SEROLOGY OF RHIZOBIA FOR TROPICAL LEGUMES

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In this presentation, the serology of rhizobia and application of serological techniques to ecological studies with rhizobia have been briefly outlined and discussed. The main points that should have emerged are :

1. Serological techniques (agglutination particularly) is an old procedure.
2. Differentiation between H and O antigens has extended the range of serological classes that can be recognized because of the greater heterogeneity distinguishable by the somatic antigen analysis.
3. There are three serological techniques available each of which has its own particular merits depending on the situation. The techniques are :(*a*) agglutination and agglutinin absorption, (*b*) gel immunodiffusion, and (*c*) fluorescent antibody.
4. Relations between rhizobial strains for tropical legumes based on those for soybean reveal a high degree of diversity and specificity in somatic antigen composition.
5. Somatic antigen constitution permits a wide range of applications of available techniques to : (i) tests of authenticity of strains, (ii) provide a means of sorting out mislabelled or otherwise mixed cultures, (iii) qualitative check in legume inoculant quality control, (iv) determination of proportion of members of mixed serotypes of known constitution (pure culture studies), (v) assessment of success of selected strains to form nodules under field conditions when in competition with native rhizobial populations, and (vi) determination of constitution of native rhizobial populations by serogrouping against a bank of antisera prepared against standard strains and the relation of serotypes to dominant soil properties such as *p*H and soil base status.

HISTORICAL REVIEW AND INTRODUCTION

The earliest recorded serological study with the root-nodule bacterium, *Rhizobium*, is that of Zipfel (1912) who was able to show a relation between the rhizobia forming nodules on *Pisum sativum* and *Phaseolus vulgaris*. Other work soon followed, e.g. Klimmer and Kruger (1914) divided 18 cultures from as many different legumes into nine groups using agglutination tests and concluded that rhizobia isolated from different species of plants were serologically distinct but related at the same time. Vogel and Zipfel (1921) observed, in addition, that the serological properties were stable and characteristic of strain. Perhaps the most significant early contributions were those of Stevens

(1923) and Jimbo (1930). These workers whilst recognizing serological differences between the rhizobia isolated from different species also recognized distinct serological groups within species. Even more important was the observation that nodules from a single plant could form distinct serological groups which may be related or unrelated to each other.

Using a collection of 55 strains from seven host groups, Stevens (1923) classified them into 18 distinct groups on the basis of cross agglutination titration checks against antisera developed against 40 of the strains. There were in fact a minimum of 20 groups (Table I). Of particular interest are the results for the 8 soybean strains for which four distinct serological groups were recognized on agglutination cross-reaction tests. Although only four groups were recognised it is obvious from the agglutination titres that strains within groups were related but not identical (Table II).

TABLE I

Serological groups in seven host groups (Stevens 1923)

Host from which isolated	No. of strains	No. of serological groups
Clovers	8	4
Medics	13	2
Pea-Vetch	10	4
Bean	3	2
Cowpea-types	4	2
Soybean	8	4
Lupin	1	1
	47	19
Unknowns	8*	1*
Total	55	20

* Eight strains (host of origin unknown) apparently did not agglutinate with any antiserum—postulate therefore a minimum of one (1) serologic group to account for them, giving a total of 20 serologic groups.

Attempts to correlate serological character with invasiveness and N-fixing ability were unsuccessful (Vogel and Zipfel 1921). Some correlation of serological and host groupings was observed by Wright, Sarles and Holst (1930); however, Bushnell and Sarles (1939) found no correlation between the ability of strains to cross-inoculate and cross-agglutinate, when working with rhizobia from the cowpea, soybean and lupin groups.

Bushnell and Sarles (1939) observed three components of the antigenic complex, the first in heated-washed cells, the second associated with the flagella

and the third the cell surface and/or its associated polysaccharide capsular gum. Ability to stimulate antibody formation in the animal and agglutination titre were significantly higher in the whole-cell unheated antigen than in the other two modified antigens. Vincent (1941) was the first to make use of this information in classifying (or grouping) rhizobial isolates. He differentiated between the flagellar (H) (relatively non-specific) and somatic (O) relatively specific) agglutinations and in addition made use of agglutinin absorption tests for differentiation between strains. Using these criteria, Vincent (1941) postulated three flagellar and seven somatic antigens to account for the cross reactions of six strains from medics. Antigenic constitution within groups of strains and serological groupings of a range of isolates for clover, medics and peas was reported subsequently (Vincent 1942 ; Hughes and Vincent 1942 ; Kleczkowski and Thornton 1944 ; Kleczkowska, Nutman and Bond 1944 ; Purchase, Vincent and Ward 1951). Little or no work was reported using these criteria for tropical type legumes until the 1960's when a number of papers on serology of soybean rhizobia appeared. However, at this time two new methods of serological assay were developed. Firstly, the gel immunodiffusion (Ouchterlony double diffusion) technique introduced by Dudman (1964), and secondly the fluorescent antibody specific staining technique (Trinick 1969 ; Schmidt, Bankole and Bohlool 1968). Each of these techniques utilizes both pure culture or bacteroids from crushed nodules as antigen (Means, Johnson and Date 1964 ; Skrdleta 1969*a* ; Trinick 1969).

TABLE II

Grouping of soybean rhizobia (Stevens 1923)

(*reciprocal of titre* $\times 10^3$)

		Antiserum							
		161	164	166	168	163	162	160	165
Antigen	161	5	2	1	7.5	—	—	—	—
	164	2	2	1	2	—	—	—	—
	166	2	2	1	7.5	—	—	—	—
	168	—	2	2	7.5	—	—	—	—
	163	—	—	—	—	20	5	—	—
	162	—	—	—	—	10	5	—	—
	160	—	—	—	—	—	—	20	·1
	165	—	—	—	—	—	—	.05	5

METHODS, ANTIGENS, ANTISERA AND INTERPRETATION OF ANTIGEN/ANTIBODY REACTIONS

Before proceeding to discuss application of serological techniques to the study of rhizobia and strain recognition it is appropriate that we discuss some details of the various serological techniques, used antigens, antisera preparation and interpretation of antigen/antibody reactions.

(a) *Methods*

(i) *Agglutination*—This depends on the exposed surface and near surface antigens of the whole cell. It is the simplest technique requiring minimal equipment and experience. It can however be used for detailed analyses by employing cross-reaction and agglutinin-absorption tests. Agglutination tests are routinely conducted at a final antiserum concentration of 1/100 (1/10 plus sufficient antigen suspension to give 1/100 in a total volume not exceeding 1 ml, in narrow tubes, e.g. Dryer agglutination tubes, Durham tubes, etc.). These are incubated in a water-bath at 52°C, although some workers prefer 37°C, for 2—4 hr depending on strain. By rule-of-thumb, the slow-growing strains require longer incubation times than the fast-growing strains, for complete agglutination.

Distinction between flagellar (H) and somatic (O) reactions is important. The H reaction is a loose flocculant clumping of cells visible in 30-60 min, while the O reaction is a compact fine-grained precipitation of bacterial cells which pack tightly in the bottom of the tube leaving a clear supernatant. The H reaction is frequently non-specific and is of little value in grouping or classifying the strains. The antigen is readily destroyed by heating to 100°C for 30 min leaving only the more specific O antigens which are thermostable. It should be noted, that in non-heated antigens H reactions can be masked by O reactions if not read sufficiently early in the incubation period. For this reason and the fact that it is a weak reaction, flagellar reactions are frequently hard to detect.

The technique of agglutinin-absorption provides a means for more detailed antigenic analysis in cases where a group of strains cross-react with each others' antisera. Common somatic antigens can be absorbed out of antiserum by reacting a concentrated (approx 10^{10}/ml) suspension of cells in equal volume of 1/5 to 1/10 diluted antiserum in the normal way (or overnight in refrigerator), centrifuging to remove agglutinated cells and decanting the clear, absorbed (diluted) serum (see outline Table III).

(ii) *Gel immunodiffusion*—Precipitation reactions using the gel diffusion technique (Ouchterlony 1958—double diffusion) provide a more definitive method based on soluble/diffusable antigens. This is usually conducted in "ion agar" (0.75 to 1.0 per cent plus 0.025 per cent sodium azide) by cutting 4 mm diameter wells in 4 mm deep agar in various patterns (e.g. hexagonal set) to which antigens and antiserum are added according to the test being made. The general technique of gel diffusion and the interpretation of

TABLE III

Agglutinin absorption

In Situation				
		Antiserum 1	Antiserum 2	Comment
	Strain 1	+	+	mutual cross
	Strain 2	+	+	reaction
then agglutinin absorption		Antiserum 1 + Strain 2		conc. cell suspension plus antiserum, incubation, centrifugation
		Antiserum 2 + Strain 1		
retest		Antiserum 1 (abs. str. 2)	Antiserum 2 (abs. str. 1)	
	Strain 1	+	—	
	Strain 2	—	+	

then interpret minimal antigenic constitution as :

Strain 1 AB with corresponding antibodies ab.

Strain 2 AC with corresponding antibodies ac.

that is		Anti ab (abs. AC) Anti b	Anti ac (abs. AB) Anti c
	Strain AB	+	—
	Strain AC	—	+

precipitin bands is described by Crowle (1961). There are three basic relations between precipitin bands when testing unknowns (Fig. 1) :

(1) Complete identity—when precipitin bands link up completely with those of the homologous control (Isolate 1), (2) Partial identity—(i) closely related to homologous strain, but different as indicated by a strong spur formation on the specific identifying band (Isolate 3), (ii) poorly related by secondary (intracellular) bands (Isolate 2), and (3) Non-identity when no precipitin bands are formed (Isolate 4).

Specific bands of identity are readily recognized as the stronger more curved bands closer to the antigen well. Non-specific bands may appear either side of the specific band, are more faint and straight and thermolabile (heating to 100°C, 30 min). Heating (or of antigens has the advantage of intensifying the precipitin band in many strains. In some instances more than one specific band may be present. As in the agglutination technique antibodies can be conveniently absorbed, by adding the appropriate antigen to the antiserum well (Humphrey and Vincent 1965).

(iii) *Fluorescent antibody* (F.A.)—This is a relatively recent technique adapted to rhizobial work by Trinick (1969) and Schmidt, Bankole and Bohlool (1968). It consists of labelling specific antiserum with a fluorescing dye (fluorescein isothiocyanate-FITC) which is used to stain a smear of the culture under test. After incubation the excess labelled antiserum is washed-off and the smear examined under the microscope using light of appropriate wavelength (for FITC

490-495 nm using a UV or 100 W quartz/iodine light source with appropriate excitation and barrier filters). This technique necessitates good microscope equipment with dark field facility for best results. It does however suffer the same lack of definitiveness as does the agglutination procedure, but with judicial dilution of antiserum cross-reactions can be eliminated or at least readily recognized by the weaker fluorescence. Non-specific fluorescence can be reduced by the use of gelatin rhodamine during the staining procedures (Bohlool and Schmidt 1968).

(iv) *Antigens*—These are generally saline (0.85 per cent) suspensions of pure cultures containing 10^8—10^9 cells per ml for agglutination and 10-–50 mg/ml dry wt (approx. 10^{10}—10^{11} ml) for gel diffusion (Dudman 1964; Dudman and Brockwell 1968). Where antigen preparation is for antiserum preparation cells can be washed in saline and recovered by centrifugation, but this may result in some loss of soluble antigenic material.

The bacteroid antigen (expressed juice of the nodules) is antigenically active. Bacteroid antigens have been exploited for species forming large (3-4 mm) nodules in the agglutination and gel diffusion (2-3 mm minimum size) test. For FA testing nodules of 0.5 mm give satisfactory results. Bacteroid antigens give almost identical results as cultures with antisera developed against culture antigens (Means, Johnson and Date 1964; Skrdleta 1969*a*, *b*; Trinick 1969).

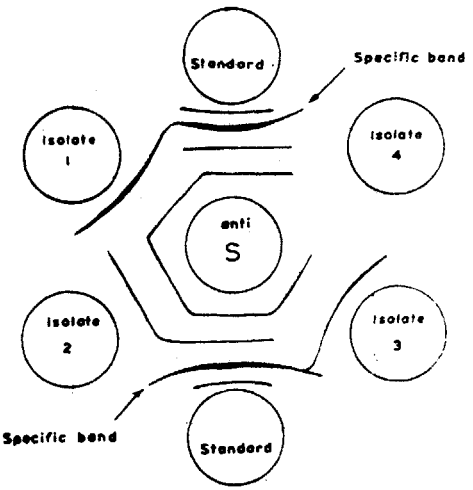

FIG. 1. Example of gel diffusiou (after Dudman & Brockwell 1968) showing typical pattern of hexagon set of wells around a central well containing antiserum prepared against standard strain. Isolate 1 was identified as identical with standard. Isolate 2 was identified as a cross-reacting strain. Isolate 3 was identified as a different type of cross reacting strain, more closely related to standard than isolate 2 because this isolate shares some of the specificity of the standard strain specific antigen. Isolate 4 was completely unrelated to the standard strains.

(v) *Antisera preparation*—The route of injection is variable and no definitive procedure is laid down. In much of the early work antigen was injected either intravenously or interperitoneally in several successive increasing doses (generally 0.5—1 ml to 3—4 ml) on successive days or with 5-7 days between injections. Most workers today use an intramuscular injection of culture and adjuvant followed by an intravenous booster 1 month later. Blood is then collected 5—10 days later by bleeding from the ear vein, cardiac puncture or slaughtering the animal. Rabbits have been found to be the most convenient animal and readily provide antisera with antibody titres ranging from 1/3000 to 1/10,000 or more depending on rhizobial strain, rabbit, route of injection, etc.

Relations between strains

There have been few studies of the serology of rhizobia for tropical legumes (Koontz and Faber 1961; Date and Decker 1965; Skrdleta 1965 and 1969*b*, *c*; Dudman 1971). All of these have included only strains for soybeans, except for Dudman (1971), who examined, in addition to soybean isolates, three strains for tropical pasture legumes.

Serological relations between the strains for soybeans were determined by somatic agglutinations and agglutinin-absorption test. All six papers reported grouping of strains but only one the antigenic constitution of the strains involved. Koontz and Faber (1961) determined a minimum of six somatic groups for 22 strains with antisera developed against 14 of them whilst Skrdleta (1965) found four main groups within 62 strains using antisera developed against 11 strains. Within the 11 strains only two somatic groups were recognized. Twenty-two of the 62 strains did not agglutinate with any antiserum, compared with 3 for Koontz and Faber (1961). Date and Decker (1965) using 28 strains observed 17 serological groups and proposed a minimal constitution of 24 antigens using antisera developed against all 28 strains. Six of these were "single" antigens the strain reacting only with its homologous antiserum (Fig.2).

The serogroups determined in this study (Date and Decker 1965) formed the basis for serotyping large numbers of field isolates. That the antigenic constitution presented is only minimal is illustrated by the finding of Johnson and Means (1963) where unknown-field isolates agglutinated with both antisera 122 and 123. These strains did not mutually cross-react in pure culture. Thus each strain must have had in addition an additional but distinct antigen (Table IV). Relations between strains using gel diffusion as determined by Skrdleta (1969*b*) and Dudman (1971) show similar antigenic constitutions. Skrdleta (1969*c*) has studied the cross reactions of 11 strains of soybean rhizobia for both thermostable and thermolabile antigens. His Table II illustrates the complexity of the relationship with numbers of precipitin bands for slow (specific) and fast (common) moving antigens. Dudman (1971) has shown similar relations with heat-treated antigens of 7 soybean strains, 1 cowpea-type legume, 1 *Desmodium* and 1 *Lotononis bainesii* strain. Contrary to this no cross reactions were detected within a group of 12 strains for tropical legumes (8 cowpea-type, 2 *Desmodium*, 2 *Stylosanthes*) and only 2 strains gave more

ANTISERA \ ANTIGEN	3	24	44	6	31	40	120	46	710	121	117	74	76	127	123	129	122	38	115	110	125	126	62	94	124	128	130	4
3	3	2	3	2																								
24	3	2	3	2																								
44	3	2	4	2	(3	2	2)																					
6	3	2	3	3																								
31					4	4	3																					
40					4	4	4																					
120					4	4	4																					
46								5	4	4																		
710								5	4	4																		
121								5	5	5																		
117											5	2	3															
74											1	2	2	1														
76											3	2	3	2														
127												2	2	5	2													
123														3	4	3												
129														1	3	3												
122																4	4											
38																		3	3									
115																		2	4	3								
110																			1	3								
125																					4	3						
126																					4	4						
62																							3					
94																								2				
124																									4			
128																										2		
130																											3	
4								(1	2	2)																		4

Numbers indicate highest antisera dilutions showing agglutination
viz. 1 = 1/100 or less, 2 = 1/200 to 1/400, 3 = 1/800 to 1/1600,
4 = 1/3200 to 1/6400, 5 = 1/12800 or greater

1. Antigen unstable but agglutination distinguishable at dilutions of 1/200 or less

FIG. 2. Somatic antigen grouping of 28 strains of *Rhizobium japonicum* (*Date and Decker 1965*)

than 1 precipitin band (Date, unpublished). The reason for this may be the differences in method of antiserum collection. For example, Dudman (1971) collects samples of blood on the 5th, 7th, 9th, and 12th days after the booster injection compared to the author's routine collection by complete bleeding

TABLE IV

Antigenic constitution of serogroup 122, 123
(isolates versus standard antisera)

Strain	Minimal antigens	Additional antigens
121	XII XIV	XXV
123	VIII X XI	XXVI
Unknown (cross reacts with antisera 122 and 123)	XXV XXVI	?

Strains 122 and 123 do not cross-react.

of the animals 5—7 days afterwards. In some strains the longer interval between booster injection and bleeding permits a greater range of antibodies to be produced, presumably because of greater breakdown of the antigen in the immunized animal. For strain CB756 (cowpea-type) length of interval between booster injection and bleeding had no influence on the number of precipitin bands as only a single specific band formed, but for CB627 (*Desmodium*) a single precipitin band was obtained when the rabbit was bled 5 days after the booster injection but 2 and sometimes 3 precipitin bands at subsequent bleeds (Fig. 3).

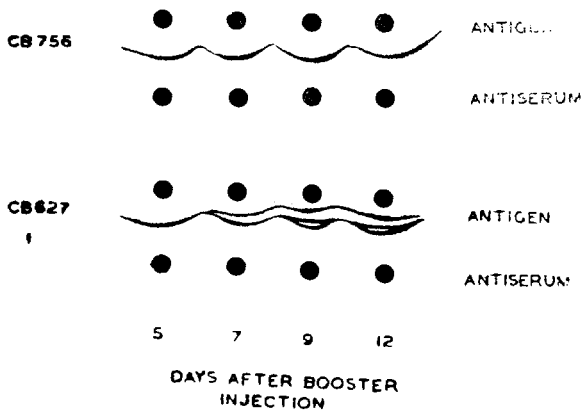

FIG. 3. Variation in number of precipitin bands between *Rhizobium* strains and number of days between booster injection and bleeding for serum collection. Note that strain CB756 formed only 1 precipitin band whereas strain CB627 formed only 1 band at 5 days but 3 bands by 9 days after the booster injection.

APPLICATION OF SEROLOGICAL TECHNIQUES TO THE STUDY OF RHIZOBIA

Serological methods can be used for strain recognition and ecological studies of natural populations. Its accuracy is dependent on being able to define the parameters of the experiment to avoid misinterpretation due to false positive identifications and groupings. Some of the uses to which serological methods can be put are :

(i) to check the authenticity of a culture which has changed its cultural or symbiotic properties. For example, Vincent (1962) has tabulated a number of characters for variants of two clover strains;

(ii) to provide evidence in sorting out accessions of cultures which have become mixed or mislabelled;

(iii) check on the trueness to label of manufactured cultures (inoculants).

(iv) as an investigational tool in identification of members of a population of mixed serotypes of known constitution, e.g. Skrdleta (1969*a* and *b*) and Means, Johnson and Erdman (1961);

(v) to assess success of strains as seed inocula in field trials, especially when in competition with other (native) populations of rhizobia able to form nodules on the particular host plants;

(vi) to gather information on the constitution of natural populations, i.e. determination of number and proportion of various serotypes in ecological studies and the relation of serotypes to a dominant soil property such as *p*H, soil base status or differences in proportions of isolates belonging to various serotypes from different hosts (e.g. Holland 1966) and the same host (Hughes and Vincent 1942).

These three latter aspects have received considerable attention, however, again for rhizobia of tropical legumes this has been restricted mostly to rhizobia for soybeans for published material and the author's unpublished work with tropical pasture legumes such as *Phaseolus atropurpureus*, *Glycine wightii*, *Desmodium intortum* and various species of *Stylosanthes*.

In mixtures of two strains Skrdleta (1969*a*) obtained between 0 and 92 per cent of nodules due to one or other strain when five strains of his serogroup B were competing against four strains of serogroup A, in pairs of approximately equal numbers. Stronger competitive effects were observed by Means, Johnson and Erdman (1961) where as little as 1.1 per cent of the competitive strain 76 accounted for 84-87 per cent of the nodules when competing against strain 38. Strain 94 was less competitive and accounted for only 19 per cent of nodules when at 0.8 per cent of the mixture with strain 38. In competition individually with five other strains, strains 76 was more competitive (71-100 per cent) than strain 94 (0-96 per cent) against four of them but was less competitive (41 per cent compared with 74 per cent) with the fifth strain. In a non-competitive field site (no native rhizobia) Caldwell (1969) confirmed Johnson and Means (1964) glasshouse data that in mixture strains $38 < 76 < 110$ in competitiveness in nodule formation. Skrdleta and Karimova (1969) also have observed such effects with two strains from Skrdleta's (1969*b*) serogroups A and B. In this case the stronger competitor (serogroup A strain) still accounted for 98 per cent of nodules when outnumbered in the inoculum by 60 to 1. Host genotype will have a strong influence on competitive ability, the proportion of nodules due to a given strain on one host may be entirely different on

a second host inoculated with the same inoculum mixture (Johnson and Means 1960).

The success of applied strains in forming nodules is not only influenced by competition between strains of the inoculum but also by their competitive ability with native rhizobial populations and their reaction to various soil properties. As little as 0 to 10 per cent of nodules only can be formed by seed applied inocula in soils known to carry effective rhizobia. This can be increased to 25 to30 per cent by increasing inoculum numbers or other seed treatment, but varies according to the nature of native population, soil (site), inoculum strain and host species (cultivar) (Johnson, Means and Weber 1965; Caldwell and Vest 1970; Ham, Caldwell and Johnson 1971). Even poorer results have been obtained with *Phaseolus atropurpureus*, which is a very promiscuously nodulating legume, on a number of sites ranging from high levels of native rhizobia effective in N-fixation to sparse, moderately effective populations. The success of the inoculum strain for *Desmodium intortum* at the same sites ranged from less than 5 per cent of nodules to almost 100 per cent, again illustrating that the specificities of the host and nature of native rhizobial population have a profound influence on the successfulness of seed applied inoculum strains (Date, unpublished).

The same bank of antisera used for assaying success of inoculum strains can be used to serogroup native populations. Such groupings can indicate a different population of bacteria in nodules of plants grown in different areas. One or two predominant serogroups are usually present, but the predominant groups differ among soils (Johnson and Means 1963). The proportion of serogroups can vary also from year to year (Vest, Caldwell and Petersen 1971) within seasons according to planting date (Caldwell and Weber 1970), host genotype (Caldwell and Vest 1968) and soil factors such as *p*H, soil type, per cent organic matter, nutrient status, mechanical analysis (Damirgi, Frederick and Anderson 1967; Ham, Frederick and Anderson 1971). As much as 80 per cent of the variation of some serotypes was attributed to soil *p*H. In areas where native legumes are present the composition of the rhizobial population does not appear to be as homogenous as in the case of the soybeans. No predominant groups have so far been detected when *Phaseolus atropurpureus* and *Desmodium intortum* have been used as the trap hosts (Date, unpublished).

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