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QUICK SEROLOGICAL METHOD OF CLASSIFYING STRAINS OF *RHIZOBIUM JAPONICUM* IN NODULES

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ABSTRACT

MEANS, URA M. (U.S. Department of Agriculture, Beltsville, Md.), HERBERT W. JOHNSON, AND R. A. DATE. Quick serological method of classifying strains of *Rhizobium japonicum* in nodules. J. Bacteriol. **87**:547-553. 1964.--A new method of classifying strains of *Rhizobium japonicum* serologically by utilizing the contents of nodules as antigens is described. The method differs from standard procedures only in the preparation of antigens, but it is less time-consuming. Agglutination reactions of culture and nodule antigens were identical for 15 of the 17 strains investigated. Nodule antigens of ineffective strain 24 failed to agglutinate with any of the 17 antisera employed, and nodule antigens of strain 46 reacted with two antisera in addition to the two with which the culture antigens reacted. Host genotypes affected the reactions of nodule antigens of strain 46, with the reactions ranging from those typical for culture antigens for some soybean (*Glycine max*) varieties to reactions with heterologous antisera only for other soybean varieties and six cowpea (*Vigna sinensis*) varieties. Although nodule antigens of most strains of *R. japonicum* reacted the same as culture antigens, the inconsistent reactions of nodule antigens of strain 46 suggest precautions in the general use of nodule antigens. Indicated precautions are discussed.

Most soils in areas of the United States where soybeans [*Glycine max* (L.) Merrill] are produced contain effective strains of nodulating bacteria [*Rhizobium japonicum* (Kirchner) Buchanan]. In experiments designed to compare strains of *R. japonicum* and inoculation techniques in such soils, some method of identifying the strains in the nodules of soybean plants is required. The established procedure of serologically identifying strains by isolating the bacteria from nodules and using whole cells in culture as antigens is so time-consuming that it precludes experiments of the scale required for adequate evaluation of strains and inoculation techniques.

It seems logical to assume that cells of bacterial origin in the nodule have antigenic properties in common with the cells of a like bacterial genotype in culture; and, if the bacterial cells in the nodule could be used directly as antigen, the time required for the serological classification of strains could be reduced. However, little information on the antigenic properties of the contents of legume nodules is to be found in the literature, and Jordan's (1962) extensive review of the literature on the bacteroids of the genus *Rhizobium* contains no reference to the antigenic properties of bacteroids.

The objectives of this paper are: (i) to describe a serological method of identifying strains of *R. japonicum* that utilizes the contents of nodules as antigens; and (ii) to present information on the serological reactions of strains taken directly from nodules, as compared with the reactions of the strains in culture.

MATERIALS AND METHODS

The method differs from standard serological procedures only in the preparation of antigens. Antigens from nodules are prepared by placing washed nodules individually in small vials and crushing each one in 3 to 4 ml of physiological saline. The resulting suspensions are then diluted to the desired turbidity with physiological saline, steamed, and pipetted into tubes. Most of the nodule debris remains in the vials. Nodules known to have originated from one strain frequently are bulked to produce larger quantities of antigen.

Reactions of culture antigens, unheated nodule antigens, and nodule antigens heated at 100 C

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(steamed without pressure) for 30 min were compared. Antigens from cultures were prepared as described by Vincent (1941).

Somatic agglutinations were carried out in the normal way, using 10 × 75 mm tubes and incubating at 52 C for 3 to 4 hr. All antisera were titrated by the double-dilution method (starting at 1:100) against the homologous culture and nodule antigens and against heterologous antigens which reacted at the 1:100 dilution. For most of the work, however, the final antisera dilutions were 1:100. All antisera were prepared from culture antigens as described by Vincent (1941).

The concentrations of viable cells in culture and nodule antigens diluted to identical optical densities were compared from plate and plant (Date and Vincent, 1962) counts of serial dilutions of a sample of the antigens employed in the serological evaluation. Nodules used in the production of antigens were produced by inoculation of soybeans or cowpeas [*Vigna sinensis* (Torner) Savi] in sterile sand in jar assemblies used in previous work (Johnson, Means, and Clark, 1958). Serological reactions of a selected group of eight strains in nodules of soybeans (Lee variety) and cowpeas (Brabham variety) were compared by inoculating plants of both species with the same cultures and preparing antigens from the resulting nodules. Similarly, the nodules of ten varieties of soybeans and six varieties of cowpeas resulting from strains 46 and 76 were compared.

Of the 28 *R. japonicum* strains employed by Date (1962), 24 were used in the current work. Four of the strains used by Date were omitted because of unstable culture antigens (74 and 128) or the special-purpose nature of the antisera (127 and 129).

RESULTS

Culture vs. soybean nodule antigens. Reactions of culture and nodule antigens (Lee variety) of 17 strains are recorded in Table 1. Data on seven strains were omitted to simplify the presentation because they were the same as those for other strains represented.

The reactions of the two types of antigens of a given strain were identical except for strains 24 and 46. The nodule antigens of strain 24 did not react with any antisera, whereas the culture antigens reacted with antisera 3, 6, and 24. Strain 24 was ineffective on both soybeans and cowpeas. Although inoculation of these species with strain 24 resulted in many small nodules, the plants appeared to be identical with those in the uninoculated checks. Culture antigens of strain 46 reacted with antisera 4 and 46. Nodule antigens gave an intermediate reaction with the same two antisera and a weak reaction with antisera 3 and 24.

Antigens from dried nodules of the Lee variety stored at room temperature for 6 months gave the same results as antigens from fresh nodules for each of the 17 strains.

The data for unheated nodule antigens were the same as those for heated antigens reported in Table 1 except for strain 46. However, the unheated antigens retained a slight turbidity, in contrast with the complete clearing of heated antigens. The turbidity was conspicuous only when heated and unheated antigens were compared directly and was not sufficient to permit a distinction on the 1 to 3 scale employed.

Unheated nodule antigens of strain 46 reacted 2+ with antisera 3 and 24, and 1+ to 2+ with antisera 4 and 46. Heated antigens reacted 1+ with antisera 3 and 24 and 2+ with antisera 4 and 46.

In general, agglutination of culture and heated nodule antigens occurred at the same maximal dilution of antiserum. Agglutination of culture antigens of strains 46 and 123, however, occurred at one dilution higher than did agglutination of nodule antigens. Titers for homologous reactions involving both types of antigens ranged from 1:800 to 1:6,400.

Soybean vs. cowpea nodule antigens. The reactions of nodule antigens from seven of eight strains were the same for Lee soybean and Brabham cowpea nodules (Table 2). For strain 46, however, the reaction characteristic of culture antigens was intermediate for soybean nodule antigens and absent from the cowpea nodule antigens. The two antisera (3 and 24) with which the soybean nodule antigens of strain 46 reacted and the culture antigens did not were the only antisera that agglutinated the cowpea nodule antigens of strain 46. Unheated nodule antigens reacted the same as the heated except for the slight residual turbidity and the change in the soybean nodule antigens of strain 46 mentioned in connection with Table 1.

Both soybean and cowpea nodule antigens of strain 24 failed to react with any of the antisera used.

Culture antigens of isolates from the soybean

TABLE 1. *Somatic cross-reactions of 17 strains of Rhizobium japonicum—culture and heated Lee soybean nodule antigens**

Antigen	Antiserum																
	3	6	24	4	46	31	38	62	76	117	94	110	122	123	124	125	130
3	+++	+++	+++	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	XXX	XXX	XXX	–	–	–	–	–	–	–	–	–	–	–	–	–	–
6	+++	+++	+++	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	XXX	XXX	XXX	–	–	–	–	–	–	–	–	–	–	–	–	–	–
24	+++	+++	+++	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
4	–	–	–	+++	–	–	–	–	–	–	–	–	–	–	–	–	–
	–	–	–	XXX	–	–	–	–	–	–	–	–	–	–	–	–	–
46	–	–	–	+++	+++	–	–	–	–	–	–	–	–	–	–	–	–
	X	–	X	XX	XX	–	–	–	–	–	–	–	–	–	–	–	–
31	–	–	–	–	–	+++	–	–	–	–	–	–	–	–	–	–	–
	–	–	–	–	–	XXX	–	–	–	–	–	–	–	–	–	–	–
38	–	–	–	–	–	–	+++	–	–	–	–	–	–	–	–	–	–
	–	–	–	–	–	–	XXX	–	–	–	–	–	–	–	–	–	–
62	–	–	–	–	–	–	–	+++	–	–	–	–	–	–	–	–	–
	–	–	–	–	–	–	–	XXX	–	–	–	–	–	–	–	–	–
76	–	–	–	–	–	–	–	–	+++	+++	–	–	–	–	–	–	–
	–	–	–	–	–	–	–	–	XXX	XXX	–	–	–	–	–	–	–
117	–	–	–	–	–	–	–	–	+++	+++	–	–	–	–	–	–	–
	–	–	–	–	–	–	–	–	XXX	XXX	–	–	–	–	–	–	–
94	–	–	–	–	–	–	–	–	–	–	+++	–	–	–	–	–	–
	–	–	–	–	–	–	–	–	–	–	XXX	–	–	–	–	–	–
110	–	–	–	–	–	–	–	–	–	–	–	+++	–	–	–	–	–
	–	–	–	–	–	–	–	–	–	–	–	XXX	–	–	–	–	–
122	–	–	–	–	–	–	–	–	–	–	–	–	+++	–	–	–	–
	–	–	–	–	–	–	–	–	–	–	–	–	XXX	–	–	–	–
123	–	–	–	–	–	–	–	–	–	–	–	–	–	+++	–	–	–
	–	–	–	–	–	–	–	–	–	–	–	–	–	XXX	–	–	–
124	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+++	–	–
	–	–	–	–	–	–	–	–	–	–	–	–	–	–	XXX	–	–
125	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+++	–
	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	XXX	–
130	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+++
	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	XXX

* Symbols: + = culture antigen; X = nodule antigen; + or X indicates minimal detectable agglutination; ++ or XX indicates intermediate agglutination; +++ or XXX indicates complete agglutination.

and cowpea nodules used in preparing the antigens that gave rise to the data in Table 2 reacted the same as the original culture antigens.

Antigenic variation of strain 46. Differences in the reactions of antigens from soybean and cowpea nodules of strain 46 suggested that the effect of host genotype on this strain should be investigated further. Ten varieties of soybeans and six varieties of cowpeas were inoculated with a culture of strain 46 and with a culture of strain 76, one of the strains of which soybean and cowpea nodule antigens reacted the same. Heated and un-

TABLE 2. *Reactions of culture, soybean nodule, and cowpea nodule antigens of selected strains of Rhizobium japonicum**

Antigen		Antiserum								
Strain	Type	3	6	24	46	31	38	76	117	110
3	Cu	+++	+++	+++	−	−	−	−	−	−
	So	+++	+++	+++	−	−	−	−	−	−
	Co	+++	+++	+++	−	−	−	−	−	−
24	Cu	+++	+++	+++	−	−	−	−	−	−
	So	−	−	−	−	−	−	−	−	−
	Co	−	−	−	−	−	−	−	−	−
46	Cu	−	−	−	+++	−	−	−	−	−
	So	+	−	+	++	−	−	−	−	−
	Co	+++	−	+++	−	−	−	−	−	−
31	Cu	−	−	−	−	+++	−	−	−	−
	So	−	−	−	−	+++	−	−	−	−
	Co	−	−	−	−	+++	−	−	−	−
38	Cu	−	−	−	−	−	+++	−	−	−
	So	−	−	−	−	−	+++	−	−	−
	Co	−	−	−	−	−	+++	−	−	−
76	Cu	−	−	−	−	−	−	+++	+++	−
	So	−	−	−	−	−	−	+++	+++	−
	Co	−	−	−	−	−	−	+++	+++	−
117	Cu	−	−	−	−	−	−	+++	+++	−
	So	−	−	−	−	−	−	+++	+++	−
	Co	−	−	−	−	−	−	+++	+++	−
110	Cu	−	−	−	−	−	−	−	−	+++
	So	−	−	−	−	−	−	−	−	+++
	Co	−	−	−	−	−	−	−	−	+++

* Cu = culture; So = soybean nodule; Co = cowpea nodule; + indicates minimal detectable agglutination; ++ indicates intermediate agglutination; +++ indicates complete agglutination.

heated antigens prepared from the resulting nodules were tested against the antisera listed in Table 1.

The antigens of strain 46 reacted with antisera 3, 4, 24, and 46. Since the reactions of antisera 24 and 4 were the same as those of 3 and 46, respectively, only the data for the last two are presented.

Host genotype had a marked influence on the reactions of the nodule antigens resulting from strain 46 (Table 3). All cowpea nodule antigens reacted 3+ with antiserum 3 but failed to react with homologous antiserum 46. Heating had little effect on the reactions.

Reactions of heated soybean nodule antigens from the ten varieties ranged from the characteristic reaction of culture antigens of strain 46 to reactions as diverse as those of cowpea nodule antigens. Heating accented the reactions with antiserum 46, but eliminated the reaction with antiserum 3 in nodule antigens of four varieties.

Soybean and cowpea nodule antigens of all varieties resulting from strain 76 reacted only with antisera 76 and 117 (same as culture antigens of strain 76), and all reactions were 3+.

Culture antigens of strain 46 prepared from isolates of nodules from the various soybean and cowpea varieties reacted the same as the original culture of strain 46.

Development of nodule antigens. Serological reac-

tions of unheated antigens prepared from nodules of strains 3, 31, 38, and 46, sampled at successive dates after planting of the soybeans, indicated that the strains differed little in the time required for the antigens to develop (Table 4). In general, antigens were detected first about 2 weeks after planting. Maximal reactivity of the antigens was attained about 3 weeks after planting and was maintained throughout the life of the plants.

Reaction of antigen of strain 46 with antisera 3 and 24 was detected about 3 weeks later and disappeared about 3 weeks earlier than the reaction of the strain with antisera 4 and 46.

Viable cells in culture and nodule antigens. Plate counts of nodule and culture antigens, diluted to the same turbidity as used in the agglutination tests, indicated that the number of viable cells in the nodule antigens of four strains (38, 46, 76, and 110) was about 20 to 30% of that in culture antigens of the same strains (Table 5). Nodule antigens of ineffective strain 24 contained only about 10% as many viable cells as did culture antigens.

The highest dilution of nodule antigens at which inoculation of soybean seeds with 1 ml of the solution resulted in nodulation was 10^8 for all strains. Comparable results were obtained with the 10^9 dilution of culture antigens of strains 24, 46, 76, and 110 and for the 10^8 dilution of strain 38.

TABLE 3. *Reactions of heated and unheated antigens of strain 46 prepared from nodules of different soybean and cowpea varieties**

Species and variety	Heated antigens with antiserum		Unheated antigens with antiserum	
	3	46	3	46
Soybean				
Grant	−	+++	+	++
Kent	−	+++	+	++
Hardee	−	+++	+	++
Harosoy	−	+++	++	++
Blackhawk	+	+++	++	+
Lee	+	++	++	+
Hood	+	++	++	+
Improved Pelican	++	++	+++	−
Otootan	+++	+	+++	−
Kingwa	+++	+	+++	−
Cowpea				
Brabham	+++	−	+++	−
Buff	+++	−	+++	−
Calva	+++	−	+++	−
Crowder	+++	−	+++	−
Rice	+++	−	+++	−
Toy Set	+++	−	+++	−

* Antigens were tested against antisera listed in Table 1, but only those listed above, plus antisera 4 and 24, reacted. Data for antisera 4 and 24 were omitted because they were the same as those for 46 and 3, respectively.

DISCUSSION

Serologically classifying the strain of *R. japonicum* in a soybean nodule directly from the antigen in the nodule is vastly quicker than the usual procedure of isolating and producing antigen in culture. A single soybean nodule provides enough antigen for testing against up to eight antisera by the tube method, but the use of eight antisera normally would require selecting the largest nodules and would preclude evaluation of a random sample. However, most soybean nodules investigated were large enough to provide sufficient antigen for evaluation against at least four antisera by the tube method. The use of available microtechniques would allow this number to be expanded sufficiently for almost any need. A composite antiserum composed of up to six antisera, each in the same concentration as when they are used individually, also is used extensively to classify nodules into a composite class. This is especially useful with samples of nodules composed primarily of two or three predominant serological types but containing several other infrequent types.

Although nodule antigens of most of the normal strains of *R. japonicum* evaluated appeared to be as reliable as culture antigens in the routine identification of strains, the effect of host genotype on the serological reaction of nodule antigen of strain 46 suggests precautions in the use of nodule antigens. If the influence of the cowpea host can be assumed to represent the extreme expected from different soybean varieties, the results from a selected group of eight strains (Table 2) suggest that for most strains host variety will have little effect.

The results from nodule antigens of strain 46 are in sharp contrast with those of other strains. The nodule environment provided by various soybean genotypes and cowpeas apparently influences the serological reactions of the strain, since isolates of strain 46 from nodules of the various genotypes reacted the same. Whether similar changes in serological reaction can be in-

TABLE 4. *Reactions of unheated antigens prepared from soybean nodules of four Rhizobium strains sampled on successive dates after planting of the soybeans*[a]

Days after planting	Strain 3 antigen with antiserum			Strain 46 antigen with antiserum				Strain 31 antigen with antiserum	Strain 38 antigen with antiserum
	3	6	24	3	4	24	46	31	38
12	−	−	−	−	−	−	−	−	−
14	−	+	+	−	+	−	+	−	++
18	+	++	++	−	++	−	++	++	++
21	++	+++	+++	−	++	−	++	+++	+++
24	+++	+++	+++	−	++	−	++	+++	+++
28	+++	+++	+++	−	++	−	++	+++	+++
33	+++	+++	+++	−	++	+	++	+++	+++
43	+++	+++	+++	+	++	++	++	+++	+++
57[b]	+++	+++	+++	++	++	++	++	+++	+++
69	+++	+++	+++	++	++	+	++	+++	+++
82[c]	+++	+++	+++	+	++	+	++	+++	+++
103[d]	+++	+++	+++	+	++	−	++	+++	+++
120[e]	+++	+++	+++	−	++	−	++	+++	+++

[a] Symbols: + indicates minimal detectable agglutination; ++ indicates agglutination; +++ indicates complete agglutination.

[b] Pods on plants beginning to develop.

[c] Pods full size.

[d] Plants almost mature; nodules sound but green inside.

[e] Plants completely mature; nodules mushy but intact.

TABLE 5. *Plate counts of soybean nodule and culture antigens of five strains of Rhizobium japoniucm (viable cells per ml × 10^6)*

Strain no.	Nodule antigens	Culture antigens
24	85	780
38	165	830
46	230	1,060
76	210	1,080
110	328	1,170

duced in culture by varying the culture medium has not been explored.

The appearance and disappearance of the nonhomologous reactions of Lee soybean nodule antigens of strain 46, at times closely approximating the times bacteroids are supposed to develop and deteriorate, suggest that bacteroids might be involved in the unusual reactions. The incomplete clearing of the antigens from nodules of some genotypes also might be interpreted similarly. However, it seems unlikely that bacteroid development in nodules of the same age on different soybean varieties varies sufficiently to explain the effect of genotype (Table 3).

The serological reactions of nodule antigens apparently are due entirely to the bacterial component of the nodule. If host cells or products of host cells were involved in the reactions, they would be expected to have a general effect regardless of the *Rhizobium* strain in the nodules, rather than to be associated with one serotype such as strain 46. The complete clearing of the nodule antigens of most strains indicates that, if bacteroid cells are present, they react the same as mature cells; otherwise, residual turbidity would be expected. The reactions of antigens prepared from nodules long past the stage at which bacteroid cells supposedly deteriorate, however, appear to be convincing evidence that bacteroid cells are not a necessary part of the serological reactions of nodule antigens.

Although difficulties in the use of soybean nodule antigens have been encountered with only one relatively infrequent serotype (strain 46), certain precautions are indicated. Only 8 of approximately 200 stock strains in the Beltsville and other collections that have been evaluated serologically react the same as strain 46, but other unknown combinations of bacterial and host genotypes that fail to give the expected reactions might occur in any given experiment. Such combinations have not been detected in extensive field experiments conducted during the past 3

years, but the following precautions are routinely employed and are recommended: (i) Inoculate proposed host genotypes in sterile culture in the greenhouse with pure cultures of *Rhizobium* strains to be used, and test serological reactions of resulting nodules against identifying antisera. (ii) Plant uninoculated seed of host genotypes in greenhouse in soil taken from proposed experimental area. (iii) Collect about 50 nodules from these plants and ascertain serological reactions of nodule antigens and of culture antigens prepared from the same nodules.

These simple precautions permit identification of test strains that might cause difficulty before they are used in field experiments, as well as strains in the soil that might complicate the identification of test strains. Step ii also permits the selection of *Rhizobium* strains that are identifiable serologically from the predominant types in the soil.

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