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Serological grouping of indigenous *Bradyrhizobium sp. (Arachis)* isolated from various soils of Thailand†

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ELISA and antibody adsorption tests were applied to determine the minimal somatic antigen constitution of 243 strains of Bradyrhizobium sp. (Arachis) using 12 antisera. The 243 indigenous bradyrhizobial isolates were from 15 sites in four regions of Thailand. A total of 29 serogroups were identified. Most (80%) of the isolates tested had at least one heat-stable antigen in common with strain 280A, forming a so-called 280A serocluster. At 11 of 15 sites tested, 53 to 100% of the isolates fell into one or two predominant serogroups. The serological properties of the indigenous bradyrhizobia were not related to the cropping history of the cultivated fields from which they were isolated.

Key words: Antigens, antisera, *Arachis, Bradyrhizobium,* ELISA, peanut, serological groups.

Peanut *(Arachis hypogaea L.)* is naturally nodulated by the slow-growing rhizobia *(Bradyrhizobium spp.)* that are widespread in tropical soils (Ezedinma 1963), the so-called 'peanut rhizobia'. Boonkerd *et al.* (1991) reported that native peanut rhizobia isolates, collected in the main peanutgrowing areas of Thailand, range widely in their ability to fix N_2. In some areas, most bradyrhizobia isolated from peanut nodules were less effective than the inoculum strain, THA 205, recommended for use in Thailand. Substitution of ineffective nodules, formed by indigenous peanut rhizobia, with nodules from a more effective inoculant strain should therefore increase the symbiotic performance of inoculated plants. To approach this problem, a better understanding of the nature of native peanut rhizobial populations is necessary. Although the antigenic specificity of peanut rhizobia has been demonstrated by several authors (Van Der Merve & Strijdom 1973; Dadarval of *al.* 1974), no data are available on the serological distribution of *Bradyrhizobium sp. (Arachis)* in different soils. The objective of this study was to determine the serological properties of indigenous peanut rhizobial populations in different peanut-growing regions of Thailand.

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Materials and Methods

Isolation of Indigenous Rhizobia

Soil samples were collected from four regions of Thailand: North, North-east, East and Central Plain. Different cropping areas were chosen in each region: (1) continuously cropped with peanuts; (2) continuously cropped with non-legumes; and (3) non-cultivated fields. Soil samples, collected from the total of 15 sites, came from one uncultivated field, two fields cropped with non-legumes, three fallow fields and nine fields cropped with legumes. The nine fields of legumes contained peanuts (five), mungbean (one), cowpea (one), soybean (one), or a mixture of other legumes (one). Soil samples were obtained by randomly collecting 10 core samples from each field. All soil cores from one site were pooled and mixed, passed through a 6.5-mm sterile sieve. A sub-sample *(3* kg) was then put immediately into an ice box (4°C) and left until tested (≤ *7* days).

Two kg of each soil sample were mixed with *2* kg autoclaved sand and put in clean plastic containers. Peanut seeds (cv. Tainan 9), surface-sterilized in *2%* (w/v) sodium hypochlorite and pre-germinated at *28*°C on 1.5% (w/v) water agar (Difco) plates for 60 h, were then planted at six seeds per container. The seedlings were thinned to three per container *7* days after germination. All pots were in a greenhouse maintained at *27* to *32*°C under sunlit conditions. Thirty days after inoculation, randomly-selected nodules (10 per plant) were detached and used for isolation of rhizobia according to the procedure described by Vincent *(1970).*

Isolates were authenticated under bacteriologically-controlled conditions on *Macroptilium atropurpureum* (Siratro) using Gibson's tubes for partly enclosed seedlings (Vincent 1970). The tubes were placed in a controlled-environment greenhouse at 27°/20°C (day/night) with the natural day length supplemented with fluorescent light to provide a 16 h photoperiod. In all, 243 isolates of bradyrhizobia were obtained from the 15 field soils selected for this study.

Serological Procedures

As somatic antigens of rhizobia are more specific than flagellar and internal antigens (Vincent 1982), they were used in this study to define strains in terms of their antigenic structure and to separate peanut bradyrhizobial isolates into different serological groups. For the preparation of culture cell antigens, the bacteria were grown at 28°C to early stationary phase in yeast/mannitol broth (YMB) (Vincent 1970). Numbers of rhizobia in pure cultures were estimated by using a plate-counting procedure (Vincent 1970). Cultures were centrifuged and washed three times in phosphatebuffered saline (PBS) (Kishinevsky *et al.* 1984). The final cell pellet was resuspended in PBS to 10^8 cells/ml and this antigen suspension heated at 100oC for 30 min to inactivate the flagellar antigens. Three (280A, 2212A and CB756) of the 12 antisera used were produced from strains isolated in Israel (280A and 2212A) and Australia (CB756) and kept in the Volcani Center *Rhizobium* Collection. The remaining nine antisera (7/20E, 31/14N, 31/9N, 22/6N, 30/4N, 34/6NE, 25/3N, 28/15N and 10/14N) were produced from *Bradyrhizobium* isolates obtained in Thailand during the course of this study, using the methods of Kishinevsky & Gurfel (1980). The indirect ELISA of Engwal & Perlmann (1971), with the modifications of Kishinevsky & Maoz (1983) and Fuhrmann & Wollum (1985), was employed for serological typing of *Bradyrhizobium* cultures.

For adsorption of antibodies, 25 ml of cells from 6-day-old YMB culture containing approximately 2 x 10^9 *Bradyrhizobium* cells/ml were packed by centrifugation (15 min, 14,000 g, 4°C). The pellet was resuspended in 5 ml 1:100 diluted heterologous antiserum and the adsorption was carried out at 37°C for 1 h with an additional 2-h agitation of the mixture at 4°C. The antigen/antiserum mixture was then centrifuged for 10 min at 14,000 g and the supernatant stored at -20°C till used. Complete adsorption was verified by ELISA testing of adsorbed antiserum with a fresh preparation of adsorbing antigen.

The minimum number of antigenic determinants occurring in a given strain was determined. Cross-reacting strains shared a common antigen. If the antiserum continued to react with the homologous strain after adsorption of antibodies by a heterologous strain, two antigenic determinants were assigned to the homologous strain. A homologous reaction of each strain after a heterologous adsorption indicated that besides common antigen(s), each strain bore specific antigens. As in the case with strains of *B. japonicum* (Robert & Schmidt 1985), Roman numerals were adopted for designation of the antigens found.

Results

ELISA Reactions of Bradyrhizobium *Strains used for Preparation of Antisera*

The results of the indirect cross-ELISA using antigens and antisera of 12 strains of *Bradyrhizobium* are presented in Table 1. The ELISA readings for homologous reactions were significantly higher than those of the heterologous ones, and ranged from as low as A_{405} = 0.64 for strain CB756 to A_{405} = 1.94 for strains 280A and 31/14N. Strain 280A was serologically-related individually to each of the following strains: 2212A, 7/20E, 31/14N, 22/6N and CB756. As strains 2212A, 7/20E, 31/14N, 22/6N and CB756 reacted with antiserum to 280A, it is reasonable to postulate that strain 280A possesses five different antigens: I, 11, V, VII, and IX, respectively, common to each of these strains (Table 1). Reciprocal adsorption tests (Table 2) showed that strains 7/20E, 2212A, and 31/14N, applied individually or as a mixture, did not adsorb all antibodies from antisenun 280A,

Table 1. Somatic ELISA cross-reactions of *Bradyrhizobium* sp. (*Arachis*).

Antigen	Indirect ELISA absorbance values (A_{405nm}) with antisera:*												Minimal antigenic constitution†
	280A	7/20E	2212A	31/14N	CB756	31/9N	22/6N	30/4N	34/6NE	25/3N	28/15N	10/14E	
280A	*1.94*	0.17	0.36	0.17	–	–	0.39	–	–	–	–	–	I, II, V, VII, IX, XI
7/20E	0.53	1.69	–	–	–	–	–	–	–	–	–	–	I, III
2212A	0.45	–	*1.28*	–	–	–	–	–	–	–	–	–	II, IV
31/14N	0.29	–	–	*1.94*	–	–	–	–	–	–	–	–	V, VI
22/6N	–	–	–	–	–	–	*1.10*	–	–	–	–	–	VII, VIII
CB756	0.31	–	–	–	*0.64*	0.20	–	–	–	–	–	–	IX, X
31/9N	–	–	–	–	0.51	*0.72*	–	–	–	–	–	–	X, XII
30/4N	–	–	–	–	–	–	–	*0.94*	–	–	–	–	XIII
34/6NE	–	–	–	–	–	–	–	–	*1.12*	–	–	–	XIV
25/3N	–	–	–	–	–	–	–	–	–	*1.4*	–	–	XV
28/15N	–	–	–	–	–	–	–	–	–	–	*1.10*	–	XVI
10/14E	–	–	–	–	–	–	–	–	–	–	–	1.20	XVII

* Values are means of duplicate wells, less the negative, control mean; results for homologous reactions are italicized. Antisera were used at a dilution of 1:500 (antiserum 30/4N, 1:100), enzyme-labelled protein A, at 1:2000 and antigen at 10^8 cells/ml.
† Based on data from Tables 1 and 2.
– —No antibody–antigen reaction.

Table 2. Antigenic heterogeneity among peanut *Bradyrhizobium* strains as revealed by cross-adsorption of antibodies.

Antibody*	ELISA reactivity with antigens:†					
	280A	7/20E	2212A	31/14N	CB756	31/9N
280A (unadsorbed)	+++	++	++	++	++	–
280A (7/20E)	++	–				
280A (2212A)	+++		–			
280A (31/14N)	+++			–		
7/20E (unadsorbed)	+	+++	–	–	–	–
7/20E (280A)	–	+++				
2212A (unadsorbed)	++	–	+++	–	–	–
2212A (280A)			+++			
31/14N (unadsorbed)	++	–	–	+++	–	–
31/14N (280A)	–			+++		
CB756 (unadsorbed)	–	–	–	–	++	++
CB756 (31/9N)					+	–
31/9N (unadsorbed)	–	–	–	–	+	++
31/9N (CB756)					–	+++

* Adsorbing antigen is indicated in parentheses.
† Visual assessment after incubation for 30 min with the enzyme substrate: +++—bright yellow; ++—yellow; +—very pale yellow; ——not detectable. For concentrations of antisera, enzyme-labelled protein A and antigens, see footnote to Table 1.

as adsorbed antiserum still reacted strongly with homologous antigen. Thus, strain 280A contains at least one additional antigen (antigen XI), as indicated in Table 1. Similarly, antisera 7/20E, 2212A, and 31/14N, adsorbed by strain 280A, continued to react with homologous antigens, indicating that each of these strains contains a second antigen (III, IV, and VI respectively). Five further strains, 30/4N, 34/6NE, 25/3N, 28/15N and 10/14E, appeared to be unique in their antigenic make-up, reacting only with homologous antisera.

There were non-reciprocal ELISA reactions in some antigen-antiserum combinations (Table 1). Strain CB756 reacted with antiserum 280A, while antiserum developed against strain CB756 failed to react with 280A antigen(s). Similarly no ELISA reaction was observed between strain 22/6N and antiserum 280A, whereas strain 280A reacted positively with antiserum 22/6N (Table 1).

Serogrouping of Indigenous Populations

The 12 antisera described above were used to examine the serological properties of indigenous peanut rhizobia from 15 sites in four different regions. Serological grouping of indigenous isolates of *Bradyrhizobium* in this study was based on the assumption that nodules of greenhouse and field plants grown in a given soil are formed by identical population(s) of rhizobia Johnson & Means 1963). From 243 isolates collected, only 13 did not react with any of the antisera used (Table 3). Based on the pattern of their ELISA reactions, the antisera-280A-positive isolates were distributed into 18 serogroups. A 'serogroup' was a group of isolates reacting with the same antiserum or antisera tested. Sixty-five isolates that reacted only with antiserum 280A formed the first serogroup. Antigen XI (Table 1) or another hypothetical antigen may be involved in the 280A reaction of this serogroup. The next four serogroups (2 to 5) were composed of 80 isolates reacting with antibodies 280A and 2212A, 31/14N, 7/20E or 22/6N, respectively. At least four different antigens (probably II, V, I, and IX) took part in these reactions. About 48% of the 280A-positive isolates tested fell into the first two groups. Twelve isolates belonging to serogroup 6 (Table 3) reacted with two antisera, 280A and 30/4N, having no antibodies in common (Table 1). A minimum of two determinants should form the antigenic constitution of these isolates. Serogroups 7 to 11 and 12 to 18 comprised, respectively, 25 and 13 isolates that were characterized by positive ELISA reactions with different combinations of three (serogroups 7 to 11) and four to six (serogroups 12 to 18) antisera having 280A in common.

Thirty-five (15%) isolates reacting mostly with single antisera (Table 4) were distributed over 11 additional serogroups (19 to 29). None reacted with antiserum 280A.

The serogroup distribution of indigenous rhizobia was not related to the cropping history of the fields from which they had been isolated. Thus, serogroup 2, dominant in peanut-grown fields N-21, N-23, N-28 in the North, was also abundant at a fallow field (E-16) in the East of Thailand (Table 3). Although identical serogroups were found in

Table 3. Indirect ELISA analysis of somatic antigens of 243 isolates of *Bradyrhizobium* sp. (*Arachis*) collected in Thailand.

Field*	Cropping history	No. of isolates analysed	Isolates (%) not reacting with any antisera	Isolates (%) reacting (A_{405} > 0.20) with antisera of serogroup:												
				1 (280A only)	2 (280A, 2212A)	3 (280A, 31/14N)	4 (280A, 7/20E)	5 (280A, 22/6N)	6 (280A, 30/4N)	7 (280A, 31/14N, 22/6N)	8 (280A, 2212A, 7/20E)	9 (280A, 22/6N, 2212N)	10 (280A, 2212N, 31/14N)	11 (280A, 2212A, 30/4N)	12 to 18 (Four or more antisera including 280A)	19 to 29 (Other sero groups)
E-7	Mungbean cultivation	17	–	18	–	18	46	12	–	–	6	–	–	–	–	–
E-10	Fallow with native legumes	16	18	–	–	6	–	–	–	12	–	–	–	–	12	52
E-16	Fallow with native legumes	13	–	46	54	–	–	–	–	–	–	–	–	–	–	–
N-21	Peanut cultivation	18	–	12	70	–	–	6	–	–	–	–	–	–	6	6
N-23	Peanut cultivation	14	13	–	53	7	–	–	–	–	20	–	–	–	7	–
N-28	Peanut cultivation	13	–	35	44	–	–	–	–	–	14	–	–	–	7	–
N-25	Cotton and corn cultivation	16	19	–	–	12	–	–	–	19	–	–	–	–	7	43
N-30	Soybean cultivation	16	–	–	–	–	–	–	76	–	–	–	–	6	6	12
N-22	Shrub, teak trees, mimosa	15	–	19	14	–	7	7	–	–	–	–	–	–	–	53
N-31	Cassava having mimosa weeds	14	6	19	25	6	6	–	–	6	6	–	–	6	–	20
NE-33	Corn rotation with peanuts	18	–	56	6	6	–	–	–	–	–	–	–	–	32	–
NE-34	Cowpea cultivation	20	20	20	10	5	–	–	–	15	–	–	–	–	–	30
NE-36	Cover legumes	19	–	64	31	–	–	–	–	–	–	–	5	–	–	–
NE-39	Fallow with native legumes	18	–	52	18	6	–	–	–	18	–	–	6	–	–	–
M-47	Corn rotation with peanuts	16	–	38	25	–	–	25	–	–	–	12	–	–	–	–

* Regions: E—East; N—North; NE—North-east; M—Central Plain.

Table 4. Serological distribution of *Bradyrhizobium* sp. (*Arachis*) isolates from seven soils in which additional serogroups were detected besides the 280A serocluster.

Site*	Isolates (%) reacting with 280A antisera	Isolates (%) reacting (A_{405} > 0.20) with antisera of serogroups:										
		19 (31/14N)	20 (30/4N)	21 (31/9N)	22 (22/6N)	23 (25/3N)	24 (10/14E)	25 (34/6NE)	26 (2212A, 10/14E)	27 (10/14E, CB756)	28 (31/9N, CB756)	29 (30/4N, 22/6N)
E-10	48	7	–	–	–	–	24	–	7	14	–	–
N-21	94	–	6	–	–	–	–	–	–	–	–	–
N-25	57	13	–	–	–	30	–	–	–	–	–	–
N-30	88	–	12	–	–	–	–	–	–	–	–	–
N-22	47	–	–	–	53	–	–	–	–	–	7	–
N-31	80	7	–	–	–	–	–	–	–	–	–	6
NE-34	70	–	22	8	–	–	–	–	–	–	–	–

* The location and cropping history of the fields are indicated in Table 3.

different soils, the frequency of a particular serogroup varied from field to field. For example, only two serogroups were found at site E-I6 in the eastern region, whereas bradyrhizobia, isolated from nodules of peanuts grown in E-7 soil of the same area, were more heterogenic and could be divided, therefore, into five serogroups. No serogroup common to all fields has been detected. At 11 of 15 sites tested, 53 to 100% of isolates fell into one or two dominant serogroups, that individually or jointly were frequent at one or several sites (Table 3). Serogroups 1 and 2 (singly or in combination) dominated the rhizobial population in eight fields, E-I6, N-21, N23, N-28, NE-33, NE-36, NE-39 and M-47, located in the eastern, northern, northeastern, and central parts of Thailand. The peanut rhizobia populations at sites E-7 and N-30 belonged mainly to serogroups 1 and 4, and to 6, respectively. One additional serogroup, lacking antigens in common with 280A, formed the majority of peanut rhizobia at site N-22 (Table 4).

Discussion

At least 17 somatic antigenic determinants were found among 12 strains that were used for antisera preparation. There was a close antigenic similarity between a well-known inoculant, strain CB756 from Australia, and strain 31/9N isolated in Thailand. Vincent (1982) reported that antigenically-similar strains of *Rhizobium* were obtained from widely separated regions of the world. The present results confirm those of Dadarval *et al.* (1974), showing that the spectrum of serological reactions of peanut rhizobia varies among strains. In our experiments, strain 280A was found to have the most complex antigenic constitution, bearing at least six antigenic determinants, compared with one or two determinants detected in the other 11 strains used for antisera preparation. It can be assumed, therefore, that more strains may react with 280A antiserum than with antisera against strains endowed with a unique antigenic make-up. The reasons underlying the inadequate ELISA reactions of some antigen-antiserum combinations (e.g. strains CB756 and 22/6N with antiserum 280A) are still not clear and need further clarification. In agglutination tests, similar failures are supposed to be due to the masking of certain antigens (Date & Decker 1965). Perhaps the most significant result of this study is the finding that 80% of the 243 isolates collected in Thailand were found to have at least one heat-stable somatic antigen in common with strain 280A, forming what might be called a 280A serocluster. Moreover, the serogroups reacting with antiserum 280A dominated the peanut rhizobial population in most of the fields tested. It can be expected, therefore, that if strain 280A were used as an inoculant strain in such fields, many nodules on the uninoculated control plants would react with the 280A antiserum. In our previous experiments (Kishinevsky *et al.* 1987), such non-specific cross-reactions were eliminated selectively by using inoculant strain gamma-globulin pretreated with bulk nodule material from uninoculated plots. The adsorbed gamma-globulin still reacted strongly with the inoculant strain.

To our knowledge, this is the first report indicating the abundance of certain serogroups within indigenous soil populations of *Bradyrhizobium sp. (Arachis).* These findings are in agreement with the results obtained for studies on *B. japonicum,* in which the well-known phenomenon of a high occurrence of serogroup 123 in different soils of soybean fields in Iowa, Indiana, Illinois and Nebraska was reported (Damirgi *et al.* 1967; Weber *et al.* 1989). One can only speculate about the reasons leading to dominance of a certain serogroup in a given soil. It was shown, however, for *Rhizobium leguminosarum* by *Viceae* (Brockman & Bezdicek 1989), that soil microclimate and topography may affect the serological distribution of indigenous rhizobia. The role of the soil microclimate in serogroup distribution of peanut rhizobia is yet to be investigated.

The antigen(s) involved in the reaction of isolates with 280A antiserum cannot be determined with certainty from our data. To figure out the antigenic structure of the isolates, antisera against each of them must be prepared. Nevertheless, the differences in extinction values among isolates within a given serogroup (data not shown) indicate the absence of full identity in their antigenic make-up. Isolates belonging to the same serogroup should be investigated further to identify and characterize the intraserogroup diversity.

Our results show the suitability of the indirect ELISA for serological typing of strains of *Bradyrhizobium*. The simplified indirect ELISA procedure (Kishinevsky & Maoz 1983; Fuhrmann & Wollum 1985) is a reliable technique which could be used in variously-equipped laboratories.

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