell and Sarles (1939), Kleczkowski and Thornton (1944), and Koontz and Faber (1961) reported a correlation between serological properties and infectivity. They used immunodiffusion to analyze the antigenic relatedness of three infective and three non-infective strains: additional antigens were found in infective strains which were not found in non-infective strains.

C. Immunofluorescence (IF)

One of the most sensitive of the serological techniques available to study rhizobia is the fluorescent antibody (FA) technique. It allows for the visualization and investigation of the antigens of individual cells with the fluorescent microscope and requires only small quantities of both antigen and

antibody (Schmidt, 1973). In contrast both agglutination and immunodiffusion require large amounts of antigen and antisera to give a visible reaction.

The FA technique originally developed by Coons et al. (1942) to visualize pneumococcal antigens in mouse tissue, was successfully adapted to studies of Rhizobium by Schmidt et al. (1968). For the first time individual cells could be identified directly in culture, in nodules, and in soil be differentiated from numerous other organisms. Using this technique, the classical approach of autecology: the study of an individual organism in its natural environment, could be applied to the study of rhizobia, or to any other soil microorganism desired.

Although immunofluorescence (IF) can be used to rapidly type the contents of root-nodules its most valuable feature is its potential ability to identify specifically bacteria directly from soil (Bohlool and Schmidt, 1979). None of the other serological techniques can be used to perform this function.

Fluorescent antibody (FA) has been used to identify strains of rhizobia (Schmidt et al., 1968; Bohlool and Schmidt, 1970, 1973b; Jones and Russel, 1972, May, 1979), to identify the nodule-bacteria (Schmidt et al., 1968; Trinick, 1969; Bohlool and Schmidt, 1973b; Jones and Russel, 1972; Lindemann et al., 1974, May 1979), to detect doubly infected nodules (Lindemann et al., 1974, May 1979), to study Rhizobium in soil (Schmidt et al., 1968; Bohlool and Schmidt, 1970, 1973b; Vidor and Miller, 1979b and c), to study population dynamics of R. japonicum in the rhizosphere (Reyes and Schmidt, 1979), and to make quantitative studies of Rhizobium in soil (Bohlool and Schmidt, 1973a; Schmidt, 1974; Reyes and Schmidt, 1979; Vidor and Miller, 1979b and c).

D. Enzyme-Linked Immunosorbant Assay (ELISA)

ELISA is the latest serological technique to be adapted for use in the identification of rhizobia (Kishinevsky and Bar-Joseph, 1978; Berger et al., 1979). The ELISA technique provides a colorimetric method for the

identification of bacteria. A strain specific antiserum is conjugated to an enzyme such as alkaline phosphatase or peroxidase; and bacteria are coated with the enzyme-labelled antibody. After a period of incubation and subsequent washing, a chromogenic substrate is applied. The formation of an antigen-antibody complex is detected visually or spectrophotometrically. The ELISA is endowed with the specificity of antigen-antibody reactions and the sensitivity of enzyme-catalyzed reactions (Berger et al., 1979). It requires very small amounts of antiserum and no microscopic equipment is necessary. ELISA does not possess the flexibility of immunofluorescence; like agglutination and immunodiffusion, ELISA requires a purified antigen preparation, either from culture or from a root-nodule.

E. Antigens of Rhizobia

In general the antigens of cultures and nodules remain stable and antigenic stability is the major premise underlying the widely used serological practices described previously for serotyping root-nodules.

However, some differences between antigens of the nodule forms of Rhizobium and their parent cultures have been reported in the literature. Means et al. (1964) used antisera against cultured cells to examine the antigens of culture and nodule forms of 17 strains of R. japonicum. They found no detectable difference between the two forms among 15 strains. However, with one strain nodule-bacteria cross reacted with a wider range of antisera than the parent culture. Both heated and unheated preparations reacted the same.

Dudman (1971) used immunodiffusion to examine the antigens of cultures and nodule-bacteria of three strains of R. japonicum. Nodule-bacteria antigens from one strain lacked the full array of antigens of the cultured cells, and repeatedly formed spur reactions of partial identity with precipitin bands from the parent culture. Using this technique, the classical approach of autecology: the study of an individual organism in its natural environment,

could be applied to the study of rhizobia, or to any other soil microorganism desired.

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D. Enzyme-Linked Immunosorbant Assay (ELISA)

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Pankhurst (1979) could detect no differences in the antigens expressed by cultures and nodules of a number of strains of Lotus rhizobia analyzed by immunodiffusion. However, he noted that there may have been a structural difference between the two forms. Whereas cultures had to be heat treated before their antigens would diffuse through the gels, the nodule forms of several strains were readily diffusable without heating. Heat treatment of the nodule suspensions generally intensified the slow diffusing somatic precipitin bands. He proposed that the readily diffusable nodule-bacteria antigens might have been due to an alteration in structure, form, or amount (though not antigenically different) of LPS as proposed by van Brussel et al. (1977) for nodule-bacteria of R. leguminosarum.

No differences have been reported between nodule-bacteria and cultured cell antigens when stained with FA's prepared from the somatic antigens of cultured cells (Schmidt et al., 1968; Bohlool and Schmidt, 1973b; Lindemann et al., 1974, May 1979). However, no investigators have used immunofluorescence-adsorption to examine differences between nodule and culture forms of Rhizobium stained with fluorescent antibodies.

Serological markers also remain stable in soil. Brockwell et al. (1977) found serological markers to be unchanged during a three year investigation of rhizobia. Diatloff (1977) studied the stability of four rhizobial characters: colony color, effectiveness, sensitivity to four antibiotics, and antigenic stability. The antigenic and colony characteristics of the strains were not changed during a residence of five to twelve years in the soil. On the other hand, effectiveness and antibiotic sensitivity underwent slight modifications.

In summary the extensive use of serology to analyze rhizobia has revealed that heat-stable somatic antigens are more strain specific than those of whole cells (Koontz and Faber, 1961; Graham, 1963; Date and Decker, 1965; Means and Johnson, 1968; Schmidt et al., 1968; Humphrey and Vincent, 1969; Skrdleta, 1969; Dudman, 1971, 1977). However, exceptions do exist, gel diffusion of the

somatic antigens from eight strains of R. meliloti (Humphrey and Vincent, 1975) revealed seven of the eight strains had identical heat-stable somatic, as well as heat-labile antigens. In this instance serology could not differentiate between strains.

II. Intrinsic Antibiotic Resistance

Josey et al. (1979) used the variation in intrinsic resistance to low levels of eight antibiotics as a characteristic (an "antibiotic fingerprint") to identify 26 strains of R. leguminosarum. The major advantage of this method is that no alterations of the strain, which might interfere with its field performance are required. The fingerprint technique is a useful supplement to serological methods since, as with nutritional and biochemical tests, further delineation of strains that serologically cross-react may be possible. However, it is necessary to choose concentrations of antibiotics that will yield maximum strain differentiation. Unlike serological techniques this method requires isolation and culture steps which are time consuming.

III. Quantitative Techniques in Rhizobium Ecology

Only by studying the rhizobia directly in their natural habitat, rather than by indirect plant-dilution infection techniques (Date and Vincent, 1962) can the questions of survival, population densities, growth responses, interactions with other organisms, nutritional substrates, host specificity, and competition between strains for host sites be truly evaluated (Schmidt, 1978, 1979; Bohlool and Schmidt, 1979).

Although the need for a selective medium to enumerate Rhizobium has long been recognized (early literature reviewed by Fred et al., 1932), the physiological diversity of both the rhizobia and the resident soil microorganisms makes the prospects for the development of a truly selective medium specific for all rhizobia, unlikely (Schmidt, 1978). The work of Graham

(1969b) and Pattison and Skinner (1973) demonstrate the difficulties inherent in making selective media for Rhizobium.

The "selective medium" most often used to detect the presence of rhizobia in soil has been the plant itself. Enumeration by the plant-dilution infection assay (or Most Probable Number, MPN) (Date and Vincent, 1962) has been the basis for virtually all ecological studies dealing with the persistence of Rhizobium in soil and their response to rhizosphere conditions (Schmidt, 1978). Unfortunately there exists a high degree of statistical uncertainty in plant-dilution infection/MPN methodology (Alexander, 1965). In addition the existence of host Rhizobium incompatibilities resulting in nodulation failures are well documented (Caldwell and Vest, 1968; Masterson and Sherwood, 1974; Sherwood and Masterson, 1974). Nodulation by only certain strains of Rhizobium might skew results when using plant-infection assays. This cannot happen with direct microscopic enumeration of FA stained cells.

Before 1973 all serological techniques, used in studies of Rhizobium, were qualitative. No means existed for the direct enumeration of rhizobia in soils at natural population levels. Breed slide counts require high numbers of rhizobia (10^6 cells/gram) to be practicable (Bohlool, 1971; Schmidt, 1978). To work with natural populations it is necessary to remove the bacteria from interfering soil particles and to concentrate them for enumeration. Bohlool and Schmidt (1973a) described a soil release procedure and a quantitative membrane-filter FA technique (SRP), in which cells, recovered from soil on non-fluorescent membrane-filters and stained with the appropriate FA, were enumerated microscopically. The development of the SRP technique was a breakthrough in methodology for quantitative studies of microbial ecology in soil.

A. Quantitative Membrane-Filter Technique

Briefly, the quantitative membrane-filter (SRP) technique consists of dispersing by blending a soil sample in water. The soil suspension is then transferred to a narrow container, flocculant is added and the soil colloids allowed to settle. After settling, an aliquot of the supernatant is passed through a membrane-filter, stained with FA and the FA reactive bacteria enumerated (Bohlool and Schmidt, 1973a; Schmidt, 1974).

Since its development, the use of the quantitative technique in microbial ecology has not often been reported in the literature. Bohlool and Schmidt (1973a) followed the growth of a strain of R. japonicum (USDA 110) in autoclaved soil (Clarion) comparing filter counts to plate counts. The filter counts tended to underestimate at low cell numbers (approximately 30% of plate counts) and overestimate the plate counts at high numbers (approximately 150%), probably due to the accumulation of dead cells. The technique was used in a field study to examine the population level of the same strain in the rhizosphere of both inoculated and uninoculated (to examine the indigenous population) soybean plants. The rhizosphere levels of the uninoculated plants remained fairly constant (approximately 5 x 10^3/gram of soil) while the rhizosphere populations of inoculated plants were both higher and more variable (attributed to uneven inoculation).

Schmidt (1974) followed the growth of Nitrobacter winogradski in a partially sterilized soil. He compared growth with nitrate formation; the two parameters correlated well during the exponential phase of growth, however, nitrate formation continued to increase when the growth of the population apparently leveled-off.

Several reports exist in the literature describing problems in implementing the membrane-filter quantitative technique. Reed and Dugan (1978) used the quantitative method, with indirect immunofluoresence, to determine the distribution of methane oxidizing bacteria in sediments from Cleveland harbor. They recovered only 10% of the methane oxidizers in the sediments. With

several minor modifications Reyes and Schmidt (1979) obtained a 44% recovery, as compared to plate counts, of a strain of R. japonicum (USDA 123) from a sterilized Minnesota soil (Waukegan). The quantitative technique was originally developed using a different soil:strain combination (Clarion:USDA 110). Reyes and Schmidt (1979) did not compare recoveries of USDA 110 in sterilized Waukegan or USDA 123 in sterilized Clarion soil to determine if their problem with recovery was methodological, or related to the soil, or the strain. To aid recovery Vidor and Miller (1979a, b and c) substituted 1% $CaCl_2$ as a flocculant.

Wollum and Miller (1980) modified the density gradient centrifugation technique, used for studies of clay particles by Francis et al. (1972), to use for quantitative studies of Rhizobium. They examined the recoveries of two strains each, of the slow grower R. japonicum, and the fast grower R. phaseoli. High recoveries were obtained when the soils contained 10^8 - 10^9 cells/gram. Recoveries were better with temperate soils than with two South American Oxisols. The authors had difficulty in clearing the Oxisols; they suggested additional studies were needed.

B. Sorptive Interaction Between Microorganisms, Clay Particles and Soils

The predominance of the solid phase is one of the main characteristics that distinguishes soil from other microbial habitats (Marshall, 1976; Stotzky and Rem, 1966). In habitats which have wide liquid:solid ratios, such as the oceans, much of the microbial activity is associated with solid, particulate materials (ZoBell, 1943: Jannasch, 1967, 1970; Pearl, 1975). In fact, attachment may be advantageous to microorganisms living in dilute environments. Nutrients may concentrate at the interface because of differences in charge between the solid surface and the surrounding solution, as well as to

differences in the hydrophobic or hydrophyllic nature of the surface (ZoBell, 1943; Stotzky, 1966a and b; Fletcher, 1979).

Of the solid phase components in soil, the smallest mineral particles are the clays. It is due to their large surface area that clays exert the greatest influence on soil microorganisms (Stotzky and Rem, 1966; Stotzky, 1966a and b). Clay minerals are usually associated as aggregates or occur as coatings on larger particles (Brady, 1974). Clays differ from other particles normally present in natural microbial habitats in that they have an overall net negative charge, but also have positive charges at the broken edges. These charges are neutralized by ions from the surrounding solution, and by interactions with adjacent minerals (Brady, 1974). In many tropical soils the minerals and clay size particles are coated with an additional layer of oxides whose charges are pH dependent (Sanchez, 1976). The charges on these coatings vary with local soil conditions. Factors such as the type of clay, saturating ions, and the organization of the clay minerals within the soil matrix may be more important than the total amount of clay present in the soil habitat (Marshall, 1971; Stotzky, 19743).

The degree of sorption between microorganisms and soil particles is broadly related to the surface area and surface charge properties of the particles (Daniels, 1972). The positive and negative charges on microbial cells observed at different pH's (Daniels, 1972; Lamanna and Mallette, 1965; Marshall, 1967) are due to the degree of ionization of surface components. Therefore the overall charge on the cell is determined by the isoelectric points or pH's of these constituents. Most organic materials of biological origin have either no charge, or they are amphoteric and have a charge dependent on pH (Lehninger, 1970; Metzler, 1977).

Fast growing strains of Rhizobium (R. trifolii, R. meliloti, R. leguminosarum) exhibit a sharp increase in negative charge at high pH values (pH 11) and a slightly positive charge at low pH's (pH 2) when assayed by

electrophoretic mobility techniques (Marshall, 1967); this is generally true of most bacteria (Lamanna and Mallette- 1965; Daniels, 1972). The slow growing strains of Rhizobium (R. japonicum, R. lupini) possess either no charge at low pH (pH 2) or a negative charge at all other pH's. The rhizobia can therefore be separated into two groups on the basis of their surface ionogenic character (Marshall, 1967, 1968), as well as by differences in growth rate, G+C, flagellation (Jordan and Allen, 1974), carbohydrate utilization (Fred et al., 1932, Graham and Parker, 1964; Vincent, 1977), glucose-6-phosphate dehydrogenase (Martinez-Drets and Arias, 1972; Martinez-Drets et al., 1977), and internal antigens (Humphrey and Vincent, 1965; Vincent and Humphrey, 1970; Vincent et al., 1973).

Marshall et al. (1971) proposed two major mechanisms for the sorption of marine bacteria to glass surfaces: (1) a random attachment concerning both motile and non-motile bacteria which involved polymeric bridging between the cells and the solid substrate; (2) an instantaneous reversible mechanism involving only motile bacteria which they believed was due to electrostatic attractions and van der Waals forces (physico-chemical). Fletcher (1977) found that the attachment of a marine pseudomonad to polystyrene petri dishes as dependent on the number of cells present, the time allowed for attachment, the growth phase of the culture (cell age and morphology), and temperature. Log phase cultures had the greatest facility for attachment, followed by stationary and death phase cultures, respectively. She found that the results could be described by a model based on physical/chemical adsorption (Langmuir adsorption isotherms). The model indicated that non-biological processes were playing a major role in initial events of bacterial adhesion. Fletcher's model is similar to those describing molecular adsorption from solutions onto surfaces, in which the process is controlled by the concentration of the solution (or bacterial culture), time, and temperature. The relationship between solution concentration and extent of adsorption is termed the adsorption isotherm. A

Langmuir Isotherm assumes: (1) adsorption is limited to a monomolecular layer; (2) adsorption is localized so that adsorbed components are confined to specific sites; and (3) the heat of adsorption is independent of surface coverage (Fletcher, 1977).

Scheraga et al. (1979) found that bacteria added to autoclaved marine sediments were adsorbed almost instantaneously. They plotted their data according to Fletcher (1977) and obtained Langmuir-type adsorption isotherms. This would indicate that the initial events in bacterial adsorption to sediments are similar to those observed with polystyrene. Thus, it seems likely that both biological and nonbiological forces are involved in sorptive interactions between microorganisms and their environment.

Few reports exist describing actual sorptive forces in soil. The problem is not necessarily one of methodology but one of deciding which fractions in soil are most influential, for example clay, organic matter, clay:organic matter complexes, oxide coatings, all fractions interacting simultaneously, or particles of larger size coated with these substances. The factors which are most influential in sorptive processes in one soil may have no bearing on those operative in another soil.

Niepold et al. (1979) advanced a model system to describe various forces which may influence the recovery of bacteria from soil. Bacteria may attach to soil particles by capillary (Hattori, 1973; Hattori and Hattori, 1976), electrostatic and adhesive forces (Marshall, 1971). Niepold et al. (1979) reasoned that detachment of bacteria from soil particles might be influenced by the chemical properties of the extraction fluid. They adsorbed the hydrogen bacterium Alcaligenes eutrophus to three types of model materials with differing capillary properties ("Mosy", a porous material used for arrangements of cut flowers, pore sizes 0.1-0.5 mm in diameter), electrostatic properties (DEAE-cellulose), and adhesive properties, i.e. physico-chemical (125-200 mm diameter glass beads). In these studies A. eutrophus was more strongly held by

capillary forces than by adhesive forces. Based on viable counts they found recovery to be lowest when extraction fluids contained detergents such as Tween 80, Triton X-100, Sodium Dodecyl Sulfate (SDS), and sodium desoxycholate. Recovery was also low when extraction fluids contained organic solvents such as DMSO, or dioxane. The highest recoveries were obtained with Tris buffer (pH 7.5). The low recoveries with detergents and organic solvents were attributed to the toxic effects these compounds had on the cells. Litchfield et al. (1975) also found SDS to be toxic when used to quantitate bacteria in marine sediments by viable counts.

Once extracted from soil, bacteria must be separated and recovered for enumeration. Niepold et al. (1979) investigated various methods for the separation of bacteria from model materials and soils. Filtration of soil suspensions through filter paper, settling for eight hours, and slow speed centrifugation (325 X g, 5 minutes) resulted in the lowest recoveries. In contrast, letting the soil suspension settle for 15 minutes prior to dilution for plate counts, resulted in greater recoveries. A flocculant to remove the soil colloids from solution was never tried. They adsorbed eight strains of hydrogen bacteria of differing size, motility, and slime formation to each of three soils. The efficiency of extraction of the adsorbed bacteria was strongly dependent on the bacterial strain used but was not significantly dependent upon soil type.

Rubertschik et al. (1936) used plate counts to demonstrate the ability of sediments to remove bacteria from suspension. The degree of sorption (e.g. removal) varied with the sediment and the species of bacteria. Shaking the mixtures for one minute did not increase desorption, i.e. the viable counts did not increase.

Hornby and Ullstrop (1965) found that for dilution plating from soil suspensions, agitation by blending or rapid stirring gave better sampling precision than shaking with a reciprocating shaker. They also found that a

viscous solution, such as 1% carboxy-methyl cellulose or 0.2% agar gave better reproducibility than soil suspended in water. Sodium hexa-meta-phosphate (Na-HMP) is a powerful soil deflocculant (Michaels, 1958; Lahav, 1962). The many negative charges of phosphate ions combine with the broken edges of clay particles (positively charged) and neutralize their charges, thus soil particles are dispersed by mutual repulsion. Gamble et al. (1952) briefly blended (45 seconds) 10 grams of soil in 100 mls of 5% NaHMP, added 400 mls of water to produce a total dilution of 1:50, and then blended for another two minutes before plating. They felt this procedure would release the bacteria for plate counts. However, they never compared their method with any others.

Both Balkwill et al. (1975) and Faegri et al. (1977) used a combination of sequential homogenizing procedures with blendors, and sedimentations by centrifugation to extract and concentrate bacteria from soils. Faegri et al. (1977) enumerated bacteria extracted from Norwegian soils by direct counts of acridine orange (AO) stained cells (AO is a non-specific fluorescent dye) recovered on black membrane-filters. Depending upon the soil type almost equal numbers of cells were obtained in the first two extractions. Fewer cells were recovered following a third extraction.

From the foregoing discussion, it appears that there are many different opinions as to what method is best to recover and enumerate bacteria from soil. Depending upon the type of counting procedure to be employed, the type(s) of microorganism(s) to be enumerated and the type of soil which the bacteria inhabit, it is problematical whether only one or more of the described procedures could work. Except for Wollum and Miller (1980) all the investigations cited above have dealt mainly with temperate soils. There is a need, therefore, to determine which methods will function in tropical soils for the quantitative recovery of Rhizobium using the FA-membrane filter procedure.

CHAPTER 3

SEROLOGICAL ANALYSIS OF CHICKPEA *RHIZOBIUM*

Introduction

Serology has been used extensively in many taxonomic and ecological investigations of the Rhizobiaceae. The fluorescent antibody (FA) method is the most flexible of the serological techniques since it permits the investigation of *Rhizobium* in its several habitats (Schmidt, 1973; Dudman, 1977; Bohlool and Schmidt, 1979). The gel diffusion technique has also been extensively applied to taxonomic (Dudman, 1964, 1971; Gibbins, 1967; Humphrey and Vincent, 1965, 1969, 1975; Vincent and Humphrey, 1968, 1970, 1973) and ecological (Dudman and Brockwell, 1968; Dudman, 1977; Vincent, 1977) investigations of rhizobia. These serological investigations have emphasized the marked somatic heterogeneity of the Rhizobiaceae. When groups of microorganisms which have not been investigated previously are to be studied serologically, it is necessary to determine their degree of serological relatedness; ecological applications of fluorescent antibody (FA) methodology require an especially high degree of serological specificity (Schmidt, 1973).

Cicer arietinum L. (chickpea, garbonzo) is an important pulse crop; and it is the third most widely-grown grain legume in the world (van der Maesen, 1972). It is grown extensively in the Middle East; and in many regions of India it is the most important legume grown (Medhane and Patil, 1974; van der Maesen, 1972). Neither *Cicer* rhizobia nor the host have received much attention in their literature.

This report describes a serological investigation of 27 strains of Cicer rhizobia with seven anti-chickpea *Rhizobium* FA's. Immunofluorescence adsorption (Belly et al., 1973) and immunodiffusion were used to more fully evaluate the relationships of four of the strains used to produce FA's. In addition, the antigenic properties of two strains and their corresponding nodular forms were

investigated using both immunofluorescence and immunodiffusion. As a further means of differentiation and identification, 15 strains were tested for their resistance to low levels of nine antibiotics (Josey et al., 1979).

Materials and Methods

I. Source and Maintenance of Cultures

Table 1 lists the strains, their origin and source, and a detailed pedigree of the Cicer rhizobia used in this study. All strains were maintained on a yeast extract-mannitol medium (YEMS) (Bohlool and Schmidt, 1970) (see Appendix Table 19a for composition). For experimental use strains were grown in YEMS broth. All media were sterilized by autoclaving at 121°C for 20 minutes.

II. Production of Root-Nodules

Seeds of Cicer arietinum L. variety JG-62 (provided by Dr. P. J. Dart ICRISAT, Hyderbad, India) were surface sterilized with 4% calcium hypochlorite for 15 minutes, rinsed six times in sterile water, and germinated aseptically in petri dishes containing 1% water agar. The seedlings were planted in modified Leonard jars (Leonard, 1943), which contained sterile vermiculite and a nitrogen-free nutrient solution (Broughton and Dilworth, 1971) and had the following composition: for each 10 liters of complete culture solution 5.0 m. each of solutions 1 to 4 (see Appendix Table 20), was added to 5.0 liters of water and diluted to 10 liters. The pH was adjusted to 7.0.

Each of three seedlings in a jar received 1 ml of a cell suspension containing 1 x 10^6 rhizobia/ml; two jars were left uninoculated. A 2 cm layer of sterile perlite was added to the surface of all jars. Plants were grown under controlled conditions in a growth chamber (Model M-31 Environmental Growth Chambers, Chagrin Falls, Ohio) with a 14 hour day and a day/night temperature of 29^0C/24^0C. The nodules were harvested after three weeks and used immediately for immunofluorescence and immunodiffusion. Nodules for immunodiffusion were

Table 1. Sources of cultures

Culture collection number	Culture and Pedigree	Source
B58	Nitragin 27A2 (USDA 691 = Rothamsted 3828 = ICRISAT 3828)[x+]	
B59	Nitragin 27A3 (USDA 3100)[x]	
B60	Nitragin 27A7 (O. N. Allen 736)[x]	Dr. J. C. Burton
B61	Nitragin 27A8 (Mexico)[x]	Nitrigin Co.
B62	Nitragin 27A11 (Mexico)[x]	Wisconsin
B107	TAL-1008 (= Dardarwall Ca 2)[Y]	
B109	TAL-1010 (= Dardarwall Ca 1002)[Y]	
B111	TAL-1012 (= Dardarwall Ca 141 St.)[Y]	
B148	TAL-263 (= Nu 191)[*]	
B149	TAL-385 (= CB 1189 = Rothamsted 3827 = ICRISAT 3827 = TAL-619-- origin USDA 3HOa1)[+*¢]	
B150	TAL-480 (= UASB 67)[*]	
B151	TAL-619 (same as TAL-385)[**]	
B152	TAL-620 (= CC 1192 = Rothamsted 3889 = ICRISAT 3889, -ex CSIRO Canberra, -ex Israel)[+*¢]	NifTAL Culture Collection University of Hawaii
B153	TAL-621 (= ICRISAT DNRa-1)[*]	
B154	TAL-622 (= ICRISAT Cicer-5)[*]	
B155	TAL-623 (= ICRISAT CA-7)[*]	
B156	Nitragin 27A5	Dr. A. H. Gibson
B158	ATCC 11444 (= USDA 3HOa1)[%]	Canberra, Australia
B160	USDA 3HOa1 (obtained from M. S. Raju)[#]	
B161	USDA 3HOa2 (Wis. No. 67, Univ. of Wisconsin)[#]	Dr. D. F. Weber U.S. Department
B162	USDA 3HOa5 (Univ. of Wisconsin, 1944)[#]	of Agriculture
B163	USDA 3HOa6 (Ithaca, N. Y.)[#]	Maryland
B164	USDA 3HOa8 (P. A. Ark, Univ. of Calif.)[#]	
B165	USDA 3HOa9 (P. A. Ark, Univ. of Calif.)[#]	
B166	USDA 3HOa10 (P. A. Ark, Univ. of Calif.)[#]	
B167	USDA 3HOa12 (Univ. of Calif.)[#]	
B185	Egypt 57 (nodule isolate, parent culture unknown)	Dr. V. G. Reyes, formerly of the NifTAL Project Hawaii

[x] (Information courtesy of Dr. J. C. Burton, Nitragin Co., Wisconsin)
[+] (Information courtesy of Dr. P. J. Dart, ICRISAT, Hyderbad, India)
[Y] (Information courtesy of Dr. P. Samasegaren, NifTAL Project, Hawaii)
[*] (NifTAL Rhizobium Catalog of Selected Strains, Reyes and Samasagaren, 1978)
[¢] (Corbin et al., 1977)
[%] (American Type Culture Collection Catalogue of Strains)
[#] (Information courtesy of Dr. D. F. Weber, USDA Beltsville, Maryland)

crushed in filtered saline containing thimerosal (1:10,000) and were either left untreated or received a heat treatment similar to culture antigens.

III. Serological Procedures

A. Immunofluorescence

Fluorescent antibodies were prepared against the somatic components of TAL-480, USDA 3HOa9, TAL-619 and TAL-620, and Nitragin strains 27A3, 27A8, 27A11. Preparation of antisera and conjugation procedures were done according to Schmidt et al. (1968) except cultures were grown in YEMS broth for three days instead of seven days. The rabbits which were first injected with somatic cell antigens were later injected with whole cells to develop antisera for immunodiffusion. No whole cell serum was prepared for strain USDA 3HOa9.

Smears from pure cultures and nodules were stained by the method of Schmidt et al. (1968) using gelatin-rhodamine isothiocyanate conjugate to control non-specific staining (Bohlool and Schmidt, 1968). Stained smears were observed with a Zeiss universal microscope equipped for epifluorescence and transmitted dark field. Incident illumination was from an HBO-200 (OSRAM) light source with a fluorescein isothiocyanate (FITC) filter. Transmitted dark field was from a l2v quartz-halogen lamp, using a Zeiss Ultra-condenser.

Cross-reactions of FA-stained smears were quantitated by subjective assessment of the degree of fluorescence, from 0 to 4+. Immunofluorescence adsorption (Belly et al., 1973) was used for finer antigenic analysis and to determine serologic relatedness. The somatic antigens used for adsorption were prepared from three-day old shake-flask cultures (Schmidt et al., 1968), distributed to three tubes and pelleted by centrifugation. The pellet from one tube was resuspended in 6 ml of undiluted FA and incubated at room temperature. Thimerosal was added as a preservative, final concentration 1:10,000). The fluorescent agglutinate was removed after four hours, the supernatant transferred to a fresh pellet, and incubated as before. The adsorption cycle was carried out

three times, with the final incubation proceeding overnight at 4^0C. The adsorbed FA was used at a dilution of 1:4.

B. Immunodiffusion

Antisera for immunodiffusion were prepared from whole cell antigens grown on solid, defined medium (Vincent, 1970) (see Appendix Table 19b) for composition. The same rabbits used for production of somatic antisera for FA were injected intramuscularly with one ml (0.5 ml each hip) of an equal mixture of antigen and Freund's complete adjuvant (Difco). After three weeks two mls of the same culture was injected intravenously without adjuvant. The rabbits were bled one week later.

Immunodiffusion was performed by the method of Dudman (1971). Cell suspensions used in gel diffusion experiments contained approximately 10^{10} cells per milliliter, equivalent to approximately 25 mg dry weight cell mass per ml. Crushed nodule suspensions were prepared in saline (Dudman, 1971) to which thimerosal was added (1:10,000) as a preservative. For the study of somatic antigens, cell suspensions were heated in tightly capped tubes (Cryotubes, Vanguard International, Neptune, N.J.) for two hours in a water bath at 100^0C (Skrdleta, 1969). The gels were incubated at room temperature (about 23^0C) for one week. Results were recorded photographically.

IV. Intrinisic Antibiotic Resistance

The method used was similar to that described by Josey et al. (1979). The use of a multiple inoculator allowed for the simultaneous inoculation of up to 27 cultures per petri plate. In practice, 15 cultures were replicated four times per antibiotic concentration used. Fresh solutions of antibiotics were added to cooled (48^0 C), melted YEMS medium (Bohlool and Schmidt, 1970, see Appendix Table 19a) to give final concentrations (mg/ml) of: chloramphenicol .012, .025; streptomycin sulfate .0025, .010; tetracycline hydrochloride .004 (antibiotics

obtained from Calbiochem); kanamycin sulfate .010; naladixic acid .010; neomycin sulfate .0025; polymyxin B sulfate .020; rifampin .006, .010; vancomycin .0015, .005 (antibiotics obtained from Sigma). In the tables the names of the antibiotics are abbreviated to the first three letters. Antibiotic stock solutions were prepared in sterile distilled water at a concentration of 10 mg/ml, except for chloramphenicol (10 mg/ml in 95% ethanol), naladixic acid (10 mg/ml in 1N NaOH, and rifampin (10 mg/ml in methanol). Petri plates contained 25 ml of medium.

Results and Discussion

All Rhizobium species or inoculation groups examined so far have been found to contain strains that are serologically distinct, as well as strains that share cross-reactive antigens, i.e. no Rhizobium species is serologically homogeneous (Dudman, 1977). The chickpea rhizobia are no exception. Twenty-seven strains of Cicer rhizobia were tested with seven anti-chickpea Rhizobium FA's. The four serogroups defined by the resulting immunofluorescent reactions are listed in Table 2. Those strains that failed to react with any FA were designated Serogroup V. No cross-reactions were detected with 19 other non-Cicer rhizobia. Serogroups I and IV were highly specific for their homologous antigens, Nitragin 27A3 and USDA 3HOa9, respectively. Serogroup II contained 15 strains which cross-reacted at maximum fluorescence (4+) with FA's TAL-619, TAL-620, and Nitragin 27A8 and 27A11.

The large number of cross-reactions between strains of Serogroup II were quite unexpected. By tracing the histories of a number of these strains (Table 1) it was determined that several of them originated from one culture. The problem arose from the use of multiple collection numbers for the same culture (a phenomenon which should be of concern to those working with Rhizobium, especially curators of culture collections). For example, six collection numbers have been applied to USDA 3HOa1 (which itself originated in India). It is the same as ATCC

Table 2. Immunofluorescence reactions of chickpea rhizobia*

Strains	Fluorescent Antibodies			
	FA Nitragin 27A3	FA Nitragin 27A8‡	FA TAL-480	FA USDA 3H0a9
Nitragin 27A2	-	4+	-	-
Nitragin 27A3	[4+]†	-	-	-
Nitragin 27A5	-	4+	-	-
Nitragin 27A7	-	4+	-	-
Nitragin 27A8	-	[4+]	-	-
Nitragin 27A11	-	4+	-	-
ATCC 11444	-	4+	-	-
TAL-263	-	-	-	-
TAL-385	-	4+	-	-
TAL-480	-	-	[4+]	-
TAL-619	-	4+	-	-
TAL-620	-	4+	-	-
TAL-621	-	4+	-	-
TAL-622	-	-	4+	-
TAL-623	-	-	-	-
TAL-1008	-	-	-	-
TAL-1010	-	-	-	-
TAL-1012	-	-	-	-
USDA 3H0a1	-	4+	-	-
USDA 3H0a2	-	4+	-	-
USDA 3H0a5	-	4+	-	-
USDA 3H0a6	-	4+	-	-
USDA 3H0a8	-	4+	-	-
USDA 3H0a9	-	-	-	[4+]
USDA 3H0a10	-	-	-	-
Egypt 57	-	-	-	-
	Serogroup I	Serogroup II	Serogroup III	Serogroup IV§

* See Table 1 for pedigree of strains of Cicer rhizobia

‡ FA27A11, FA TAL-619, FA TAL-620 gave the same reactions

† [ ] = Homologous

§Serogroup V = nonreactive strains

11444, Rothamsted 3827, ICRISAT 3827, CC-1189, TAL-385, and TAL-619. Several of the other strains in this serogroup may be related, but an incomplete pedigree precludes discerning such a relationship. Therefore six of the cross-reactions were only apparent.

Nitragin strains 27A8 and 27A11 (isolated in Mexico in 1971) and NifTAL strains TAL-619 (source = USDA 3Hoa1, ex-India M.S. Raju, origin ?), and TAL-620 (ex-Israel, origin ?) (see Table 1) although from different sources and isolated in different parts of the world are serologically identical by immunofluorescence (Table 2), immunofluorescence-adsorption (Table 3) and immunodiffusion (Figure 1A, B). These strains, therefore, constitute one serotype. Normally serological cross-reactions are of a lower titer than homologous reactions (Eisen, 1974). Since these four strains had identical cross-reactive titers, and since they produced identical intrinsic antibiotic sensitivity patterns (Table 4) they are all probably derived from the same strain. This effective nodulator may have been disseminated around the world by various researchers. Further biochemical tests, or perhaps ribosomal protein mapping could elucidate the relationships of these strains. A more complete history of a number of these strains would be the easiest way of determining potential relationships between those that cross-react.

The antigenic relationship between the cultured cells and bacteroid forms of rhizobia is a subject of great interest. Means et al. (1964), and Dudman (1971) working with strains of R. japonicum, and Pankhurst (1979) working with strains of Lotus rhizobia have examined this relationship. Means et al. (1964) examined the cultured and nodule forms of seventeen strains of R. japonicum by agglutination and found no detectable difference between the two forms in fifteen strains. However, in one strain the nodule-bacteria failed to cross-react with the homologous antiserum. In another strain, the nodule form cross-reacted with a wider range of anitgera than the cultured cells. Dudman (1971) compared the antigens of nodule-bacteria and cultured forms of three strains of R. japonicum

Table 3. Measure of similarity of the somatic antigens of 4 strains of chickpea Rhizobium from Serogroup II by FA/Cross-adsorption

FA Adsorbed	Absorbing Antigen	Immunofluorescent Reactions of Test Antigens N/Adsorbed FA			
		Nitragin 27A8	Nitragin 27A11	TAL-619	TAL-620
	Control‡	3+	3+	3+	3+
	27A8	-	-	-	-
FA-27A8*	27A11	-	-	-	-
	TAL-619	-	-	-	-
	TAL-620	-	-	-	-

* FA-27A11 and FA-TAL-619 produced identical patterns (FA-TAL-620 was not used)

‡ Control - no adsorbing antigen

Figure 1. A - Identical immunodiffusion patterns of four cross-reacting strains of chickpea rhizobia from Sero-group II, used for antiserum production.

Center well = Nitragin 27A8 antiserum, Well 1 = Nitragin 27A8, Well 2 = Nitragin 27A11, Well 3 = TAL-619, Well 4 = TAL-620. (All antigens heat treated 2 hours, 100^0C).

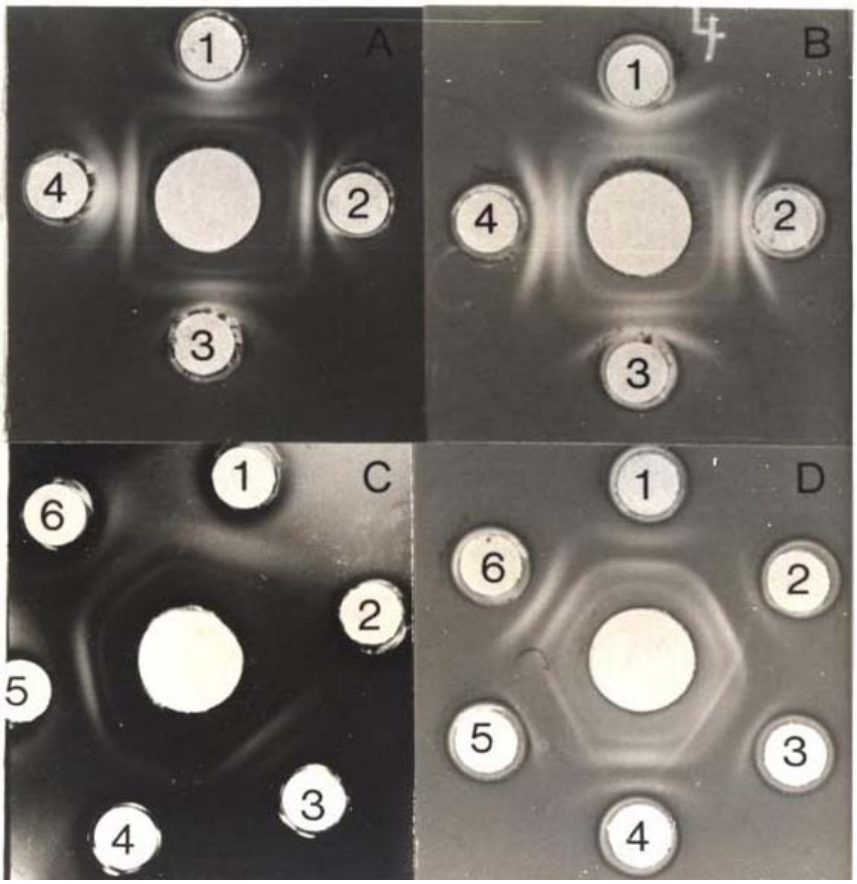
B - Antigen wells same as for A. Center well = TAL-620 antiserum.

C - Comparison of Nitragin 27A3 antigens from culture and from nodules.

Center well = Nitragin 27A3 antiserum, Wells 1 & 4 = antigens from solid defined medium (heat-treated 2 hours, 100^0C), Well 2 = antigens from untreated broth cultures, Well 3 = broth culture (heat treated 2 hours, 100^0C), Well 5 = antigens from crushed nodules (untreated), Well 6 = antigens from crushed nodules (heat treated 2 hours, 100^0C).

D - Comparison of TAL-620 antigens from culture and from nodules.

Center well = TAL-620 antiserum, Wells 1 & 4 = antigens from solid defined medium (heat treated 2 hours, 100^0C), Well 2 = antigens from crushed nodules (heat treated 2 hours, 100^0C), Well 3 = antigens from crushed nodules (untreated), Well 5 = antigens from broth culture (untreated), Well 6 = antigens from broth culture (heat treated 2 hours 100^0c).


A
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using gel diffusion. The nodule form of one of the strains lacked the full array of antigens associated with the cultured form. With a second strain no antigenic differences were detected between the two, while the nodule form of a third strain occasionally yielded an extra precipitin band. Pankhurst (1979), investigating Lotus rhizobia, found no differences in the total array of antigens expressed by the two forms. However, in contrast to the cultured cell forms of these strains, he found that the nodule form of several strains required no pre-treatment to give strong somatic precipitin bands.

The immunofluorescence reactions of nodules containing Nitragin 27A3 or TAL-620 paralleled the reactions of their parent cultures. Although no differences were apparent in the amount of fluorescence between nodules and cultures (i.e. 4+) a difference was detected in the quality of fluorescence. Whereas the fluorescent outline of cells from culture was sharp and well defined, the fluorescent surface of the nodule-bacteria appeared diffuse and thick, perhaps indicating a difference in cell wall structure between the two forms. Nodule bacteria have long been known to be pleomorphic (Fred et al., 1932). The shapes of the Cicer nodule-bacteria were different from their parent cultures. The parent culture of TAL-620 contained rods (dimensions 3 x 1 µm, ± 0.7 x 0.1 µm) (see Figure 2) while the nodule bacteria were spherical (diameter = 1.7 ± 0.5 µm) (see Figure 3). The cultured form of Nitragin 27A3 had dimensions 2.6 x 1 µm ± 0.7 x 0.1 µm (see Figure 4) while the nodule bacteria tended to be thickened rods slightly larger than the parent culture 3 x 1.3 ± 0.5 x 0.2 µm (see Figure 5).

Serological differences between Rhizobium in culture and in nodules have been shown to occur (Means et al., 1964; Dudman, 1971; Pankhurst, 1979), and van Brussel et al. (1977) showed that the cell walls of nodule-bacteria of R. leguminosarum have different amounts of LPS than cell walls obtained from cultures grown on regular media. The two strains of chickpea Rhizobium from nodule-bacteria and from culture exhibited differences in quality of

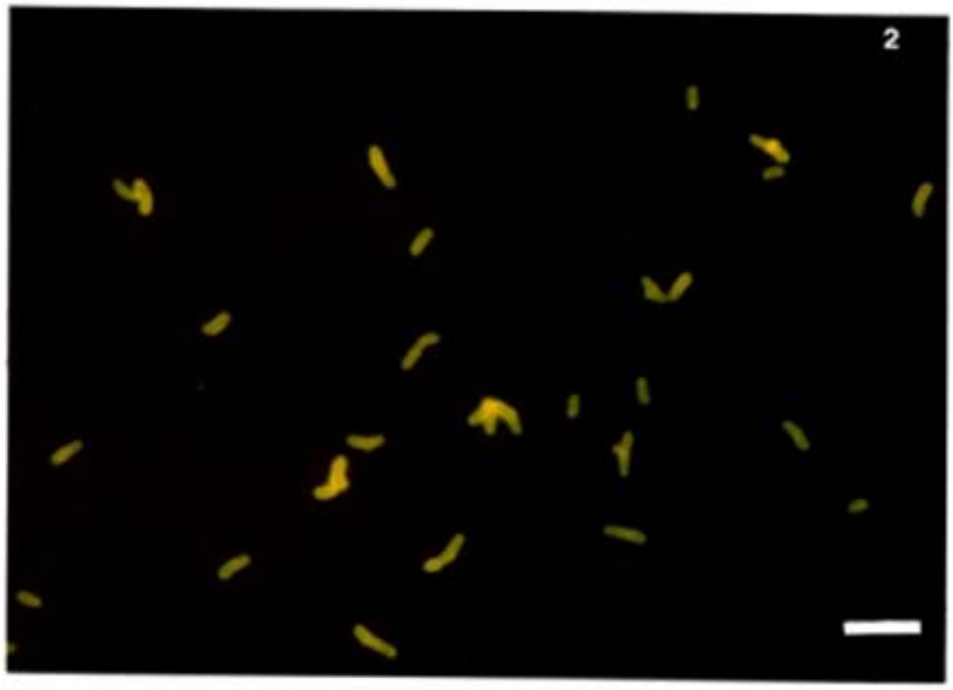
immunofluorescence. For these reasons a study, using immunodiffusion, was undertaken to investigate the antigens of these two strains of chickpea Rhizobium from culture and from nodules.

If Nitragin 27A3 antigens (Serogroup I), grown on solid defined medium (Appendix Table 19a), were left untreated no precipitin bands developed. However, untreated TAL-620 antigens (Serogroup II) produced one precipitin band close to the antigen well. After Nitragin 27A3 antigens were heat treated for two hours, three precipitin bands developed. Similarly, heat-treated TAL-620 antigens produced three precipitin bands after the two hour heat-treatment; the outermost band produced reactions of identity with the single band from the unheated antigen wells. For both Serogroups I and II no differences were noted in precipitin patterns of heat-treated antigens obtained from YEMS broth (Appendix Table 19a), solid defined medium (Appendix Table 19b), or a heated saline suspension of crushed nodules (see Figure 1C,D). Unheated broth cultures produced variable patterns depending upon the age of the culture. Young broth cultures (unheated) produced patterns similar to unheated cultures grown on solid defined media (not shown in Figures). Older cultures, especially TAL-620, yielded one band close to the antigen well and a unique heat labile band. Unheated TAL-620 nodule suspensions also produced a unique pattern (Figure 1D). In addition to the three bands found in both heated nodules and cultures unheated nodule suspensions released a unique heat labile band. Since somatic antigens are known to be heat stable (Koontz and Faber, 1961; Date and Decker, 1965; Skrdleta, 1969; Dudman, 1977), this additional band may have been an internal, heat labile antigen released from lysed nodule bacteria. The TAL-620 broth culture may have been too old and many cells may have lysed. Unheated antigens from defined agar media formed one band close to the antigen well and no heat labile, fast diffusing antigens were observed.

The identification of strains of Rhizobium in soil or nodules using serology is only reliable when no cross-reactions are known to occur. Where

Figure 2. TAL-620 broth culture, mid-exponential phase. Cells stained with homologous FA. Note typical rod shape. (scale = 4 µm)

Figure 3. TAL-620 nodule smear, stained with FA prepared against somatic antigens of TAL-620 from culture. Note round shape of the nodule-bacteria. (scale = 4 µm)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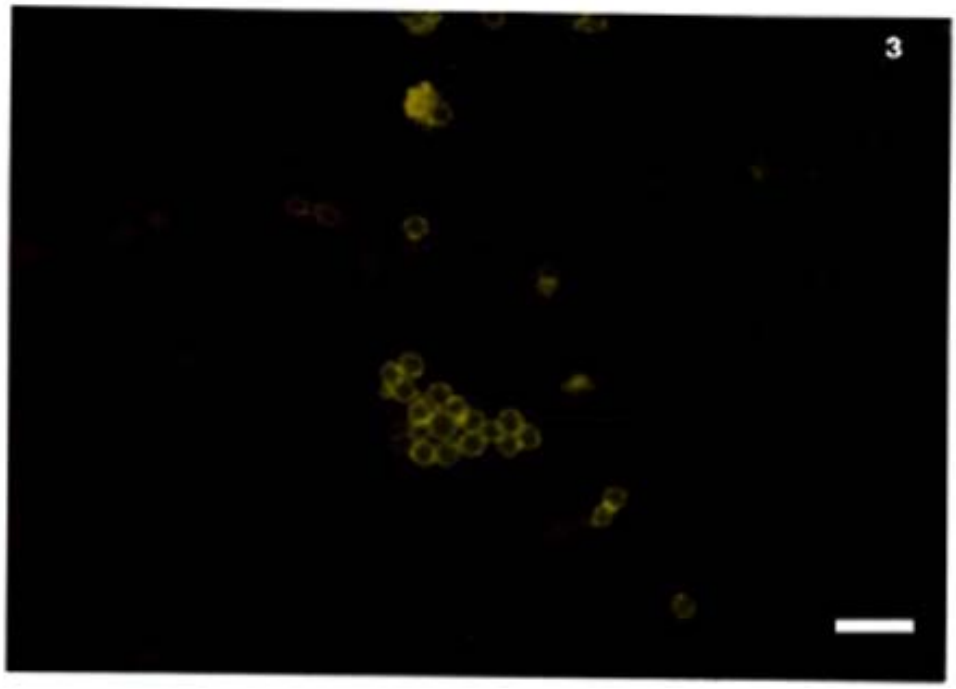
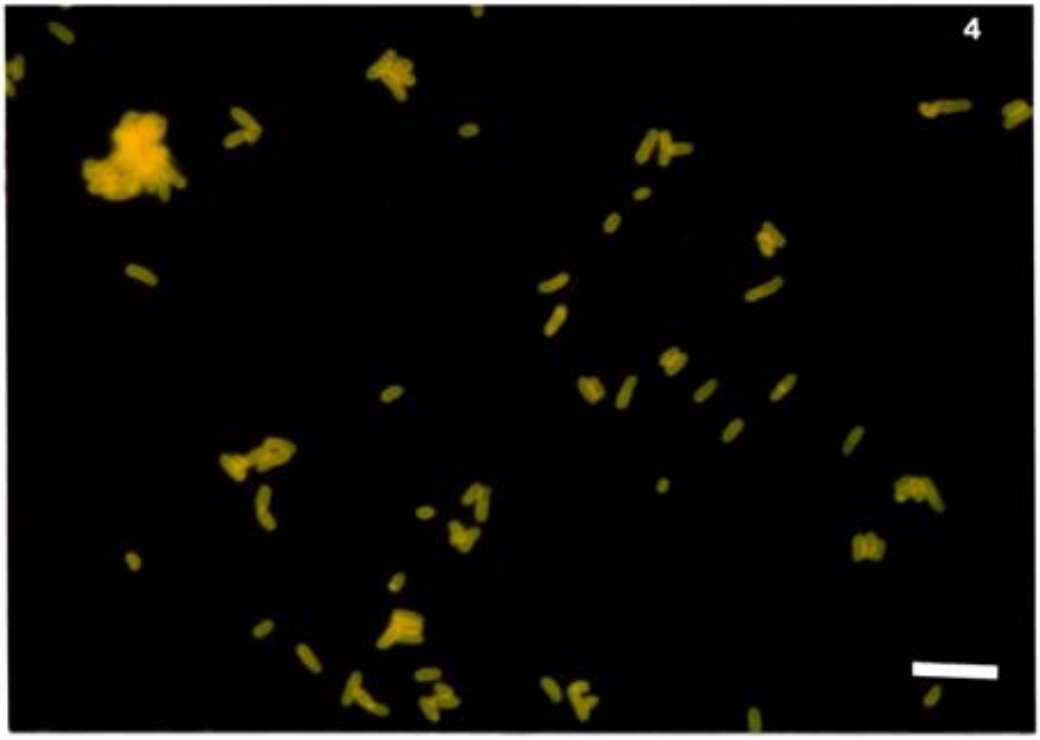

3

Figure 4. Nitragin 27A3 broth culture, mid-exponential phase. Cells stained with homologous FA. Note typical rod shape. (scale = 4 µm)

Figure 5. Nitragin 27A3 nodule smear, stained with FA prepared against somatic antigens of Nitragin 27A3 from broth culture. Note pleomorphic shape of nodule-bacteria. (scale = 4 µm)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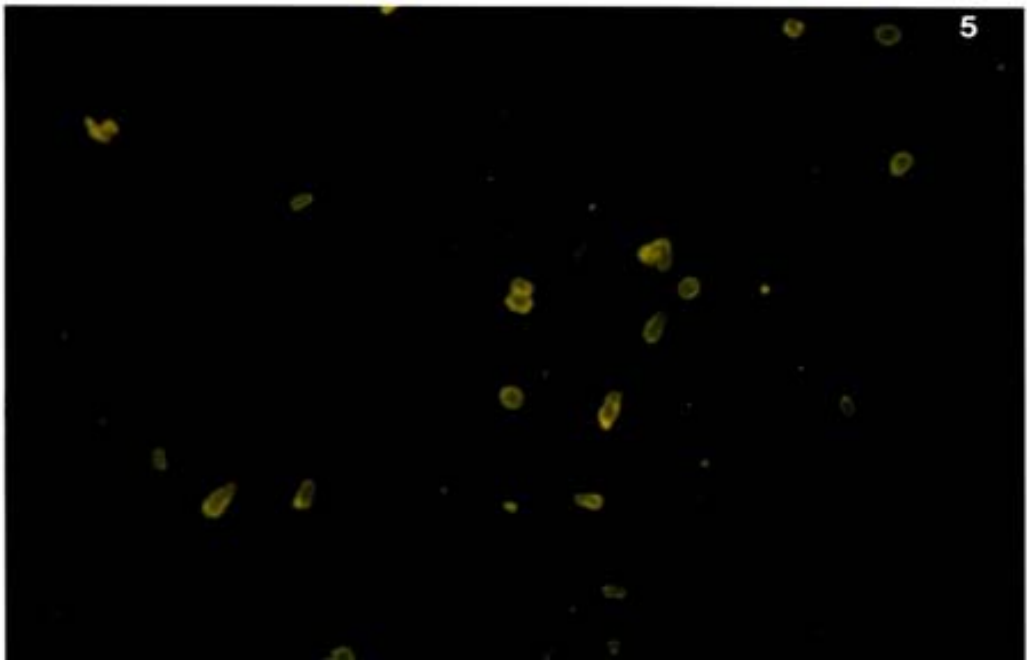

5

strains do cross-react another method of identification is required. The use of intrinsic antibiotic resistance (Josey et al., 1973) is a simple and useful technique to determine the similarity of strains. The results of such tests with Cicer rhizobia (Table 4) agree with observations made by Vincent (1977) that strains identical serologically can be different in other properties. Serology should not be the sole criterion to determine strain interrelationships. Within Serogroups II and III, where strains appeared similar or identical by immunofluorescence, different antibiotic fingerprints were obtained which indicated physiological dissimilarities between strains (Table 4). In some cases several strains produced identical resistance patterns and were grouped together.

Selection of the optimum antibiotic concentration to permit maximum differentiation between members of a species of Rhizobium is important. Josey et al. (1979) suggest that concentrations of antibiotics useful to differentiate between strains of R. leguminosarum were not as useful to differentiate between strains of R. phaseoli; to achieve this, higher antibiotic concentrations may have been required. The simultaneous testing of several antibiotic concentrations should permit selection of the proper concentrations. The concentrations of several antibiotics in this study were not optimum, as can be judged from the number of completely resistant and sensitive strains tested. For resistant strains higher concentrations of antibiotics would have been necessary to observe sensitivity. Where all strains were sensitive to a given concentration, a lower level of antibiotic should have been used.

Summary and Conclusions

The serological reactions of Cicer rhizobia were species specific and generally strain specific. The occurrence of a large number of apparent cross-reactions was due to the same strain (USDA 3Hoal) having multiple accession numbers from various Rhizobium collections.

Table 4. Summary of antibiotic resistance patterns for some chickpea *Rhizobium* strains used in this study

Serogroup #	Pattern #	Culture	Chl .012*	Chl .025	Kan .010	Nal .010	Neo .0025	Pol .020	Rif .005	Rif .010	Str .0025	Str .010	Tet .004	Van .0015	Van .005
II	1	Nitragin 27A2 Nitragin 27A8 Nitragin 27A11 TAL-385/-619 TAL-620	+	-	+	+	+	+	+	-	+	+	-	+	+
I	2	Nitragin 27A3	+	-	+	+	+	+	sl.	-	+	+	-	+	+
II	3	Nitragin 27A7	sl.	-	+	+	+	+	+	-	+	+	-	+	+
II	4	Nitragin 27A5 USDA 3H0a1	+	-	+	+	+	+	+	+	+	+	-	+	+
III	5	TAL-480	-	-	+	+	+	+	-	-	-	-	-	+	+
III V	6	TAL-622 USDA 3H0a10 USDA 3H0a12	-	-	+	+	+	+	+	-	-	-	-	+	+
IV	7	USDA 3H0a9	-	-	+	+	+	+	+	+	+	+	-	+	-
V	8	TAL-1012	+	-	+	+	-	+	-	-	+	+	-	sl.	-

* Antibiotic concentration (mg/ml)

sl. = slight growth

\+ = growth -- resistant

\- = no growth -- sensitive

The antigens from cells in nodules of two strains, Nitragin 27A3 and TAL-620, had different structures than their corresponding cultures. Nodule antigens were freely diffusable in immunodiffusion gels while antigens from culture required a preparative heat treatment. Pankhurst (1979) found Lotus nodule-bacteria to behave similarly.

A number of strains were screened for resistance to low levels of nine antibiotics. Strains which cross-reacted serologically in some cases produced identical patterns while in others they produced unique patterns. This would indicate a physiological heterogeneity exists within these serologically cross-reactive strains.

CHAPTER 4

PROBLEMS IN RECOVERING FAST-GROWING RHIZOBIA FROM TROPICAL SOILS FOR IMMUNOFLUORESCENT (IF) ENUMERATION

Introduction

Microbial ecologists require accurate techniques to quantify soil microorganisms. Such techniques help provide estimates of biomass, growth rates in soil and growth responses to environmental variables. Although plate counts are one of the simplest and perhaps most highly exploited of the quantitative techniques, they are particularly inadequate for soil microorganisms. It is impossible even to approximate natural environmental parameters in culture media (Brock, 1971; Schmidt, 1978). In addition there exists a discrepancy between numbers of microorganisms indicated by colony counts and those obtained by direct microscopic counts. Direct counts of soil bacteria usually result in total numbers at least three times greater than those obtained by plate counts (Stotzky, 1972; Faegri, et al., 1977). In one case up to 100 times more bacteria and six times more fungi were estimated by a direct microscopic examination of soil (Skinner, et al., 1952). Similar discrepancies have been observed for the natural bacterial flora in seawater (Jannasch and Jones, 1959).

Attempts have been made to produce media for the selective enumeration of Rhizobium directly from soil (Graham, 1969b; Pattison and Skinner, 1973; Barber, 1979). However, the biochemical diversity of these agriculturally important bacteria prevents the formulation of a medium truly selective for all strains (Schmidt, 1978). Because of the lack of a selective medium or other adequate procedures, the plant-infection technique has been the basis for virtually all ecological studies of the root-nodule bacteria (Schmidt, 1978). This method relies on the legume to act as a selective agent for Rhizobium. Plant-infection is both cumbersome, since a large number of seedlings is required, and time consuming, since one has to wait for the seedlings to grow and nodulate.

Bohlool and Schmidt (1973a) developed a soil release procedure (SRP) for the rapid quantification of specific strains of Rhizobium directly from soil. This procedure takes advantage of the specificity of immunofluorescence to detect and identify the desired organism from others present, and the ability of membrane filters to concentrate bacteria which are released into solution from the soil matrix. When applied to studies of slow-growing Rhizobium in several soils of the midwestern United States a 100% efficient release of the cells was not always attained (Vidor and Miller, 1979a; Reyes and Schmidt, 1979). Inefficient release has also been documented with other bacteria such as Azotobacter (Schmidt, 1974), and Methane oxidizers (Reed and Dugan, 1978). In addition Kingsley and Bohlool (Proc. 7th No. American Rhizobium Conf., p. 8, 1979) found this technique was not satisfactory to recover strains of fast-growing Rhizobium from tropical soils for IF enumeration. The problem of inefficient release must be solved since it greatly limits the ability to study the ecology of free-living Rhizobium in tropical soils.

Materials and Methods

Source and Maintenance of Cultures

Two strains of fast-growing Rhizobium were used in this series of experiments: TAL-620 (Rhizobium for Cicer, see Chapter 1, Table 1), and Hawaii-5-0 (Rhizobium leguminosarum, for lentils, May, 1979). Both strains were maintained on a modified YEMS medium (see Appendix Table 19a) and when required were grown in broth of the same composition. Exponential phase broth cultures were enumerated by counting with a Petroff-Hauser chamber. The cultures were diluted in filtered (0.45 µm) saline to the desired inoculum size. For experiments one or two ml of the inoculum was added to moistened soil.

Chemical Reagents

Reagent grade chemicals were purchased from: J. T. Baker, Phillipsburg, N. J.; Mallinckrodt, St. Louis Missouri; Matheson, Coleman & Bell, Norwood, Ohio. Antifoam C Emulsion, Thimerosal, and Tween 80 were obtained from Sigma Chemical Company, St. Louis, Missouri. Disodium-EDTA was obtained from Eastman Organic Chemicals, Rochester, N. Y. Nonidet P40 (BDH), a non-ionic detergent, was purchased from Gallard-Schlesinger Chemical Mfg. Corp., Carle Place, N.Y. Peptone (Bacto-Peptone) and gelatin (Bacto-Gelatin) were purchased from Difco, Detroit, Michigan.

Soil Samples

Table 5 lists the ten soils used, their pH, cation exchange capacity (CEC), and percent organic matter (% O.M.) (for place of origin see Table 6, except for Clarion and Hubbard--soils from Minnesota). All soils were air-dried, sieved through a #25 mesh (710 µm) sieve, and ten gram portions were dispensed to 25 x 200 mm screw-cap tubes. Prior to inoculation the soils were moistened with distilled water such that the volume of liquid in the inoculum would give a final soil moisture content of approximately 60% of the water holding capacity (Bohlool, 1971.).

Preparation of Fluorescent Antibodies (FA, FA Staining, Epifluorescence Enumeration

Fluorescent antibodies were prepared by the method of Schmidt et al. (1968). The basic protocol for the release of strains inoculated into soil, and their subsequent concentration and immunofluorescent enumeration on membrane-filters, followed the procedures outlined by Bohlool and Schmidt (1973a) and Schmidt (1974) (see Appendix Table 21). Several modifications to these procedures should be noted. Polycarbonate membrane-filters (Huclepore) stained with Irgalan Black (Hobbie et al., 1977) were substituted for india-inkstained Millipore filters. The gelatin-rhodamine isothiocyanate conjugate (Bohlool and Schmidt, 1968) was let

Table 5. Properties of soils used in Chapters 2 and 3

Soil		pH*	CEC*	% OM*
	Inceptisols			
Burabod		4.8	45.05	12.89
LPHS	Hydric Dystrandept	5.7	53.3	6.41
PLP		4.6	47.8	5.68
Makiki	Andic Humic Humitropept	6.1		
Waimea	Typic Eutrandept	6.2	46.9	13.00
	Mollisols			
Clarion	Typic Haploboroll	6.9	--	--
Hubbard	Udorthentic Haploboroll	5.2	--	--
	Vertisols			
Lualualei	Typic Chromustert	7.1	34.1	0.66
	Oxisols			
Molokai	Typic Torrox	6.5	19.56	3.71
Wahiawa	Tropeptic Eustrustox	5.4	22.89	2.27

* Data compiled from: Ikawa, 1979; Soil Conservation Service, 1972, 1976

dry completely on each filter prior to FA staining (May, 1979). Gelatin coated filters could be stored dry, either in a desiccator, or left in a drying-oven (60^0C) for long periods of time prior to staining. This greatly simplified the processing of large numbers of samples. All FA enumerations were made with a Zeiss universal microscope. Incident illumination was from an HBO-200 (OSRAM) light source, and a Zeiss Fluorescein Isothiocyanate (FITC) filter.

Recovery of TAL-620 from Eight Tropical Soils

To determine the sorptive nature of various tropical soils for a strain of chickpea Rhizobium, eight soils, representative of three soil orders common to the tropics, were chosen: Wahiawa (Oxisol); Molokai (Oxisol); Lualualei (Vertisol); PLP, Burabod, LPHS, Makiki, and Waimea (all Inceptisols). An exponentially-growing culture of TAL-620, enumerated with a Petroff-Hauser chamber, was adjusted to 2 x 10^7 cells/ml with saline (= Inoculum). The actual inoculum size was verified by FA-membrane-filter counts of the inoculum. Each tube of ten grams of non-sterile soil received two ml of inoculum
(= 4 x 10^6 cells/gram soil). Two tubes containing ten grams of silica sand served as inoculated non-soil controls. After incubation for two hours at room temperature (24^0C) the recovery of TAL-620 was assayed using the procedure of Bohlool and Schmidt (1973a) with the modifications previously described (see Appendix Table 21).

Soil Titrations

This series of experiments was designed to illustrate the sorptive capacities of soils, both tropical (5 soils) and temperate (2 soils), for fast growing rhizobia. In these experiments the number of bacteria added to each treatment was identical; the variable was the increasing quantity of

soil. The six treatments used are listed below: Treatment 1 served as a non-soil control; each treatment was run in duplicate.

Amount/Tube	Treatment Number 1	2	3	4	5	6
Sand (grams)	10	9.5	9.0	8.0	5.0	0
Soil (grams)	0	0.5	1.0	2.0	5.0	10
Percent Soil	0	5	10	20	50	100

The following soils were tested: Burabod, Clarion, Hubbard, Lualualei, Molokai, Wahiawa, Waimea. Both the TAL-620 and Hawaii-5-0 strains were used. The cultures were adjusted to approximately 2 x 10^7 cells/ ml with a Petroff-Hauser counting chamber; one ml was added to the non-sterile soils and incubated for two hours at room temperature (24^0C). The number of cells contained in the inoculum was verified by IF-membrane-filter counts. The influence of the various treatments, i.e. increasing soil, on the recovery of strains was assayed by SRP (see Appendix Table 21).

Use of Different Diluents/Extractants and Flocculants to Increase Recovery of TAL-620 from Wahiawa Soil

The Wahiawa soil was chosen as the model problem soil. It consistently gave one of the poorest recoveries. In these experiments different extracting solutions were substituted for the H_2O-Tween 80 extractant normally used in the SRP procedure. Mid-exponential phase cultures of TAL-620 were enumerated by Petroff-Hauser counts and adjusted to give 3 x 10^6 to 1 x 10^7 cells/gram of non-sterile soil. The final inoculum size was confirmed by FA-filter counts.

The inoculated soils incubated two hours at ambient temperature (24^0C) prior to assay for recovery by SRP.

The nonionic detergent Nonidet P40, (0.5% solution) was substituted for Tween 80. Solutions of salts at different concentrations were tried (1M KCl, 3M NaCl), low pH (0.4M HCl), organic solvents (100% methanol, 10% ethanol), two different anion extractants $CuSO_4$: Ag_2SO_4 (used for NO_3^- and NO_2^- extraction from soils, Jackson, 1962), and $NaHCO_3$ (used for the extraction of soil phosphorus, Jackson, 1962), solutions of the chelator EDTA (0.01 and 0.1M), two solutions of peptone (1%, 2%), and two solutions containing partially hydrolyzed gelatin (PHG) were also substituted.

To prepare PHG a 1% solution of Bacto-Gelatin was adjusted to pH 10.3 with 1N NaOH and autoclaved for ten minutes at 121^0C to partially hydrolyze the gelatin. When required the 1% solution was diluted to 0.1% either with water, or with 0.1M phosphate buffered saline (PBS) (see Appendix Table 22); thimerosal was added (final concentration 1:10,000) to prevent bacterial growth in all gelatin solutions.

Results and Discussion

Reports in the literature indicate that the soil release procedure (SRP) (see Appendix Table 21) does not fully release and extract Rhizobium and other bacteria from temperate soils (Reyes and Schmidt, 1979; Vidor and Miller, 1979a; Schmidt, 1974). In addition, seven soils representative of those commonly found in the tropics were highly sorptive for strains of fast-growing rhizobia when assayed by this procedure. The recovery of TAL-620, a strain of chickpea Rhizobium, from eight tropical soils is summarized in Table 6. All soils except one (Waimea) were highly sorptive for the added bacteria; recoveries were extremely low, in several cases less than 1% of the added level.

The Wahiawa Oxisol was the most highly sorptive of the eight soils and gave the poorest recovery. Poor recovery of Rhizobium was not related to soil order as both Oxisols (highly weathered, "old" soils) and Inceptisols (little weathered, "young" soils) retained the bacteria. (The soil order is the broadest level in soil classification and is equivalent to the phylum in biological classification.) Recovery of bacteria from soil may be related to the finer classification of soils such as at the sub and great group level, where both mineralogy and soil forming conditions are considered: from a typic eutrandept (Waimea) 100% of the inoculated bacteria were recovered, while recovery from hydric dystrandepts (PLP, LPHS, Burabod) and an andic ustic humitropept (Makiki) were much poorer.

The capacity of 5 tropical and 2 temperate soils to sorb bacteria and their recovery and enumeration by SRP was shown by the soil titration experiments. Figures 6, 7, and 8 show the inverse relationship between increasing soil content and recovery; as the quantity of soil in each treatment increased, recovery of added Rhizobium decreased. The strain of lentil Rhizobium Hawaii-5-0 was strongly sorbed by the Wahiawa soil as shown in Figure 6. This indicates that the poor recovery of TAL-620 was not a strain specific phenomenon. In fact Hawaii 5-0 was more strongly adsorbed by the Wahiawa soil than TAL-620. The Waimea Inceptisol (see Figure 7) and the two midwestern soils (Figure 8) had less sorptive capacity relative to the other soils; the decrease in recovery of added bacteria was not as great.

Seventeen different extractants were substituted for H_2O in the SRP recovery procedure, these included 2 nonionic detergent, salts, low pH, alcohols, solutions of peptone, EDTA, and two solutions of partially hydrolyzed gelatin. Of these 17 extractants (see Tables 7 and 8) four gave recoveries greater than 30% (see Table 8): 0.5M $NaHCO_3$ (33% recovery), 1% peptone (33% recovery), and 0.1% PHG in phosphate buffered saline (60% recovery). It was important to select the proper concentration for the

Table 6. Recovery of TAL-620 from 8 different tropical soils using SRP

Soil				Recovery		
Series	Sub-great-group	(Order)	Origin	Added (#/g)	Recovered (#/g)	% Recovery
Wahiawa	Tropeptic Eutrustox	(Oxisol)	Hawaii	4×10^6	8×10^2	<1
Molokai	Typic Torrox	(Oxisol)	Hawaii	4×10^6	6×10^4	1.5
Lualualei	Typic Chromustert	(Vertisol)	Hawaii	4×10^6	3×10^4	<1
PLP	Hydric Dystrandept	(Inceptisol)	Indonesia	4×10^6	2×10^4	<1
Burabod	Hydric Dystrandept	(Inceptisol)	Philippines	4×10^6	2×10^4	<1
LPHS	Hydric Dystrandept	(Inceptisol)	Indonesia	4×10^6	5×10^5	13
Makiki	Andic Ustic Humitropept	(Inceptisol)	Hawaii	4×10^6	5×10^5	13
Waimea	Typic Eutrandept	(Inceptisol)	Hawaii	4×10^6	4×10^6	100
Control						
Silica Sand				4×10^6	4×10^6	100

Figure 6. Soil Titrations - recovery of TAL-620 (chickpea Rhizobium) from two Hawaiian Oxisols, Molokai (-●--●-) and Wahiawa (-o--o-), and recovery of Hawaii-5-0 (R. leguminosarum) from Wahiawa (-◘--◘-) using Soil Release Procedure (SRP) methodology— both strains strongly affected by increasing percent of soil--the greater the content of soil the fewer cells recovered.

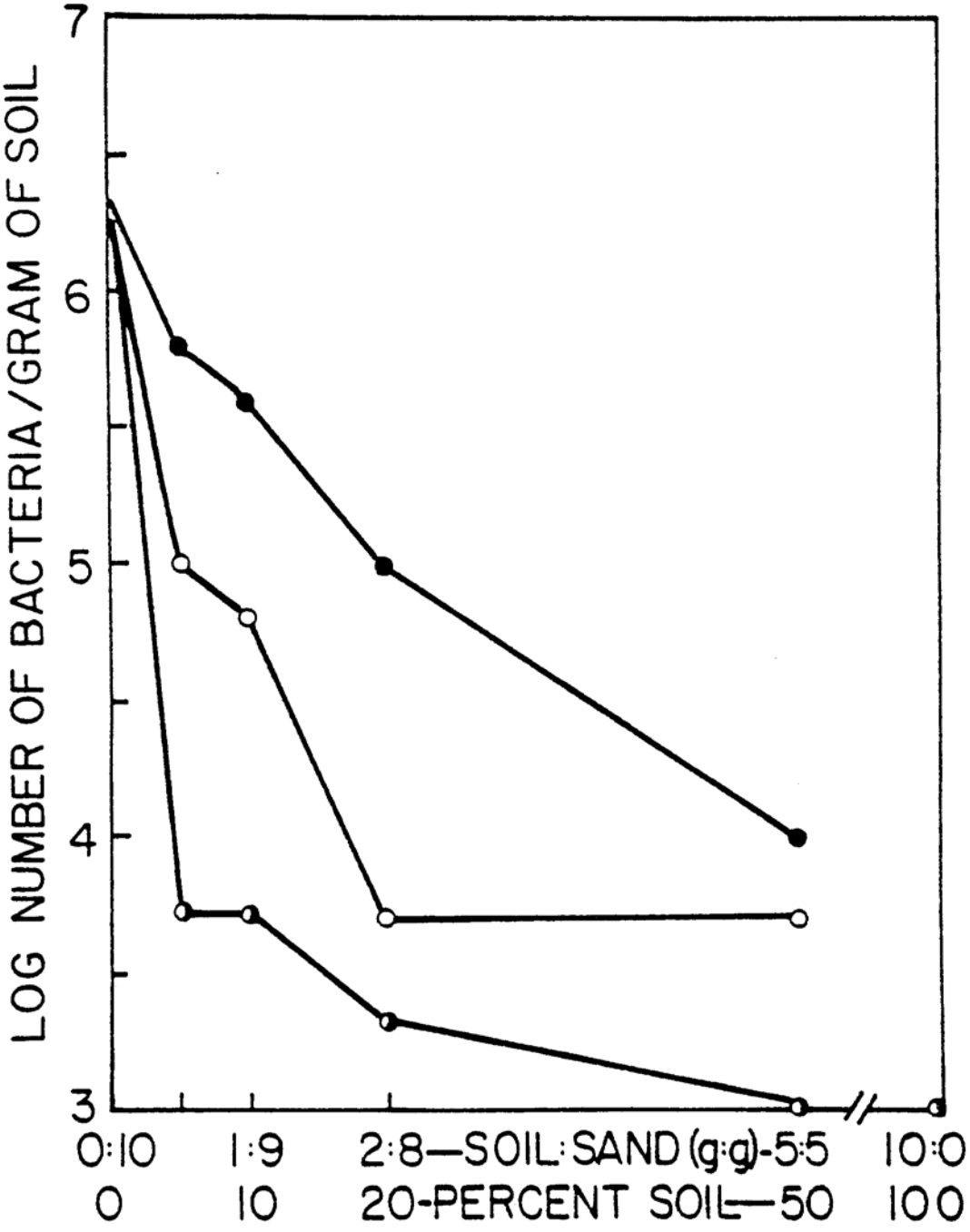
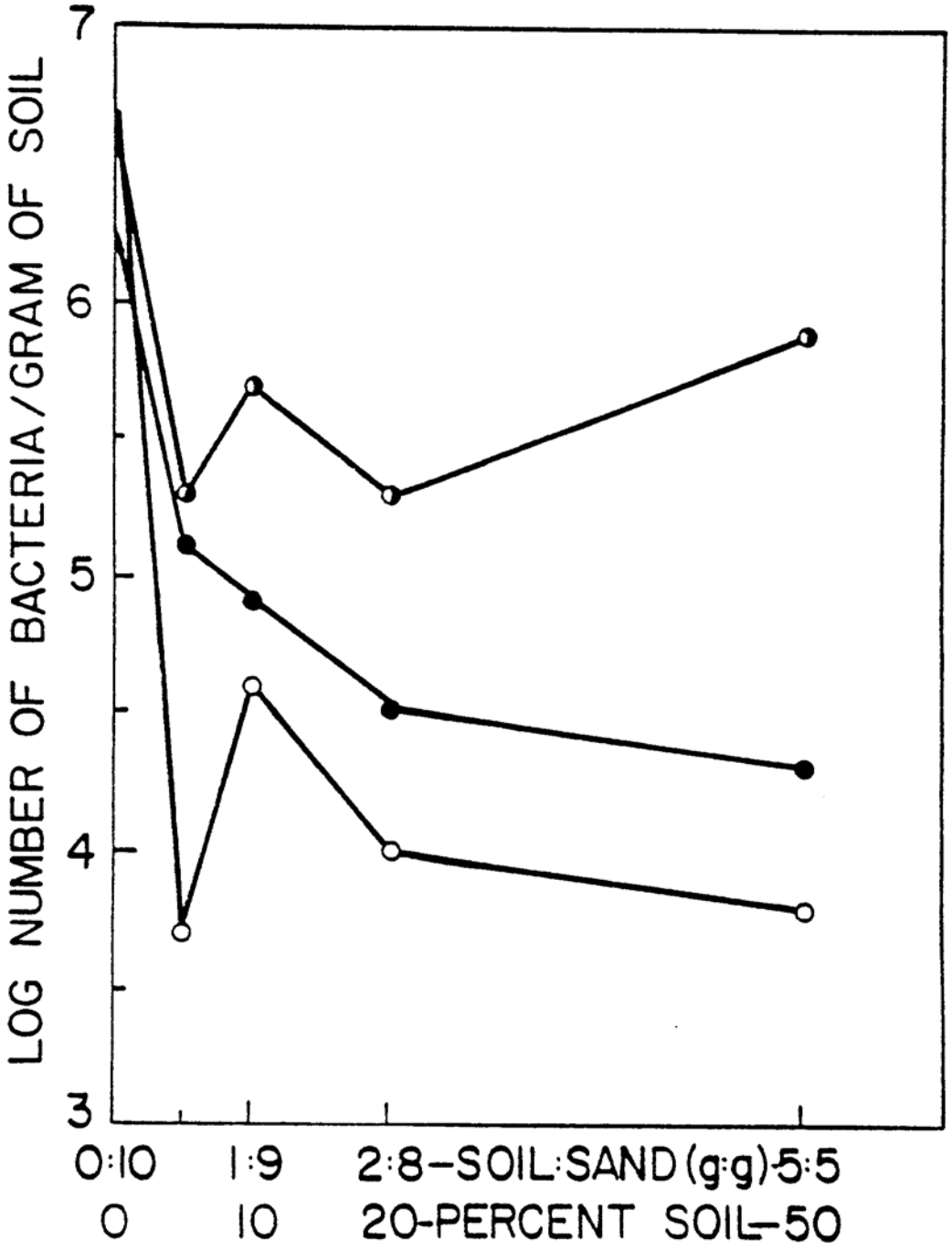

LOG NUMBER OF BACTERIA/GRAM OF SOIL
7
6
5
4
3
0:10
1:9
2:8—SOIL:SAND (g:g)-5:5
10:0
0
10
20-PERCENT SOIL—50
100

Figure 7. Soil Titrations - recovery of TAL-620 from 3 tropical soils Waimea (-◘--◘-), Lualualei (-●--●-), and Burabod (-o--o-) using Soil Release Procedure (SRP) methodology. The Waimea Inceptisol has less sorptive capacity than the other soils.


LOG NUMBER OF BACTERIA/GRAM OF SOIL
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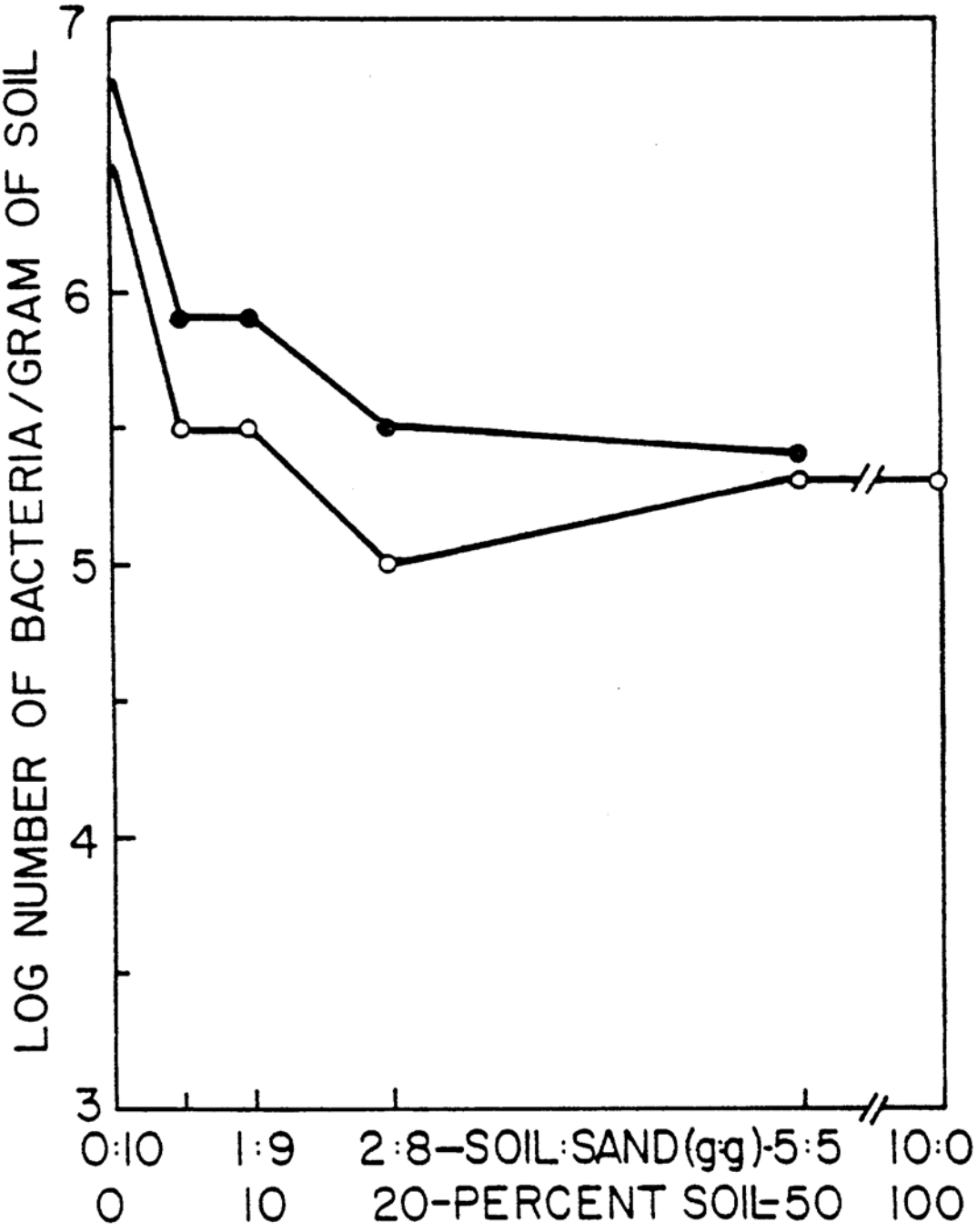
6
5
4
3
0:10
1:9
2:8-SOIL:SAND (g:g)-5:5
0
10
20-PERCENT SOIL-50

Figure 8. Soil Titrations - recovery of TAL-620 from two mid-western Mollisols Clarion (-●--●-), and Hubbard (-o--o-) using soil release procedure methodology. These soils are less strongly sorptive than most of the tropical soils tested.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5
4
3
0:10
1:9
2:8—SOIL:SAND(g·g)-5:5
10:0
0
10
20-PERCENT SOIL=50
100

Table 7. Recovery of TAL-620 from Wahiawa soil (Oxisol/Hawaii): Evaluation of extractants for increasing recovery. I. Extracts yielding <1% recovery

Extractant	Tween 80	Antifoam	Flocculant	Recovery		
				Added #/g	Recovered (#/g)	% Recovery
H_2O (Bohlool and Schmidt, 1973)	+	+	$Ca(OH)_2$:$MgCO_3$	1×10^7	1.5×10^4	<1
1M KCl	+	+	$Ca(OH)_2$:$MgCO_3$			
0.4M HCl	+	+	1% $CaCl_2.2H_2O$			
1% Sucrose	+	+	$Ca(OH)_2$:$MgCO_3$			
Methanol	+	+	None			
Nonidet P40 (25% aqueous soln.) (Nonionic detergent)	-	+	$Ca(OH)_2$:$MgCO_3$	$(3 \times 10^6 - 1 \times 10^7)$	$(6 \times 10^2 - 4.6 \times 10^4)$	<1
$CuSO_4$:Ag_2SO_4 (0.02M:0.002M) (NO_3^- + NO_2^- Extractant)	+	+	$Ca(OH)_2$:$MgCO_3$			
10% ETOH	+	+	$Ca(OH)_2$:$MgCO_3$			

Table 8. Recovery of TAL-620 from Wahiawa soil (Oxisol/Hawaii): Evaluation of extractants for increasing recovery. II. Extractants yielding >1% recovery

Extractant	Tween 80	Antifoam	Flocculant	Recovery		
				Added #/g	Recovered (#/g)	% Recovery
3 M NaCl	+	+	1% $CaCl_2 \cdot 2H_2O$	3×10^6	9×10^4	3
0.01 M Na-EDTA	+	+	0.7 g $Ca(OH)_2$:$MgCO_3$	1×10^7	1.5×10^6	15
0.1 M Na-EDTA	+	+	"	1×10^6	3×10^4	3
0.5 M Na HCO_3	-	+	"	1×10^7	3×10^6	33
1.0 M Na HCO_3 (P extractant)	-	+	"	1×10^6	8×10^4	8
1% Peptone	-	+	"	1×10^7	3×10^6	33
2% Peptone	-	+	"	1×10^6	1.5×10^5	15
Partially Hydrolyzed Gelatin (PHG) in 0.1% H_2O	-	+	"	1×10^6	3.5×10^5	35
PHG 0.1% in 0.1M PBS	-	+	"	1×10^6	6×10^5	60

extractant solution. In three cases (see Table 8) use of higher concentrations of the extractant gave poorer recovery than with the less concentrated solution. This was also true of PHG solutions. In preliminary experiments with solutions of partially hydrolyzed gelatin greater than 0.1%, the soil colloids would not flocculate out of suspension. Less than 0.1 ml of the solution could be filtered. Because of this, PHG was used at a final strength of 0.1%. The composition of the solution with which the 1% partially hydrolyzed gelatin was diluted, to give the final 0.1% working mixture, was important: gelatin in O.1M PBS gave higher recovery than gelatin diluted in H_20.

Niepold et al. (1979) proposed that the chemical composition of the extraction fluid should influence recovery. My results, obtained with the different extractants, support this proposal. However, nonionic detergents, high ionic strength, low pH, and polar organic solvents were ineffective. Organic extractants containing protein digests (peptone) or proteins (gelatin) were the most successful. The extractants containing PHG gave the best results. Therefore the failure to recover rhizobia from tropical soils was a function of inadequate extraction of the bacteria; recovery was increased simply by altering the chemistry of the extraction fluid.

Those extractants with complex charge chemistries, such as solutions of PHG, led to the extraction of more bacteria. In fact, PHG solutions in phosphate buffered saline gave the greatest recovery. This might indicate that ion exchange processes (Na^+) as well as charge neutralization (PO_4^{3-} ions and charges of PHG molecules) are important in the recovery of bacteria from the soil matrix. The phosphate ions might aid in neutralizing the positive charges at the broken edges of the clay plates; the sodium ions, due to mass action can displace divalent cations, bridging bacteria to soil colloids, or bridging clay particles together and prevent adequate dispersion. The gelatin ions may also combine and neutralize charges within

the soil matrix. Since soils are highly complex ion exchange systems it seems logical that a solution with a complex charge status is required to recover the bacteria. If cation exchange is an important phenomenon in the recovery of bacteria from soils, a cation with more exchange power such as K^+ or NH_4^+ might lead to increased recovery of Rhizobium.

CHAPTER 5

MODIFIED MEMBRANE FILTER - IMMUNOFLUORESCENCE FOR ENUMERATION OF *RHIZOBIUM* FROM TROPICAL SOILS

Introduction

Immunofluorescence (IF) provides a direct method for *in situ* autecological studies of microorganisms; it allows for the simultaneous detection and identification of the desired organism in its natural habitat. The technique can be made quantitative by separating the bacteria from soil particles and concentrating them on non-fluorescent membrane-filters for IF enumeration (Soil Release Procedure, SRP, see Appendix Table 21). In applying SRP to study *Rhizobium* in tropical soils I encountered great difficulty in releasing bacteria from soil particles and recovering them for IF enumeration (see Chapter 2).

Most tropical soils were highly sorptive for *Rhizobium* when assayed by SRP. However, as discussed by Niepold et al. (1979) the chemical composition of the extraction fluid greatly influenced the degree of sorption. Extraction solutions containing proteins (Partially Hydrolyzed Gelatin, PHG) were most successful in increasing recovery of rhizobia from a Hawaiian Oxisol, chosen as the model problem soil. Therefore, the failure to recover rhizobia sorbed to soil is directly related to the techniques used to extract and enumerate them. The bacteria are not irreversibly bound. All that is required is the proper methodology to recover the cells. In addition, results obtained with partially hydrolyzed gelatin solutions indicated that ion exchange may be important in the recovery of bacteria from tropical soils.

The present work describes efforts to optimize recovery of *Rhizobium* from tropical soils. Experiments to select the best diluting solution to use with PHG were undertaken. In the course of these studies I realized that blending the soils (SRP, Appendix Table 21) especially the well aggregated Oxisols led to a breakdown of the stable aggregates and to a very large increase in surface area. This increased surface, by creating more area for sorptive interactions,

probably led to lower recoveries. I reasoned that the chemical extractants might work better when used with a less disruptive dispersion method. The final outcome of these investigations was the development of a modified soil release procedure (MSRP). The soils were dispersed by shaking on a wrist-shaker in flasks containing glass beads and a chemical extractant using PHG.

Materials and Methods

Source and Maintenance of Cultures

Two strains of fast growing rhizobia, TAL-620 (*Rhizobium* for chickpea, see Chapter 1, Table 1), Hawaii-5-0 (*Rhizobium leguminosarum*, May, 1979), and two strains of slow-growing rhizobia, USDA 31 and USDA 110 (both *R. japonicum*) (Dr. D. F. Weber, USDA Beltsville, Md.) were used in these experiments. All strains were maintained on a modified YEMS medium (see Appendix Table 19a), and when required were grown in broth of the same composition. Exponential phase cultures were enumerated by direct counts with a Petroff-Hauser chamber. The cultures were diluted in saline to the desired inoculum size. For experiments, one or two ml of the inoculum was added to moistened soil. Strict aseptic techniques were followed at all steps when autoclaved soils were to be inoculated.

Chemical Reagents

The chemical reagents have already been described (see Chapter 2, Materials and Methods). Granulated gelatin was obtained from three sources: Difco Bacto-Gelatin (2 lots, control #459821, and #464194), Difco, Detroit, Michigan; Fisher Bacterilogical Gelatin (Lot number illegible), Fisher Scientific, Fairlawn, N. J.; U.S.P. Granular Gelatin (No lot number), Pioneer Chemical Co., Inc., Long Island City, N. Y.

Soil Samples, Soil Sterilization

The soils used in these experiments, and their method or preparation and distribution were described previously (see Chapter 2, Materials and Methods). Soil sterilization, when needed was done by autoclaving for 1.5 hours at 121^{0}C (Bohlool, 1971).

Preparation of Fluorescent Antibodies, FA Staining, Epifluorescence Enumeration

Fluorescent antibodies were prepared by the method of Schmidt et al. (1968). The soil release enumeration procedure (SRP) (Bohlool and Schmidt, 1973a; Schmidt, 1974) is described in Appendix Table 21). Modifications to the published procedures were described previously (see chapter 2, Materials and Methods).

Preparation of Partially Hydrolyzed Gelatin (PHG)

A 1% solution of gelatin (10 g/L H_20) was adjusted to pH 10.3 with 1N NaOH, and autoclaved 10 minutes at 121^{0}C to partially hydrolyze the gelatin. The hydrolyzed solution was stored at 4^{0}C until used; thimerosal was added, 1:10,000 final concentration, as a preservative. All experiments unless indicated otherwise used Difco Bacto-Gelatin Lot No. 464194 (will be referred to as gel #1).

SRP - Effect of Different Strength Gelatin Solutions

To determine what concentration of gelatin would give maximum recovery of added bacteria, a 1% solution of PHG was used either undiluted (1%) or was diluted with water to give solutions containing 0.01%, 0.05%, 0.08%, 0.1%, 0.15% PHG. All solutions were adjusted to pH 7, and thimerosal was added, 1:10,000, to prevent bacterial growth. A mid exponential phase culture of TAL-620 was enumerated by direct counts with a Petroff-Hauser chamber, and adjusted to

1 x 10^7 cells/ml; one ml portions were inoculated into 25 x 200 mm screw-cap tubes containing 10 g of non-sterile Wahiawa soil. Duplicate tubes were inoculated for each gelatin concentration tested (12 tubes). The soils, after incubating for two hours at 24^0 C, were assayed with SRP for recovery of inoculated Rhizobium. The procedures were the same as described in Appendix Table 21 except that the various PHG solutions were substituted for H_2O-Tween 80.

SRP - Recovery of TAL-620 from Wahiawa Soil, 0.1 PHG (in H_2O) at Different pH's

Solutions of 0.1% PHG (1% diluted to 0.1% with H_2O) were adjusted to pH 4, 6, 7, 8, and pH 9 with 1N HCl and 1N NaOH. Thimerosal was added, 1:10,000, to prevent bacterial growth. A mid exponential phase culture of TAL-620 was enumerated by direct counts with a Petroff-Hauser chamber, and adjusted to 1 x 10^7 cells/ ml. One ml was inoculated into 10 g of non-sterile Wahiawa soil to give approximately 1 x 10^6 cells/g soil; duplicate tubes were used for each PHG solution. The inoculated soils were incubated for two hours at 24°C prior to assay for recovery. The assay procedure was the same as that in Appendix Table 21 except that PHG solutions were substituted for the H_2O-Tween 80 extractant.

SRP - Recovery of TAL-620 from Wahiawa Soil, 0.1% PHG, Use of Different Diluents

A 1% solution of PHG was prepared as described previously. After autoclaving, the solution was adjusted to pH 7 and thimerosal was added 1:10,000. The following solutions were used to test for percentage of recovery in conjunction with PHG (final strength of PHG - 0.1%): H_20; 0.1M Na-EDTA; 0.5M $NaHCO_3$; 0.001M NaHMP; 0.1M PBS. A mid exponential phase culture of TAL-620 enumerated, as described above, was adjusted to 1 x 10^7 cells/ml; one ml was inoculated into 10 g of non-sterile Wahiawa soil to yield approximately 1 x 10^6

cells/g of soil; duplicate tubes were used per each PHG solution tested. After a two hour incubation at 24°C the soils were assayed for recovery with SRP. Procedures were the same as those described in Appendix Table 21 except that PHG solutions were substituted for H_2O-Tween 80.

Development of a Modified Soil Release Procedure (MSRP)

Rather than blending the soil, the modified procedure required shaking the soil and extracting solution together on a wrist-action shaker, in a screw-cap flask with glass beads. Several PHG-diluent combinations were tested: 0.1% PHG - H_20; 0.1% PHG - O.1M Na_2HPO_4 (pH 9); 0.1% PHG - 0.1M $(NH_4)_2HPO_4$ (pH 8.3); 0.1% PHG - 0.1M PBS (pH 7.1). Thimerosal was added to all gelatin solutions, final strength 1:10,000. A mid exponential phase culture of TAL-620 was adjusted to 1 x 10^7 cells/ml as described previously; one ml was inoculated into 10 g of non-sterile Wahiawa soil to give approximately 1 x 10^6 cells/g of soil. Duplicate tubes were used for each PHG solution tested. The inoculated soils were incubated for two hours at 24°C prior to assay for recovery.

The assay procedure was the same as that described in Appendix Table 21 with the following modifications: the soils and extracting solution were added to 250 ml screw cap flasks containing 25 - 30 g of 3 mm glass beads. The flasks were shaken for five minutes on a Burrel wrist-action shaker to disperse the soil, all other procedures (flocculation, filtration, enumeration, etc.) were the same as those described in Appendix Table 21.

MSRP - Effect of the Decreasing Hydrated Radius of Four Monovalent Cations Upon Recovery of TAL-620 from Wahiawa Soil

Solutions of 0.2M strength prepared from LiCl, NaCl, KCl and NH_4Cl were mixed with 1% PHG (prepared as described previously) to yield the following extractant solutions: 0.1% PHG - 0.2M LiCl; 0.1% PHG - 0.2M NaCl; 0.1% PHG - 0.2M KCl; 0.1% PHG - 0.2M NH_4Cl. A mid exponential phase culture of TAL-620 was

adjusted to 3 x 10^7 cells/ml as described previously; one ml was added to 10 g of non-sterile Wahiawa soil, in duplicate tubes. After a two hour incubation at 24°C the soils assayed for recovery by MSRP.

MSRP - Effect of Gelatin Type (Manufacturer) and Shaking Time on Recovery of TAL-620 from Wahiawa Soil

Four 1% solutions were prepared from each of the granulated gelatin preparations listed above under Chemical Reagents; thimerosal was added as a preservative and the solutions were stored at 4°C until used. All gelatin solutions were adjusted to 0.1% PHG with O.1M $(NH_4)_2HPO_4$ (pH 8.3). A mid exponential phase culture of TAL-620 was adjusted to 2 x 10^6 cells/ml, one ml was inoculated into duplicate tubes containing 10 g of non-sterile Wahiawa soil. A two hour incubation at 24°C preceeded the recovery assay. The soils and extractants were shaken for five minutes.

The effect of shaking time was evaluated using Difco gel #1. A 1% PHG solution was prepared as described previously; this solution was adjusted to 0.1% with O.1M $(NH_4)_2HPO_4$ (final pH of extractant = 8.3). Four dispersion times were tried. The soils and extractant were shaken for 5, 15, 30, and 60 minutes.

MSRP - Soil Titrations

The procedures used to construct the titrations were described in the Materials and Methods section of Chapter 2. The SRP assay discussed in Chapter 2 and this assay (MSRP) were conducted simultaneously. For these experiments the MSRP extractant consisted of 0.1% PHG - O.1M $(NH_4)_2HPO_4$ (final pH = 8.3); the soil and extracting solution were shaken for five minutes.

Growth of Fast and Slow Growing Rhizobium in Sterile Wahiawa Soil

The growth of TAL-620 was followed for 20 days in sterile Wahiawa soil. The bacteria were enumerated directly from the soil by both viable counts and

MSRP (MSRP = 0.1% PHG - O.1M $(NH_4)_2HPO_4$, shaking for 5 minutes). One set of data was analyzed by viable counts, SRP and MSRP. For viable counts soils were mixed with 95 ml of sterile H_2O in sterile screw cap flasks containing 25 - 30 g of 3 mm glass beads, and shaken for five minutes. After dispersion, the appropriate dilutions were plated onto YEMS medium (see Appendix Table 19a) using the Miles and Misra drop plate technique (Vincent, 1970). The procedure was modified to use a Pipetman P-20 adjustable micropipeter (Rainin Instrument Co., Inc., Woburn, Mass.) and disposable tips (sterilized by autoclaving). The pipetor was adjusted to dispense 20 pl of solution; one disposable tip was used per dilution.

A mid exponential phase culture of TAL-620 was adjusted to 1 x 10^6 cells/ml; and one ml was inoculated into 10 g of sterile Wahiawa soil to yield approximately 1 x 10^5 cells/g. When necessary the soils were moistened with several drops of sterile distilled water. The tubes were sampled randomly, four tubes were assayed per data point.

The growth of two strains of R. japonicum was followed for seven days (USDA 31) and fourteen days (USDA 110) in sterile Wahiawa soil. The bacteria were enumerated by viable count (as described), SRP, and MSRP. Mid exponential phase cultures of USDA 31 and USDA 110 were enumerated in a Petroff-Hauser chamber, adjusted to approximately 10^5 cells/ml, and one ml was aseptically inoculated into the soils to give approximately 10^4 cells/g. Population levels were determined after five days incubation at 28^0C (USDA 31, USDA 110), seven days (USDA 31), and fourteen days (USDA 110). Duplicate tubes were used for each measurement.

Growth of USDA 110 in Sterile Clarion Soil

The growth of USDA 110 was followed in sterile Clarion soil for 4 days using plate counts, SRP and MSRP. A mid exponential phase culture of USDA 110 was enumerated with a Petroff-Hauser chamber and adjusted to 2 x 10^6 cells/ml;

one ml was inoculated aseptically into tubes containing 10 g of autoclaved Clarion soil to give approximately 2 x 10^5 cells/g. The cells were counted three and five days after inoculation. The tubes were incubated at 28^0C; duplicate tubes were used for each measurement.

Statistical Methods

A one way analysis of variance (ANOVA) (Sokal and Rohlf, 1969) was used to determine if the monovalent cation treatments had a significant effect on extraction of Rhizobium from soils and to determine if the type of gelatin was important, as well as the shaking/extraction period.

Results and Discussion

The results discussed in Chapter 2 indicated the difficulty in quantitatively recovering bacteria from tropical soils when using SRP (see Appendix Table 21); seven of eight tropical soils were highly sorptive for added rhizobia. Several other investigators have also experienced difficulty in recovering bacteria quantitatively from soil and sediments (Vidor and Miller, 1979a; Reyes and Schmidt, 1979; Reed and Dugan, 1978). However, the substitution of solutions of partially hydrolyzed gelatin (PHG) led to increased recovery of added bacteria.

Solutions of gelatin, due to their protein origins are amphoteric, i.e. they have pH dependent charges. The charges on the gelatin molecules can interact with both soil colloids and microbial cells. Partial hydrolysis of the gelatin increases the number of small peptides which can interact with the soils exchange complex and therefore satisfy charges within the matrix. By satisfying charges on both bacteria and soil particles the two should not bind due to any charge interactions. Preliminary evidence indicated that ion exchange phenomena might be involved in the extraction of bacteria from the soil matrix. PHG solutions mixed with phosphate (sodium) buffered saline gave greater recovery

than PHG mixed with H_2O (see Table 8). Therefore, experiments were designed to establish the optimum concentration of PHG; to establish the importance of the extracting solution, and to determine the proper combination of ions for increasing recovery, as well as to determine if ion exchange processes were involved.

A solution of 0.1% PHG gave the best quantitative recovery of TAL-620 from the Wahiawa soil (see Table 9). Concentrations of gelatin greater than 0.1% prevented flocculation of the soil colloids. I did not determine if the lack of flocculation was due to the increased viscosity of the more concentrated PHG solutions, relative to H_2O or to an increase dispersive effect. The addition of more flocculant did not precipitate the soil colloids.

In addition to selecting the proper PHG concentration, it was necessary to select the optimum pH for extraction (see Table 10). A low pH gelatin solution (pH 4) gave poorer quantitative recovery than solutions at higher pH. Solutions at pH 8 and pH 9 gave quantitative recovery of added Rhizobium. However, good recovery was obtained at pH 6 and pH 7. Preliminary results had demonstrated that high pH would disperse the Wahiawa Oxisol. However, high pH alone did not lead to quantitative recovery of Rhizobium (Table 8, $NaHCO_3$ solutions, pH 8.2 - 8.3).

Previous results (see Chapter 2, Table 8) demonstrated the importance of selecting the proper diluent to mix with the gelatin solution, e.g. H_2O vs. PBS. I decided to mix several of the solutions which had given slightly increased recoveries when substituted for H_2O-Tween 80 in SRP with solutions of PHG. Both sodium-EDTA and sodium bicarbonate solutions were not satisfactory when mixed with PHG (see Table 11) as recoveries were below 50% of the inoculum. A combination of PHG and the soil deflocculant sodium hexa-meta-phosphate did not increase recovery of added Rhizobium. In addition, extractant solutions of PHG and water gave quite variable results from 20% recovery up to 80%.

During the course of these experiments, trends in the data indicated that

Table 9. SRP[a] - Effect of different strength Partially Hydrolyzed Gelatin (PHG) solutions on increasing recovery of TAL-620 from Wahiawa soil[b]

% PHG	pH	Recovery		
		Added (#/g)	Recovered (#/g)[c]	% Recovery
0.01	7	1×10^6	1×10^4	1.0
0.05	7	1×10^6	7×10^4	7.0
0.08	7	1×10^6	5×10^5	50.0
0.10	7	1×10^6	1×10^6	100.0
0.15	7	1×10^6	Soil colloids did not settle	
1.00	7	1×10^6	" " " " "	

[a] Soil Release Procedure, described in Appendix 3, gelatin solutions substituted for H_2O-Tween 80 extractant

[b] Mid exponential phase culture inoculated into nonsterile soil and incubated for two hours (24°C) prior to recovery

[c] Mean of duplicate tubes, 2 filters counted/tube, total of 4 filters per gelatin solution

Table 10. SRP[a] - Influence of pH of a 0.1% Partially Hydrolyzed Gelatin (PHG) solution to recover TAL-620 from Wahiawa soil

pH of 0.1% PHG[b]	Recovery		
	Added (#/g)	Recovered (#/g)	% Recovery
4	1×10^6	1×10^5	10
6	1×10^6	9×10^5	90
7	1×10^6	9×10^5	90
8	1×10^6	1×10^6	100
9	1×10^6	1×10^6	100

[a] Soil release procedure, described in Appendix 3, gelatin solutions substituted for H_2O-Tween 80

[b] 1% solution of PHG diluted to 0.1% with H_2O, pH adjusted with 1N NaOH and 1N HCl

[c] Mean of duplicate tubes, 2 filters counted/tube, total of 4 filters per gelatin solution

there was a difference the blendor cups. The percentage recovery obtained from extractions in one blendor cup were consistently higher than those obtained from another cup. However, the level of recovery could be decreased simply by installing new bearing and blade assemblies in the cups. Apparently, the bearings in the older blade assembly slowed the blending speed, and greater recoveries were obtained than with blendors with new assemblies. The decrease in recovery might have been a function of increased soil area brought about by high speed blending. Oxisols are well aggregated soils; however, the aggregates are composed of small clay size particles that bind together (Sanchez, 1976). Strong blending results in high shear forces that destroy the aggregates, consequently leading to a very large increase in surface area. This increased surface area can interact with microorganisms and bind or trap them in floccules.

I decided that a modified soil dispersion method was needed; one that did not fully deaggregate the soils yet also gave consistently good recovery. The procedure finally adopted was shaking the soils and extracting solutions with a wrist-action shaker, employing glass beads to increase agitation. Four 0.1% PHG-diluent combinations were tested (Table 12). The combination offering the most promise was 0.1% PHG - 0.1M $(NH_4)_2HPO_4$ (pH 8.3).

These experiments gave more concrete evidence that an ion exchange-type of process might be important; poorer recoveries were obtained with sodium salt - PHG combinations than with ammonium salt - PHG combinations. Sodium is known to be a poorer exchanger than ammonium (Brady, 1974). For this reason I decided to see if the cations themselves could influence recovery. The results in Table 13 show that the cation does have a significant influence on recovery ($F = 33$, $F_{.01}(3,22) = 4.87$). It may also be inferred that the phosphate radical is important, as greater recoveries were obtained with ammonium and sodium phosphate-PGH combinations than with

Table 11. SRP[a] - Effect of different diluents to increase recovery of TAL-620 from Wahiawa soil when mixed with Partially Hydrolyzed Gelatin (PHG)

Extracting Solution	Recovery		
	Added (#/g)	Recovered (#/g)	% Recovery
0.1% PHG - 0.01M Na-EDTA	1×10^6	8×10^4	8
0.1% PHG - H_2O (pH 8.5)	1×10^6	2×10^5	20
0.1% PHG - 0.001M NaHMP	1×10^6	3×10^5	30
0.1% PHG - 0.5M $NaHCO_3$	1×10^6	3×10^5	30
0.1% PHG - H_2O (pH 7)[b]	1×10^6	3.5×10^5	35
0.1% PHG - 0.1M PBS[b]	1×10^6	6×10^5	60
0.1% PHG - H_2O (pH 8.5)	1×10^6	6×10^5	80

[a] Soil release procedure, described in Appendix 3, gelatin solutions substituted for H_2O-Tween 80

[b] Data from Table 7

their corresponding chloride salts.

The PHG extracting solution adopted as the standard MSRP extractant consisted of 0.1% PHG - 0.1M $(NH_4)_2HPO_4$, pH 8.3. The extraction time was not important (see Table 14) and a five minute extraction was adopted as the standard. However, the type of gelatin (manufacturer) used in the PHG solutions was important (see Table 15). The Difco Bacto-Gelatins (Gels 1 and 2) gave the best recovery. Granulated gelatins are prepared by both acidic and basic digests of animal biproducts. This results in different products. In addition the starting materials composing any one lot of granulated gelatin might vary and this can influence the final product. Even though the pH of the four solutions varied prior to hydrolysis (pH Difco gel 1 and 2 6.2, pH Fisher 4.6, pH Pioneer 7.6) each solution had an identical pH after hydrolysis and mixing with O.1M $(NH_4)_2HPO_4$ (pH 8.3). The differences in recovery were therefore not due to any pH effects. Due to the differences in the granulated gelatins several different lots and manufacturers should be tested. The lot giving the greatest recovery should then be used.

The MSRP procedure finally adopted as the standard method for the extraction of Rhizobium from tropical soils is described in Table 16. The major difference between this procedure and the SRP method is the extraction step, a chemical extractant is employed, and the soil is dispersed by rapid shaking rather than blending. All other procedures are the same as those described in Appendix Table 21.

The efficiency of the gelatin technique compared to SRP is illustrated in Figures 9, 10 and 11. The SRP technique was greatly affected by soil content and did not possess the ability to desorb the bacteria; therefore, as the soil content increased the number of bacteria recovered decreased. The gelatin MSRP method was hardly affected by the amount of soil and was able to desorb and extract the added Rhizobium. The Waimea Inceptisol (see Figure 10) and the other two Midwestern soils (Figure 11) were less sorptive for rhizobia than the

Table 12. MSRP[a] - Development of a modified soil release procedure - effect of different Partially Hydrolyzed Gelatin (PHG) extractants on recovery of TAL-620 from Wahiawa soil

PHG Extractant	Recovery		
	Added (#/g)	Recovered (#/g)[b]	% Recovery
0.1% PHG - H_2O	7×10^5	7×10^2	<1
0.1% PHG - PBS	7×10^5	2×10^5	28
0.1% PHG - 0.1M Na_2HPO_4	7×10^5	2×10^5	28
0.1% PHG - 0.1M $(NH_4)_2HPO_4$	7×10^5	5×10^5	71

[a] Modified soil release procedure, soils and extracting solution shaken for five minutes on wrist-action shaker

[b] Mean of 8 tubes, except for PHG-H_2O mean of 4 tubes

Table 13. MSRP[a] - Effect of the hydrated radius of four monovalent cations upon recovery of TAL-620 from Wahiawa soil

PHG Extractant	Hydrated Radius ($\mathring{A}$)[b]	Recovery		
		Added (#/g)	Recovery (#/g)[c]	% Recovery
0.1% PHG - 0.2M LiCl	6	3×10^6	1×10^4	1
0.1% PHG - 0.2M NaCl	4	3×10^6	3×10^4	1
0.1% PHG - 0.2M KCl	3	3×10^6	6×10^4	2
0.1% PHG - 0.2M NH_4Cl	2.5	3×10^6	2×10^5	7
	d_F = 33./F.01 (3,22) = 4.87 S.D.			

[a] Modified soil release procedure, soils and extracting solution shaken for five minutes on a wrist action shaker

[b] Reference: Butler, J. N. 1964. Ionic Equilbria, A Mathematical Approach. Addison-Wesley Publishing Co., Palo Alto.

[c] Mean of six tubes/treatment

[d] ANOVA performed on data from filter counts (#/field), significant difference between the PHG-Cation solutions

Table 14. MSRP[a] - Effect of shaking time on recovery of TAL-620 from Wahiawa soil

Shaking Time (minutes)	Recovery	
	Added (#/g)	Recovered (#/g)[b]
5	2×10^5	7×10^4
15	2×10^5	5×10^4
30	2×10^5	6×10^4
60	2×10^5	5×10^4

$c_F = 0.87$ F.01 (3,12) = 5.95 N.S.D.

[a] Modified soil release procedure, soils and extracting solution (0.1% PHG - 0.1M $(NH_4)_2HPO_4$, pH 8.3) shaken for five minutes

[b] Mean of duplicate tubes, two filters counted per tube

[c] ANOVA performed on data from filter counts (#/field), no significant difference between the shaking periods

Table 15. MSRP[a] - Effect of gels from different sources: Recovery of TAL-620 from Wahiawa soil

PHG Extractant[b]	Recovery	
	Added (#/g)	Recovered (#/g)[c]
Gel 1	2×10^5	8×10^4
Gel 2	2×10^5	6×10^4
Gel 3	2×10^5	5×10^4
Gel 4	2×10^5	4×10^4

$d_F = 6.7\ F_{.01}\ (3,12) = 5.95$ S.D.

[a] Modified soil release procedure, soils and extracting solution shaken for five minutes on wrist-action shaker

[b] Gel 1 = Difco Control #464194; Gel 2 = Difco Control #459821; Gel 3 = U.S.P. Gelatin (Pioneer Chemical Co.); Gel 4 = Fisher Scientific's gelatin

[c] Mean of duplicate tubes, two filters counted per tube

[d] ANOVA performed on data from filter counts (#/field), significant difference between the different manufacturers of gelatin

Table 16. Procedure for the use of gelatin in the quantitative procedure

Preparation of Gelatin

1) Prepare 1% solution of gelatin in water (adjust to pH 10-11, autoclave 10 minutes)

2) Dilute 1:10 with 0.1M $(NH_4)_2HPO_4$

Extraction

1) 1:10 dilution of soil in gelatin solution (add antifoam)

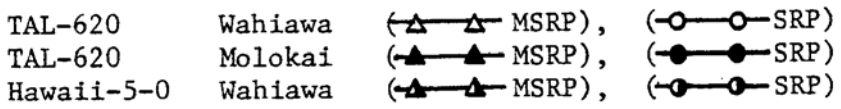
2) Shake 5 minutes with wrist-action shaker

3) Transfer to narrow container (add 0.7 g flocculant [$Ca(OH)_2$:$MgCO_3$ 2:5], shake

4) Allow to settle 1 hour

5) Filter, stain, enumerate

Figure 9. Soil Titrations - comparison of the Modified Soil Relese Procedure (MSRP) (triangles)and the Soil Release Procedure (SRP) (circles) for the recovery of Rhizobium from tropical soils. The MSRP Procedure (two Oxisols) is not affected by soil content, while recovery with the SRP procedure is soil dependent. (SRP data same as in Figure 6).



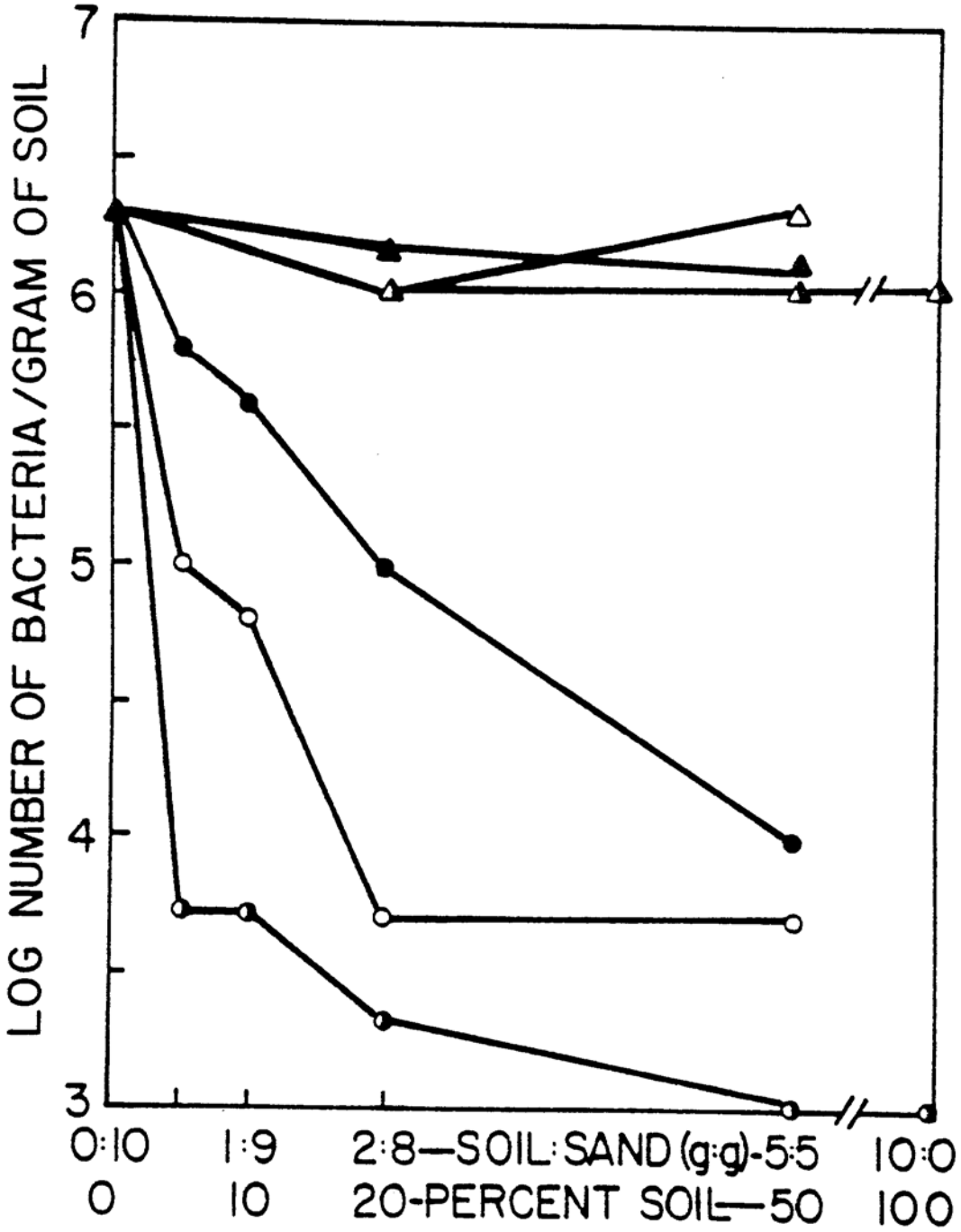
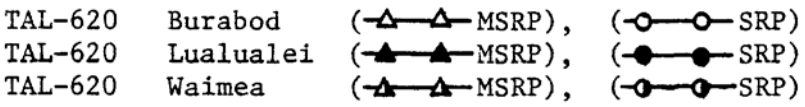

LOG NUMBER OF BACTERIA/GRAM OF SOIL
7
6
5
4
3
0:10
1:9
2:8—SOIL:SAND (g:g)-5:5
10:0
0
10
20-PERCENT SOIL—50
100

Figure 10. Soil Titrations - comparison of the Modified Soil Release Procedure (MSRP) (triangles) and the Soil Release Procedure (SRP) (circles) for the recovery of a strain of chickpea Rhizobium from tropical soils. The MSRP procedure is not affected by soil content, while recovery with the SRP procedure is soil dependent. (SRP data same as in Figure 7)



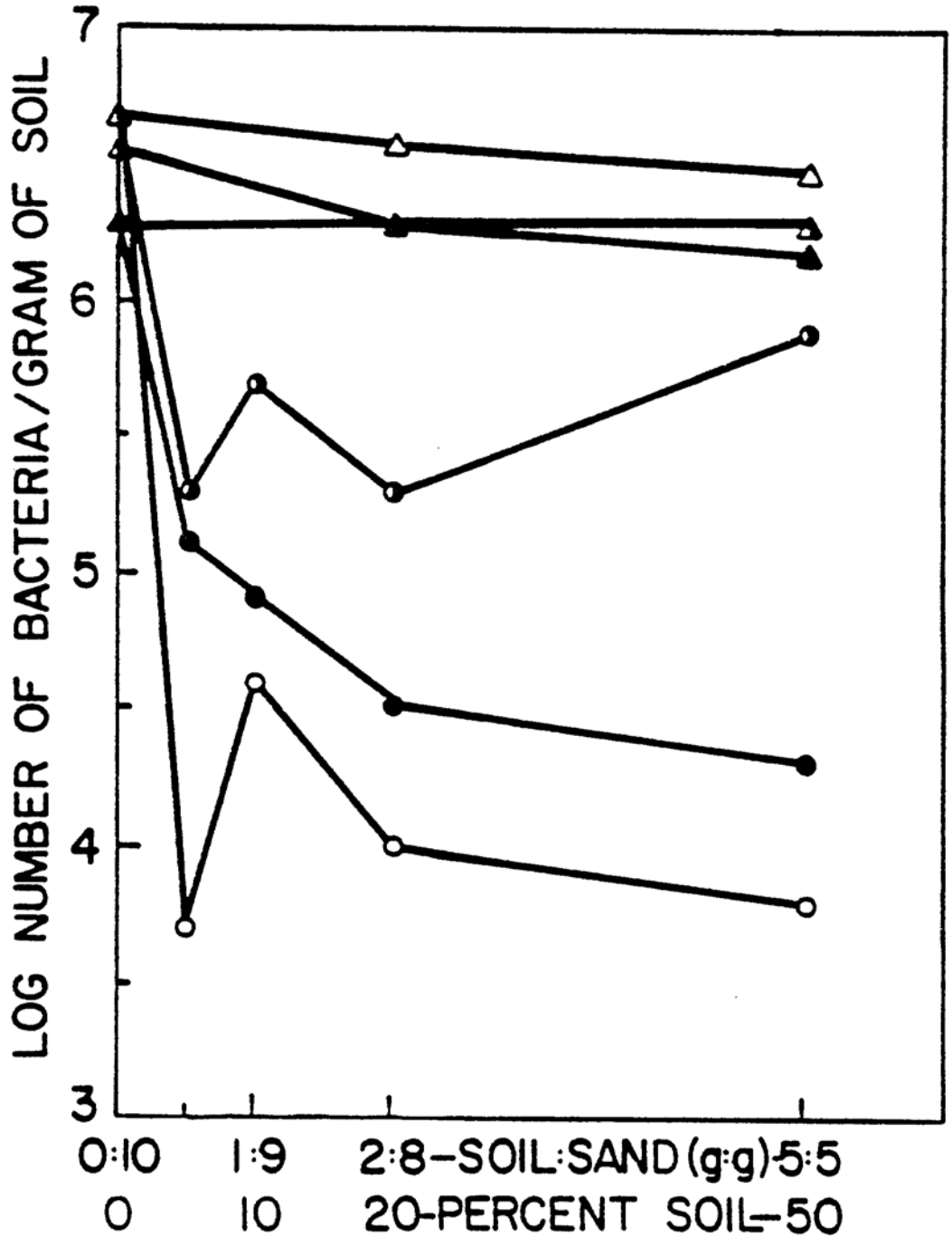
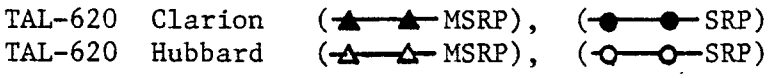
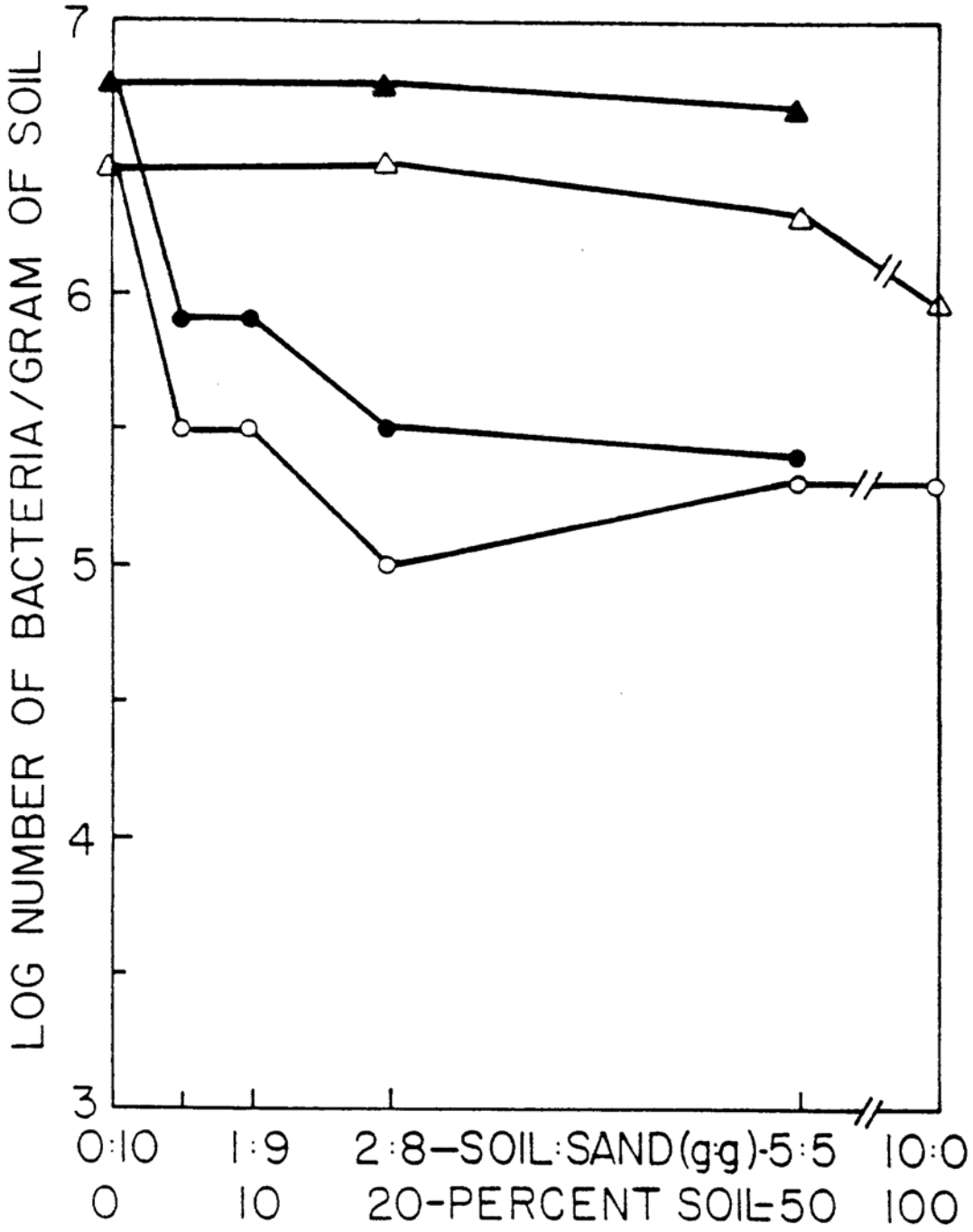

LOG NUMBER OF BACTERIA/GRAM OF SOIL
7
6
5
4
3
0:10
1:9
2:8-SOIL:SAND (g:g)-5:5
0
10
20-PERCENT SOIL-50

Figure 11. Soil Titrations - comparison of the Modified Soil Release Procedure (MSRP) (triangles) and the Soil Release Procedure (SRP) (circles) for the recovery of a strain of chickpea Rhizobium from two midwestern Mollisols. The MSRP procedure is not affected by soil content, while recovery with the SRP procedure is more soil dependent. (SRP data same as in Figure 8)




LOG NUMBER OF BACTERIA/GRAM OF SOIL
7
6
5
4
3
0:10
1:9
2:8—SOIL:SAND(g:g)-5:5
10:0
0
10
20-PERCENT SOIL=50
100

other tropical soils when the SRP assay was employed. This was discussed previously in Chapter 2. Even though these three soils were less sorptive, the MSRP procedure was able to recover more Rhizobium cells.

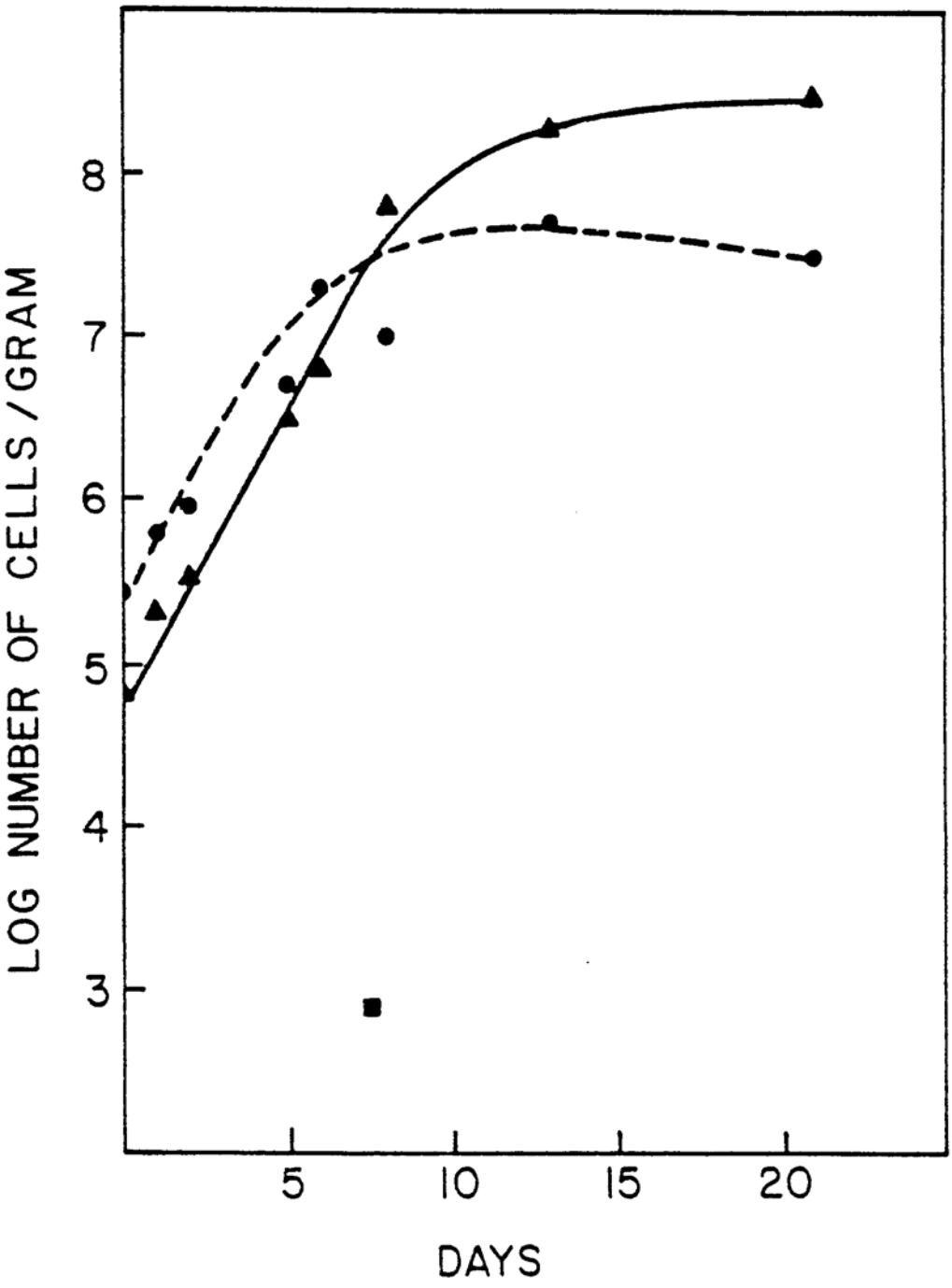
Bacteria growing in soil may attach to soil particles by different mechanisms than bacteria that are added from culture and extracted shortly thereafter. Scheraga et al. (1979) found that bacteria added to marine sediments were rapidly sorbed. The sorption phenomenon followed a Langmuir plot (Fletcher, 1977) and therefore indicated physico-chemical adsorption. Although Marshall (1980) stated that no definite evidence exists that would indicate polymeric bridging as important in the binding of bacteria to soils and sediments, polymer exudations from bacteria could bind them to soil particles.

In the previous experiments, where attempts to modify the soil release procedure (SRP) (see Appendix Table 21) resulted in a modified method of dispersion, the bacteria were added to the soil and recovered two hours later. Since the bacteria were not growing in the soil, physico-chemical binding was predominant. The MSRP method was successful in overcoming these forces and extracting the bacteria from the various soils. To determine the feasibility of using MSRP for studies of Rhizobium growing in nature, where polymeric briding might be important, several sterile systems were established. The advantage of the sterile systems is that it allows the various IF enumeration procedures to be checked against viable counts. Under non-sterile conditions, as discussed previously, it is extremely difficult to enumerate Rhizobium by plate counts.

The growth of TAL-620 was followed for 20 days in sterile Wahiawa Oxisol by plate counts and the modified soil release procedure. One set of data was analyzed by plate counts, the soil release procedure and the modified soil release procedure (see Figure 12).

The MSRP method underestimated the plate counts at the lower numbers of

Figure 12. Growth of TAL-620 in sterile Wahiawa Oxisol followed by plate counts (-●--●-), the modified soil release procedure (-▲--▲-), and the soil release procedure (■) (one data point only).


LOG NUMBER OF CELLS / GRAM
8
7
6
5
4
3
5
10
15
20
DAYS

cells and overestimated at the higher numbers. Bohlool and Schmidt (1973a) observed a similar phenomenon when the growth of USDA 110, a strain of R. japonicum, was followed in a sterile midwestern soil. They attributed the overestimation of cells by the IF enumeration procedure (SRP) to the accumulation of dead bacteria. In a natural environment dead cells would be degraded fairly rapidly by other organisms present in the environment.

In contrast to the relatively close correlation between the MSRP-IF enumeration procedure and plate counts, the SRP procedure and viable counts did not correlate. While both plate counts and viable counts indicated a population level of approximately 10^7 cells/g of soil the SRP method indicated only 10^3/g. The MSRP technique, as well as being able to quantitatively extract bacteria added to soil was able to quantitatively extract Rhizobium growing in soil.

Marshall (1969a,b) has shown that slow growing rhizobia have a different surface charge character than the fast growing rhizobia. The differences in surface charge might affect the ease with which slow and fast growing rhizobia are extracted from tropical soils. To ensure that the MSRP method was satisfactory for the enumeration of both fast and slow growing rhizobia two strains of R. japonicum were inoculated into sterile Wahiawa soil. The growth of USDA 31 and USDA 110 was followed by plate counts, the soil release procedure (SRP), and the modified soil release procedure (MSRP) (see Table 17). Although the MSRP method generally recovered more cells than the SRP method, neither procedure approached the levels indicated by the viable count. This problem is most likely soil dependent, however. When USDA 110 was followed in sterile (Table 18) Clarion soil (the soil:strain combination used to develop SRP) there was very close agreement between the viable count and the population level indicated by the modified procedure. However, in contrast to what I expected, the SRP method did not correlate closely with the plate counts or with MSRP. This is rather

Table 17. Growth of two strains of *Rhizobium japonicum* in sterile Wahiawa soil, followed by Plate Counts (PC), Soil Release Procedure (SRP), and Modified Soil Release Procedure (MSRP)

USDA 31 (Inoculated 1×10^4/g)

Days After Inoculation	PC	SRP	MSRP
5	-	2×10^4/g	6×10^5/g
7	2×10^7/g	8×10^4/g	3×10^6/g

USDA 110 (Inoculated 1×10^4/g)

Days After Inoculation	PC	SRP	MSRP
5	4×10^4/g	2×10^4/g	3×10^3/g
14	2×10^6/g	6×10^4/g	4×10^5/g

puzzling as close correlation was reported in the literature (Bohlool and Schmidt, 1973a). However, the problem may be related to the use of blendors, as described earlier.

In general the modified soil release procedure was able to recover Rhizobium from soils more efficiently than the previously published immunofluorescence enumeration procedure (SRP, Appendix Table 21). Most likely the success of the modified method in recovering bacteria was due to the use of a complex, multicomponent chemical extractant (0.1% PHG - O.1M $(NH_4)_2HPO_4$) and a method of dispersion that did not create high shear forces such as occur in blending. The lack of high shear force is important when working with Oxisols since the well aggregated subunits will disintegrate into clay sized components if subjected to too much stress. This can lead to a large increase in the surface area available for microbes to interact with the clays. Without a complex chemical extractant to reduce the number of charges within the soil matrix, it seems likely that the bacterial cell would become rapidly sorbed to clay particles, thus reducing the level of recovery.

Ion exchange may be an important phenomenon in the extraction and recovery of bacteria by the MSRP procedure. However, more experiments need to be performed under carefully controlled conditions to fully elucidate the relationship of cation (and perhaps anion) exchange to recovery of Rhizobium when using MSRP. For the present, however, the method successfully extracts Rhizobium from tropical soils more efficiently than previously published procedures. This should not permit the study of the ecology of free-living Rhizobium in tropical soils.

Table 18. Growth of USDA 110 in sterile Clarion soil, followed by Plate Counts (PC), Soil Release Procedure (SRP), and Modified Soil Release Procedure (MSRP)

(Inoculated 2 x 10^5/g)

Days After Inoculation	PC	SRP	MSRP
3	7 x 10^6/g	7 x 10^4/g	2 x 10^6/g
5	2 x 10^8/g	1 x 10^7/g	1 x 10^8/g

APPENDICES

Appendix Table 19a. Yeast extract-mannitol medium (YEMS) (Bohlool and Schmidt, 1970)

Difco Yeast Extract	1.0	grams
Mannitol	10.0	grams
$K_2HPO_4 \cdot 3H_2O$	0.65	grams
$MgSO_4 \cdot 7H_2O$	0.2	grams
NaCl	0.1	grams
Difco Agar	15.0	grams
H_2O	1	liter

Final pH adjusted to 7.0 - 7.2

Appendix Table 19b. Defined agar medium (Vincent, 1970)

K_2HPO_4	1.0	gram/liter
KH_2PO_4	1.0	gram/liter
$FeCl_3 \cdot 6H_2O$	0.01	gram/liter
$MgSO_4 \cdot 7H_2O$	0.25	gram/liter
$CaCl_2 \cdot 6H_2O$	0.1	gram/liter
Sodium Glutamate	1.1	gram/liter
Mannitol	10.0	gram/liter
Difco Agar	15.0	gram/liter
Biotin	50.0	microgram/liter
Panothenic Acid	100.0	microgram/liter
Thiamine HCl	100.0	microgram/liter
H_2O	1	liter

Final pH adjusted to 7.0

Appendix Table 20. Plant nutrient solution (Broughton and Dillworth, 1971)

Solution 1	$CaCl_2 \cdot 2H_2O$	294.1	grams/liter
Solution 2	KH_2PO_4	136.1	grams/liter
Solution 3	Fe Citrate	6.7	grams/liter
Solution 4	$MgSO_4 \cdot 7H_2O$	123.3	grams/liter
	K_2SO_4	87.0	grams/liter
	$MnSO_4 \cdot H_2O$	0.338	grams/liter
	H_3BO_3	0.247	grams/liter
	$ZnSO_4 \cdot 7H_2O$	0.288	grams/liter
	$CuSO_4 \cdot 5H_2O$	0.100	grams/liter
	$CoSO_4 \cdot 7H_2O$	0.056	grams/liter
	$Na_2MoO_4 \cdot 2H_2O$	0.048	grams/liter

Appendix Table 21. Protocol for the release of soil bacteria for enumeration by immunofluorescence microscopy (Soil Release Procedure SRP) (Bohlool and Schmidt, 1973a; Schmidt, 1974, as modified by May 1979, and Kingsley)

Step	Procedure
1. Dispersion:	In a blendor place 10 grams of soil, 93 mls of H_2O, 5 drops of Tween 80, 2 mls (1:20 dilution in water) of antifoam (= 1:10 dilution of soil). Blend 5 minutes.
2. Flocculation:	Transfer mixture to narrow container, add 0.7 g of flocculant mixture ($Ca(OH_2)$:$MgCO_3$, 2:5), shake vigorously several seconds, allow mixture to settle 30-60 minutes.
3. Filtration:	Pass desired volume of supernatant fluid over pre-blackened polycarbonate membrane-filter. Remove filter from assembly and place on glass microscope slide. Cover effective filtering surface with 3 drops of gelatin-rhodamine conjugate. Dry in an oven at 50-60°C. Dried, conjugate-stained filters may be either kept in the oven or stored in a desiccator until staining.
4. Staining:	Cover effective filtering surface with 2-3 drops of filtered (0.45 µm) FA incubate in moist chamber 20 minutes. Replace filter on filtering assembly and rinse with copious amounts of filtered (0.45 µm) saline (at least 150 mls.).
5. Examination:	Place filter on microscope slide, add a drop of FA mounting fluid (PBS:glycerol, 1:10), cover with a coverslip. Count reactive bacteria in 20-100 fields (or more if numbers are very low) using incident light fluorescence microscopy.
6. Calculations:	Number/g = $(N_f \cdot A \cdot D)/aV$ where: N_f is average number/microscope field; A is effective filtering area filtering area (cm^2); D is dilution factor; a is area of microscope field in cm^2, and V is volume in mls of supernatant fluid passed through filter.

Appendix Table 22. Formulation for phosphate buffered saline (PBS) 0.1M pH 7.2

to 81. H_2O add	1)	35.8 g $NaH_2PO_4 \cdot H_2O$
		allow to completely dissolve
add	2)	92.4 g Ha_2HPO_4
		allow to completely dissolve
add	3)	72.0 g NaCl
		adjust pH to 7.1 - 7.2
add	4)	0.8 g Thimerosal

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