

# ANTIGENIC ANALYSIS OF SOYBEAN RHIZOBIA BY IMMUNODIFFUSION

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## ABSTRACT

Immunodiffusion reactions were studied with twelve strains of *Rhizobium japonicum*. Antisera were prepared against the four *R. japonicum* strains which are presently used in the Nitragin 'S' inoculant. Five local isolates from soybean plants grown in different locations of the country along with four Nitragin strains were used to react with the inoculum antisera. The Nitragin strains were the parent strains of the sub-strains used in the present Nitragin 'S' inoculant. Strong precipitin bands were formed by homologous antigens. Out of the inoculum strains only 61A101c showed an antigenic affinity with the locally isolated strains. As strains 61A101 and SLM 112 gave similar type of precipitin bands with sera of 61A101c, it could be deduced that both these strains are the same.

## INTRODUCTION

Inoculation is a practice of introducing viable bacteria into the soil at the time of planting (Frederic, 1976). The bacteria infect the root zone and fix atmospheric nitrogen effectively under favourable conditions. Soybean (*Glycine max* (L.) Merrill) is an introduced crop to Sri Lanka and its establishment has largely depended on *Rhizobium* inoculation (Herath, 1975). The Nitragin 'S' inoculant supplied by the Nitragin Company, U. S. A. is the widely used inoculant for soybean in Sri Lanka. This inoculant is a multi-strain one and contains four efficient *R. japonicum* strains (Smith, Per. comm.).

A correct evaluation of the effectivity of inoculation largely depends on the recognition of inoculant strains in the root nodules by some means. The immunodiffusion technique is a widely accepted procedure for the recognition of strains (Ikram and Broughton, 1980). In Sri Lanka, this

aspect of strain recognition in the field has been widely over looked and the present study was conducted to investigate the antigenic relationships of the *R. japonicum* strains found in the inoculum and in the field.

#### MATERIALS AND METHODS

Recently, three sub-strains have been selected from the original parent strains of the Nitragin 'S' inoculant. These sub-strains are reported to be significantly better nitrogen fixers than the parent strains and are used for commercial inoculum production presently by the Nitragin Company, U.S.A, (Woolfenden, Per. comm.).

Antisera were prepared for the present Nitragin 'S' strains and reacted with the parent strains and the locally isolated *R. japonicum* strains..

Table 1 depicts the rhizobial strains used in the study. All organisms were flow-growing alkali-producers.

Table 1. Rhizobia

| <i>Strains</i> | <i>Origin</i>                   | <i>Host Plant</i> |
|----------------|---------------------------------|-------------------|
| 61A124         | New Zealand                     | <i>G. max</i>     |
| 61A118         | Mississippi, U. S. A.           | <i>G. max</i>     |
| 61A101         | Illinois, U. S. A.              | <i>G. max</i>     |
| 61A148         | India.                          | <i>G. max</i>     |
| 61A124a        | Sub-strain selected from 61A124 | <i>G. max</i>     |
| 61A118b        | Sub-strain selected from 61A118 | <i>G. max</i>     |
| 61A101c        | Sub-strain selected from 61A101 | <i>G. max</i>     |
| SLM 101        | Haragama, Kandy                 | <i>G. max</i>     |
| SLM 111        | Gannoruwa, Kandy                | <i>G. max</i>     |
| SLM 112        | Gannoruwa, Kandy                | <i>G. max</i>     |
| SLM 115        | Mathurata, Nuwara Eliya         | <i>G. max</i>     |
| SLM 113        | Karadiyan Aru, Batticaloa       | <i>G. max</i>     |

#### Maintenance of cultures

The rhizobial isolates were maintained on ceramic beads (Norris, 1963) and on yeast mannitol medium (Vincent, 1970). In place of 100 ml yeast water as in the original formulation, 0.4 g/litre of Difco Yeast Extract was substituted. At least once in every 3 months, rhizobia were checked for purity using phase contrast microscopy or by Gram staining. Incubation of stock cultures were at room temperature.

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### Preparation of antigens for immunodiffusion

Rhizobia from YMA slants were streaked on to fresh slants prepared in 100 ml capacity milk dilution bottles. The inoculated slants were incubated for 7-10 days at room temperature. Rhizobia were harvested aseptically in 0.85% (W/V) sterile saline and centrifuged at 8,000 rpm for 20 minutes. The concentrated cells were resuspended in small volumes of saline and freed off of clumps by vigorous shaking on a "whirlmixer". The final cell suspension was adjusted to  $10^9$ – $10^{10}$  cells per ml.

### Preparation of antisera

Albino rabbits were immunized (Schmidt *et al.*, 1968) against strains 61A124a, 61A118b, 61A101c and 61A148. When test bleeds showed satisfactory titres in agglutination reactions, the animals were bled through the marginal ear vein. Blood collected was held overnight at 4°C, the serum drawn off from the clot and kept in small aliquots at -15°C without addition of preservatives.

### Immunodiffusion technique

The immunodiffusion plates were prepared on glass petri dishes 9 cm in diameter. The gels were prepared with 0.75% (W/V) Difco Noble Agar in 0.85% sodium chloride containing 0.025% (W/V) Sodium azide as a preservative (Dudman, 1964). A gel layer of 4 mm in thickness was prepared on the glass plates. A hexagonal pattern of wells (5 mm apart from edge to edge, 4 mm in diameter) was cut using a cork borer. The gel plugs were removed with the help of a sharp pin. The bottom of the wells were sealed off with a drop of molten agar. To facilitate identification of the antigens the wells were labelled at the bottom of the petri dishes. Two drops (0.04ml/drop) of a saline suspension of the rhizobia ( $10^{10}$  cells/ml) heated at 100°C for 30 minutes, was used in the appropriate wells of the hexagonal pattern. After 2 hours of the addition of antigens the central wells were filled with 2 drops of the respective undiluted antiserum. A water saturated atmosphere was improvised by placing wet rubber sponges in a plastic tray and covering with a tight plastic lid. The immunodiffusion plates were incubated in this unit at room temperature (26°C–28°C). After 24–72 hours immuno-precipitation bands were recorded by drawing. All experiments were performed in triplicate.

## RESULTS AND DISCUSSION

Homologous titres of antisera ranged from 1600 to 3200. Fig. 1 shows the results of immuno-precipitation reactions developed with homologous and heterologous antigens. Only heat stable somatic antigens were studied. Antisera were in the centre wells and the respective antigens in the peripheral wells. Immuno-precipitation bands ranged from one to three. All strains formed strong precipitin bands when tested against their homologous antisera. Out of the sera produced for the four inoculum strains only serum of strain 61A101c reacted with the locally isolated SLM strains. As shown in Fig. 1.1, 1.2 and 1.3 except for strain 61A124a and 61A148, serum of strain 61A101c has produced internal thin bands with all other strains tested. However, specific strong external bands have been formed only by 61A101 and SLM 112 other than its homologous antigens. The precipitin bands formed in agar diffusion have been broadly divided into slow diffusible strain specific somatic antigen bands and fast diffusible non-specific internal antigen bands (Vincent and Humphery, 1970). As shown in Fig. 1.1, 1.2 and 1.3 formation of the thin internal bands suggests that strain 61A 101c has some antigenic properties related to strains 61A118b, 61A124, 61A118, SLM 101, SLM 111, SLM 113 and SLM 115 although they cannot be called identical by any means. The precipitin reactions of strains 61A101c, 61A101 and SLM 112 with serum 61A101c seemed identical except for a slight positional difference of the strong outer band formed by 61A101 which was closer to the antigen well compared to 61A101c. When the combinations in reciprocal were studied similar type of reactions were not observed. The serum of 61A118b did not react with 61A101c. This serum formed strong precipitin bands which were identical (Fig. 1.4) with its homologous antigen and its parent strain 61A118. However, a weak internal band was also formed with serum 61A118b and its homologous antigen. This second internal band did not appear with 61A118 antigen. No reactions were observed with serum 61A118b and other strains tested.

As depicted by Fig. 1.5 the serum of strain 61A148 reacted with its homologous antigen giving three close bands to each other. However, with strain 61A124a, serum 61A148 gave a single band which did not join with the other bands. Therefore, though these two strains have some common antigens they cannot be identified as similar strains. Serum 61A148 did not react with any other antigens tested.

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The most interesting feature of this study was that sera of strain 61A124a did not give any reaction with its parent strain 61A124. Therefore the selected strain has lost its antigenic similarity with its parent strain through the selection process. The cross reactions observed with serum 61A148 occurred reciprocally giving a single band with strain 61A148 and serum 61A124a and three close bands with homologous antigen and antibody (Fig. 1.6).

### CONCLUSIONS

This investigation has shown that strain 61A101c has antigenic affinities with many strains studied. As the precipitin reaction observed of serum 61A101c and strains 61A101 and SLM 112 were identical, it would be fair to conclude that these two strains are the same. SLM 112 has been isolated from Gannoruwa where inoculation with Nitragin 'S' has been conducted in previous seasons.

As no precipitin reactions were observed with serum 61A148 and the local isolates it is evident that these strains are not 61A148. This shows that strain 61A148 is not a good competitor or survivor in local soils. The sera of 61A124a and 61A118b also have not yielded positive reactions with the local isolates. However, it should be noted that the reactions between the parent strains and substrains of these two strains (61A124a and 61A118b) were not identical. The Nitragin 'S' inoculant used in Sri Lanka so far has contained only the parent strains. Therefore, until sera is produced for the parent strains and reciprocal reactions are observed, one cannot conclude the identity of the remaining local isolates. Also an interesting phenomenon of the investigation is the occurrence of cross reactions between parent strains and the selected strains. This could be resolved by producing antisera for the parent strains and observing gel precipitations of different combinations.

The occurrence of cross reaction is common and many workers have had similar experiences (Dadarwal *et al.*, 1974; Ikram and Broughton, 1980; Dadarwal and Sen, 1973). In gel diffusion however, it is not difficult to distinguish between homologous and cross-reacting strains based on the number and intensity of bands produced and band position within the gel when matched with those formed by the homologous antigens. This technique could reliably be used in field experiments in identifying inoculant strains.

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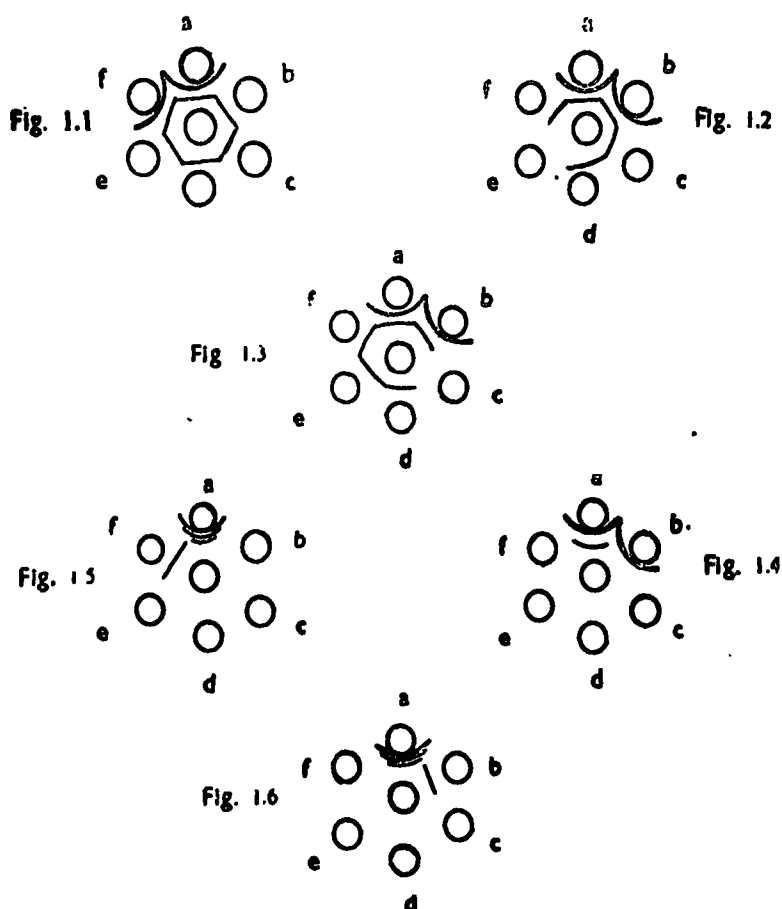


Fig. 1. Pattern of precipitin bands with homologous and heterologous antisera.

|                           | <i>Fig. 1.1</i> | <i>Fig. 1.2</i> | <i>Fig. 1.3</i> | <i>Fig. 1.4</i> | <i>Fig. 1.5</i> | <i>Fig. 1.6</i> |
|---------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Centre well<br>(antisera) | 61A101c         | 61A101c         | 61A101c         | 61A118b         | 61A148          | 61A124a         |
| Well a (Ag.*)             | 61A101c         | 61A101c         | 61A101c         | 61A118b         | 61A148          | 61A124a         |
| Well b (Ag.)              | SLM 115         | 61A101          | 61A101          | 61A118          | 61A118b         | 61A148          |
| Well c (Ag.)              | SLM 101         | 61A118          | 61A148          | 61A148          | 61A118          | 61A124          |
| Well d (Ag.)              | SLM 111         | 61A118b         | 61A118          | 61A124a         | 61A101c         | 61A118b         |
| Well e (Ag.)              | SLM 113         | 61A124a         | 61A118b         | 61A124          | 61A101          | 61A118          |
| Well f (Ag.)              | SLM 112         | 61A124          | 61A124          | 61A101c         | 61A124a         | 61A101c         |

\* — Antigen