

Polyclonal antisera production by immunization with mixed cell antigens of different rhizobial species

H.J. Hoben, P. Somasegaran,* N. Boonkerd and Y.D. Gaur

Somatic antigens of *Bradyrhizobium japonicum*, *Rhizobium* sp. (*Cicer arietinum*) and *Rhizobium* sp. (*Leucaena leucocephala*) were prepared as standard, single-species type from cultured cells. Equal numbers of the cells of these rhizobia were then combined to obtain a mixed-rhizobial-species antigen preparation. Rabbits were immunized either with the standard, single-species type or with the mixed-rhizobial-species antigen preparations. The antisera developed from the mixed antigen immunization contained antibodies for all three rhizobial species, detectable at agglutination titres of over 800. The mixed-rhizobial-species antisera were made species specific by cross-absorption. The cross-absorbed and the mixed-rhizobial-species antisera were generally similar in quality for strain identification by agglutination, fluorescent antibodies, immunoblot and ELISA. A 66% reduction in cost was estimated for the production of antisera by immunization with mixed-rhizobial-species antigen.

Key words: Antiserum, *Bradyrhizobium*, mixture, polyclonal, *Rhizobium*, somatic antigens.

The specificity of antigen-antibody reactions is the basis for the serological identification of microorganisms. Application of serology for the study of rhizobia dates back to Zipfel (1912), who showed, by using simple agglutination, the serological relationship between rhizobia that nodulate *Pisum sativum* and *Phaseolus vulgaris*. Since then other serological procedures for work with rhizobia, such as gel-immuno-diffusion (Humphrey & Vincent 1965; Holland 1966; Dudman & Brockwell 1968), the fluorescent antibody (FA) technique (Schmidt *et al.* 1968; Trinick 1969), and ELISA (Berger *et al.* 1979; Kishinevsky & Gurfel 1980), have been successfully used.

Currently, it has become common practice in research involving rhizobia and in rhizobial inoculant production to apply one or more serological techniques to monitor rhizobia in ecological studies, check the authenticity of cultures in collections, control inoculant quality and to help in the taxonomy and classification of rhizobia. With a few exceptions, where there is a need to develop and use

monoclonal antibodies (Wright *et al.* 1986), polyclonal antisera (henceforth referred to as antisera) are sufficiently analytical for work with rhizobia.

The traditional approach to antisera development is to immunize a single rabbit with the antigen preparation of a single rhizobial strain (Vincent 1970). However, there are no reports which describe the use of a mixture of antigenically distinct rhizobia for immunization of a single animal as an alternative and economic approach to develop high quality antisera. It would, of course, be most cost-effective if one rabbit could be used to produce antisera by immunization with an antigen mixture composed of several selected species or strains of rhizobia which are known not to share common somatic antigens. Strains or species of rhizobia or other bacteria which do not share common antigens are termed antigenically distinct.

Even though no published information is available, it is widely acknowledged by researchers working with rhizobia that the development of antisera is an expensive undertaking. Major costs are incurred mainly in the purchase of the rabbits and their maintenance during antisera development. The objectives of the present study are to evaluate the production of antisera by immunizing a single rabbit with an antigen preparation composed of antigenically distinct species of rhizobia and to test the antisera by selected serological techniques.

H.J. Hoben and P. Somasegaran are with the NIFTAL Center and MIRCEN, University of Hawaii, 1000 Holomua Road, Paia, Maui, HI 96779-9744, USA; fax: 808 579 8516. N. Boonkerd is with the School of Agricultural Technology, Suranaree University of Technology, University Avenue, Nakorn Racharsima, Thailand. Y.D. Gaur is with the Division of Microbiology, Indian Agricultural Research Institute, New Delhi-110012, India. *Corresponding author.

Materials and Methods

Rhizobia and Antigen Preparation

The three species of rhizobia used, previously tested for antigenic distinctness, were *Bradyrhizobium japonicum* strain TAL 102 (= USDA 110); *Rhizobium sp. (Cicer arietinum)* strain TAL 620 (= CB 1189) and *Rhizobium sp. (Leucaena leucocephala)* strain TAL 1145 (= CB 3060; CIAT 1967). Each was cultured separately on yeast extract/mannitol/agar (YMA) slants in flat medicine bottles (Vincent 1970) at 28 °C for 3 (*Rhizobium*) or 5 days (*Bradyrhizobium*). Bacteria were harvested aseptically in sterile saline (0.85% NaCl), washed three times in sterile saline by centrifugation (4000 × g, 20 min) to remove soluble somatic antigens and then heat-treated (steam) for 45 min to inactivate flagellar H-antigens (Vincent 1970; Somasegaran & Hoben 1994). The antigen preparation of each rhizobial species was adjusted to 1×10^9 cells/ml.

Based on the cell concentrations, two levels of the mixed-rhizobial-species antigens (henceforth referred to as Ag-I and Ag-II) were made. Ag-I, with a total cell concentration of 1×10^9 cells/ml (3.3×10^8 cells/ml/species), was prepared by mixing 10 ml each of the antigen preparations of the three species in a sterile plastic centrifuge tube (40 ml capacity). Ag-II, containing 3×10^9 cells/ml (1×10^9 cells/ml/species), was prepared by centrifuging 30 ml of Ag-I (4000 × g, 20 min) and removing and discarding 20 ml of the supernatant with a pipette (without disturbing the pellet). The pellet was resuspended (vortex) in the remaining supernatant to obtain Ag-11.

Immunization and Antisera Development

Fifteen 6- to 9-month-old New Zealand white female rabbits (3 kg average weight) were used for the development of antisera. Three rabbits were used for the development of each antiserum. For immunization, the antigens were injected as saline suspensions via the intravenous (IV) and subcutaneous (SC) routes. Rabbits were immunized with single-rhizobial-species antigen preparations of TAL 102, TAL 620 and TAL 1145 or the mixed rhizobial species antigens (Ag-I and Ag-II). The immunization schedule was as follows: Day 1, 0.5 ml IV; Days 2 and 3, 1.0 ml IV; Day 7, 1.5 ml IV; Days 8 and 9, 2.0 ml IV; Day 17, 1.0 ml SC; Day 24, blood removal by cardiac puncture. The blood samples were incubated at 37 °C for 1 h to facilitate clotting and then at 4 °C overnight to facilitate extrusion of the serum (Vincent 1970).

Antisera titres were determined by tube agglutination using cultured cells as the source of antigen (Vincent 1970; Somasegaran & Hoben 1994). The antisera that were obtained from mixed rhizobial-species antigen immunizations were titrated against each of the rhizobial species which were included in the mixed antigen preparation.

Cross-absorption of Antisera

Rhizobial species-specific antisera were prepared by cross-absorption of the antisera against the component rhizobial species. Only antiserum Ab-I was used in the cross-absorption. For example, to obtain cross-absorbed antisera specific to TAL 620, antigen suspensions (2×10^9 to 5×10^9 cells/ml) of TAL 102 (2 ml) and TAL 1145 (2 ml) were added to 4 ml of Ab-I. The reaction mixture was incubated, centrifuged and tested as described by Vincent (1970). Six absorptions were necessary before Ab-I showed negative agglutination against antigens TAL 102 and TAL 1145 but

Polyclonal antisera production remained positive to TAL 620. Similarly, species-specific, cross-absorbed antisera were also obtained for TAL 102 and TAL 1145 by selective cross-absorption. The resulting increases in the volume of the antisera after absorption were noted for consideration in subsequent assays. Cross-absorbed antisera were concentrated by dialysis against polyethylene glycol and adjusted to protein concentration of 10 mg/ml (Somasegaran & Hoben 1994).

Serology and Immunoassay of Antisera

Antisera from single-species immunization were designated Ab102, Ab620, and Ab1145, mixed-rhizobial-species antisera were AM and Ab-11, and species-specific cross-absorbed antisera were Ab102A, Ab620A and Ab1145A. All were tested by agglutination, FA, ELISA and immunoblot. Cultured cells and bacteroids from root nodules were used as sources of antigens. Bacteroid antigens for the various tests were prepared from oven-dried root nodules (Somasegaran *et al.* 1983). The agglutination titres were determined for each of the 15 antisera produced. A representative antiserum sample (i.e. the antiserum from one rabbit from the three replications in each treatment) was then selected for further serological analysis.

Tube agglutination titres were performed as described above and only cultured cells were used in the evaluation. Fluorescent antibody production procedures, smear preparations (cultured cells and bacteroids), staining, and microscopy were according to Schmidt *et al.* (1968) and Somasegaran & Hoben (1994). For this purpose, samples of all antisera (i.e. Ab102, Ab620, Ab1145, AM, Ab-II, Ab102A, Ab620A, and Ab1145A) were conjugated with fluorescein isothiocyanate (FITC).

Indirect ELISA (Olsen & Rice 1984) were performed using goat-anti-rabbit-gammaglobulin alkaline phosphatase (GARGAP) conjugate (Bio-Rad) diluted 1:4,000 in saline. Two-fold dilutions (1:2000 to 1:32,000) in saline of the primary antisera were prepared. Cells were immobilized in each well of microtitre plates by placement of 200 µl of the antigen preparation. The primary antiserum (rabbit) was used at the rate of 200 µl/well. The primary antiserum was excluded in the control treatments. Tests were replicated three (cultured cells) or five times (bacteroids). Results were assessed by measuring the absorbance at 405 nm using a microplate reader (Dynatech).

The immunoblot assay was carried out following instructions provided in the Bio-Rad immunoblot kit. As with ELISA, the GARGAP conjugate was diluted 1:4000 in saline. Cultured cells and bacteroids were placed in microtitre plates (200 µl/well). The cells were then transferred onto nitrocellulose filters (Schleicher & Schuell Inc., Keene, NH) by spotting with a multiple inoculator (West Coast Scientific, Emeryville, CA). Controls were not treated with primary antisera. Tests were replicated as described for the ELISA. Results were recorded by visual observation.

Results and Discussion

The agglutination titres of Ab-I and Ab-II were generally similar to the titres of the antisera obtained by immunization with single-rhizobial-species antigens (Table 1). However, two-fold differences in the agglutination titres were observed if comparisons were made on a case-by-case basis though these differences fell within the acceptable range for most practical applications. Agglutination titres of cross-absorbed Ab-I against antigens of TAL 620 and TAL1145 were two-fold lower (1:800) than the unabsorbed Ab-I (1:1600).

Table 1. Agglutination titres of single, mixed, and cross-absorbed rhizobial-species antisera.

Antigen	Antisera							
	Single*			Mixed†		Absorbed Ab-I‡		
	Ab102	Ab620	Ab1145	Ab-I	Ab-II	Ab102A	Ab620A	Ab1145A
TAL 102	1600	0	0	1600	3200	1600	0	0
TAL 620	0	3200	0	1600	1600	0	800	0
TAL 1145	0	0	1600	1600	3200	0	0	800

* Antisera from single rhizobial species immunization.

† Antisera from mixed rhizobial species (Ag-I and Ag-II) immunization.

‡ Antisera Ab-I made strain-specific by cross-absorption.

However, Ab102A did not show any difference in agglutination titres compared with Ab-I.

A staining titre of 3 + (bright-yellow-green) was scored for the FA prepared from the species-specific Ab102A, Ab620A, and Ab1145A, the unabsorbed Ab-I and Ab-II, and the antisera obtained by immunization with single-rhizobial-species antigens. The dilutions of the FA at which these 3 + fluorescence reactions were observed ranged from 1:16 to 1:32 (Table 2).

As with antisera produced by immunization with single-rhizobial-species antigen preparations, the immunoblot assay indicated that Ab-I, Ab-II and the cross-absorbed AbI species-specific antisera can be used at dilutions ranging from 1:8000 to 1:32,000 (Table 3). Positive reactions appeared as distinct dark purple spots at locations on the nitrocellulose filters where the antigen was applied. The control treatments did not show any reactions and no cross-reactions were observed. As observed in agglutinations and the FA reactions, a slight decrease in activity of the cross-absorbed antisera was noticeable. In the immunoblot assay this decrease was seen in the cross-absorbed species-specific antisera Ab620A and Ab1145A. However, these decreases do not diminish the usefulness of these antisera as lower dilutions of the antisera can be used for the analysis. A 1:1000 diluted antisera was used successfully in the immunoblot analysis of *R. meliloti* in commercial peat inoculants (Olsen & Rice 1989).

The A405 values for the ELISA utilizing the various primary rabbit antisera which were diluted 1:4000 are shown in Table 4. This dilution was the maximum for the detection of the bacteroids of TAL 1145 using Ab1145, AM, Ab-11 and Ab1145A. At higher dilutions of the antisera of TAL 1145, the A values of the specific homologous reactions were similar to those of the non-specific reactions. However, the antisera of TAL 102 and TAL 620 could be further diluted before use as the dilution limit was not reached even at 1:32,000; this was attributed to the low non-specific reactions. Non-specific reactions are caused mostly by residual nodule tissue which is not completely

removed during the rinsing/washing steps when performing the ELISA. The A values for non-specific and homologous (positive) reactions ranged from 0.02 to 0.3 and 0.52 to 1.12, respectively (Table 4). The positive A values were well within the acceptable range, as it is recommended that positive A values be set at two (or three) times above the mean negative A values (Kishinevsky & Jones 1987).

The results of this study demonstrate clearly that antigenically distinct rhizobia belonging to different genera and species can be mixed to prepare mixed-rhizobial-species antigen for immunization. The polyclonal antisera that are developed following this procedure are of similar quality to that obtained when a single strain is used in the antigen preparation and immunization. Further, the mixed-rhizobial species antisera can be made species-specific by cross-absorption without serious or total loss of activity. Also, antisera developed as described here can be used in the analysis of occupant rhizobia (bacteroids) in nodules.

In this investigation we cross-absorbed the antisera to demonstrate that strain-specific antisera may be prepared from the mixture following standard procedures. The antisera need to be absorbed only when high specificity is needed, such as in ecological studies conducted in the presence of indigenous rhizobia and for the serogrouping of new and previously uncharacterized rhizobia where cross-reactions are anticipated.

Selection of species or strains of rhizobia for the preparation of mixed-rhizobial antigen for antisera production will require prior knowledge of their antigenic distinctness. Therefore, laboratories which have serological information on the rhizobia in their collections may decide to produce antisera by mixing cultures which do not share common antigens. Sets of three antigenically distinct *Bradyrhizobium* spp. and *Rhizobium* spp. for at least 12 different species of legumes have already been assembled and are available for inoculation and ecological studies (Somasegaran & Hoben 1994).

The technique of mixing antigenically distinct rhizobia for antisera production is significant to researchers working with rhizobia. It is conceivable that this approach

Table 2. Titres of single, mixed, and cross-absorbed rhizobial-species fluorescent antibodies.

Antigen*	Fluorescent antibodies							
	Single			Mixed		Absorbed Ab-I		
	Ab102	Ab620	Ab1145	Ab-I	Ab-II	Ab102A	Ab620A	Ab1145A
TAL 102	32	0	0	32	32	16	0	0
TAL 620	0	16	0	16	32	0	16	0
TAL 1145	0	0	16	16	16	0	0	16

* Bacteroid antigens from dried root nodules.

Table 3. Highest dilutions of single, mixed, and cross-absorbed rhizobial-species antisera that gave positive reactions in the immunoblot assay.

Antigen*	Antiserum							
	Single			Mixed		Absorbed Ab-I		
	Ab102	Ab620	Ab1145	Ab-I	Ab-II	Ab102A	Ab620A	Ab1145A
TAL 102	32,000	0	0	32,000	32,000	32,000	0	0
TAL 620	0	16,000	0	32,000	16,000	0	8000	0
TAL 1145	0	0	32,000	32,000	32,000	0	0	16,000

* Bacteroid antigens from dried root nodules.

Table 4. Bacteroid antigen reactions with single, mixed, and cross-absorbed rhizobial-species antisera as determined by ELISA A_{405} values.

Antigen*	Antiserum							
	Single			Mixed		Absorbed Ab-I		
	Ab102	Ab620	Ab1145	Ab-I	Ab-II	Ab102A	Ab620A	Ab1145A
TAL 102	0.99	0.06	0.03	0.91	0.52	0.88	0.02	0.02
TAL 620	0.25	0.97	0.24	1.12	0.80	0.20	0.94	0.04
TAL 1145	0.20	0.30	0.96	0.71	0.58	0.18	0.22	0.66

* Bacteroid antigens from dried root nodules.

may also be applied to other bacterial species as long as the criterion of antigenic distinctness among the component strains in the antigen preparation is satisfied. The data presented in this investigation demonstrate that the immune system of the rabbit does not show a selective preference or affinity towards any one particular rhizobial species over another in initiating antibody formation. It appears that as long as species or strains of rhizobia in the mixture have similar immunogenicity, the different rhizobial species in the mixed rhizobial-species antigen preparation have the same potential in inducing antibody formation. Although three species of rhizobia were mixed in the present study it should be possible to mix more than three provided the lower limits of the cell concentrations that will yield acceptable antibody titres are established. The present results indicate that the low concentration of 3.3×10^8 cells/ml/species was of similar effectiveness to that of

1×10^9 cells/ml/species in producing antisera of acceptable quality.

One of the objectives of this investigation was to reduce the cost of antisera production. In the USA, researchers are required to follow mandatory government guidelines set by the United States Department of Health and Human Services (1985). These guidelines require that only animals from certified laboratory animal suppliers are accepted. The animals must be kept at 16 to 21°C and 40 to 60% relative humidity with an air exchange of 10 to 15 room volumes/ h. Cages must be cleaned and sterilized periodically and extra sets of cages must be available to house the animals during cleaning. If an animal facility is already available, the major components of the cost of antiserum production are the cost of the animal, feed, electricity for climatic control and the labour for feeding, cleaning and sanitation. On a

cost re-imbursement basis, the animal facility at the University of Hawaii charges US\$1.47/day for each rabbit. A total of 50 ml of antiserum is usually collected from each rabbit over a period of 60 days. With an average price of US\$48 for a laboratory-grade rabbit, the total cost of antiserum production in one rabbit is US\$[(1.47 x 60) + 48] = US\$136.20. Traditionally, two rabbits are used for each antigen preparation because it is not uncommon that one animal will perish. Production of one antiserum then costs US\$272.40. To produce three traditional antisera, six rabbits are required, at a cost of US\$817.20. Since only two rabbits are required if all three strains are used as a mixed antigen, there is a difference of US\$544.80 or a reduction of 66% in cost when mixed rhizobial antisera are produced in one rabbit instead of using antigen from a single strain in each rabbit. Besides rabbits (New Zealand white females or other breeds), other homothermic animals such as chickens, guinea pigs, rats, mice, sheep and horses can also be used for the production of antisera (Ball *et al.* 1990). In the authors' laboratory (Hawaii), goats were immunized to produce goat-anti-rabbit-gammaglobulins (GARGG) for application in the indirect fluorescent antibody technique. Goats, which may be more easily available, especially in developing countries, may be substituted for rabbits for developing antisera against rhizobial antigens. However, suitable immunization procedures for antisera development in goats need to be developed.

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