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Suitability of oven-dried root nodules for *Rhizobium* strain identification by immunofluorescence and agglutination

P. SOMASEGARAN, R. WOOLFENDEN, & J. HALLIDAY *NifTAL Project, Department of Agronomy and Soil Science, University of Hawaii, Honolulu 96822*

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Legume root-nodules, dried at oven temperature (70°C for 48 h) were suitable for *Rhizobium* strain identification by immunofluorescence and agglutination. The fluorescence of bacteroids of *R. japonicum*, *R. leguminosarum*, *R. meliloti*, *R. phaseoli*, and *Rhizobium* spp. from oven-dried nodules was the same as those from frozen, desiccated, or nodules dried at room temperature (28°C). Oven-dried nodules did not require further steaming for agglutination. Bacteroid agglutinations gave 2–16 fold lower titres than those of the cultured cells. Fresh and oven-dried soybean rhizobia from a mixed inoculation gave exactly the same results when identified by immunofluorescence or agglutination.

In studies with inoculated legumes, the dry weight of oven-dried nodules has been shown to be a good indicator of symbiotic nitrogen fixation (Martinez *et al.* 1970). Nodule dry weight is frequently measured because of its high correlation with other parameters of effectiveness (Ahmad *et al.* 1981).

In addition to measuring yield parameter of the nodulated legumes, identification of the occupant strains in the nodules may be done to assess strain performance. Since the bacteroids in the nodules share common antigenic properties with the bacterial genotype in culture (Zipfel 1912), bacteroids from fresh nodules have been used directly for nodule identification by tube agglutination (Means *et al.* 1964), immunodiffusion (Skrdieta 1969), and immunofluorescence (Trinick 1969). Nodules which could not be identified immediately have been frozen (Russell & Jones 1975; Wagner *et al.* 1978; Berger *et al.* 1979), dried over $CaCl_2$, or suspended in 0·2% formalin prior to storage at 4°C (Parker & Grove 1970).

It is obvious, therefore, that to determine nodule dry weight and identify occupant strains in nodules from the same experiment, two separate samples would be needed. However, much time and labour could be saved if the same oven-dried nodules, after dry weight determination, could also be used for strain identification. This, however, would only be possible if the bacteroid antigens in oven-dried and fresh-nodules gave similar serological reactions with the homologous antibodies developed against its corresponding cultured genotype.

This work describes experiments designed to verify the suitability of oven-dried nodules for use in strain identification by immunofiorescence and agglutination.

Materials and Methods

STRAINS OF *Rhizobium*

The different strains of *Rhizobium* (Table 1) used in this investigation were obtained from the NifTAL *Rhizobium* germplasm resource and maintained on slants of yeast-mannitol agar (Vincent 1970).

Table 1. Sources of rhizobial strains

TAL no.	Original designation	Cross-inoculation group	Host legume
102	USDA 110	*R. japonicum*	*Glycine max*
169	176A22 (Nitragin)	*Rhizobium* sp.	*Vigna unguiculata*
182	TAL 182	*R. phaseoli*	*Phaseolus vulgaris*
209	TAL 209	*Rhizobium* sp.	*Vigna unguiculata*
309	CB 756	*Rhizobium* sp.	*Macrotyloma africanum*
379	CB 1809	*R. japonicum*	*Glycine max*
380	SU 57	*R. meliloti*	*Medicago sativa*
620	ICRISAT 3889	*Rhizobium* sp.	*Cicer arietinum*
634	B-13, NZP-5400	*R. leguminosarum*	*Lens culinaris*
638	I-2	*R. leguminosarum*	*Lens culinaris*
640	I-11	*R. leguminosarum*	*Lens culinaris*
1145	CIAT 1967	*Rhizobium* sp.	*Leucaena leucocephala*
1148	27A3 (Nitragin)	*Rhizobium* sp.	*Cicer arietinum*
1371	T-1	*Rhizobium* sp.	*Arachis hypogaea*

ANTISERA

Rhizobia were cultured and harvested in saline as described by Vincent (1970). Harvested cells were washed three times, standardized to *ca* 1×10^9 cells/ml and heated (steam without pressure) at 100°C for 1 h to inactivate flagellar antigens. Young adult, female, New Zealand rabbits were immunized according to the following schedule: day 1, intramuscular injection with 1 ml of antigen (rhizobial suspension emulsified with an equal volume of Freund's complete adjuvant (Difco Labs, Detroit, Michigan. USA)); day 20, subcutaneous injection with 2 ml saline suspension; day 28, similar as in day 20; day 42, trial bleed from marginal ear-vein and titre determination. Blood (30–50 ml) was obtained by cardiac puncture from rabbits with titres of >800. Antisera were conjugated with fluorescein isothiocyanate (FITC) as described by Schmidt *et al.* (1968).

PLANT CULTURE FOR NODULES

Seeds were surface sterilized with 3% (v/v) commercial sodium hypochlorite and pregerminated on 0·75% water agar at 28°C. Three pregerminated seeds were planted per Leonard jar (Leonard 1944) containing N-free nutrient solution (Broughton & Dilworth 1971) and inoculated with 2 ml of a turbid suspension of the appropriate *Rhizobium*. Nodules were harvested at 38 d. Samples of nodules were desiccated (over silica-gel), dried (in an oven for 48 h at 70°C and at room temperature) and frozen. An oven temperature of 70°C was chosen for drying the nodules as this temperature is commonly used for drying plant material for dry weight determination (Agboola 1978; Ham & Dowdy 1978). Dried nodules were further treated by storing at room temperature (28°C) for 12 months without desiccant in open vials, aluminium weighing boats, or in loosely capped tubes.

SMEAR PREPARATION, IF-STAINING AND MICROSCOPY

Wells of Microtiter U-plates (Cooke Laboratory Products, Alexandria, Va., USA) were used for squashing nodules for smear preparation. Frozen nodules were thawed for 1 h prior to placement in the wells. Dried and desiccated nodules were rehydrated by placing one nodule per well with one drop of sterile distilled water. Wells were sealed with cellophane tape and nodules were left to imbibe water for 1 h at room temperature or stored overnight in a refrigerator. Nodules were then squashed with fine forceps or pierced with flat toothpicks to obtain material for smear preparation. Twelve smears of each treatment were examined on each slide along with a cultured cell control. Smears were stained as described by Schmidt *et al.* (1968). Microscopy was performed with a Zeiss Standard Microscope 14, with incident light fluorescence illuminator equipped with an Osram HBO 50 W mercury vapour light source. Observations were made by epifluorescence alone or in combination with the phase contrast system.

AGGLUTINATION TITRES OF OVEN-DRIED NODULES

Nodules were placed in a Petri-dish with some water and imbibed overnight in a refrigerator. Five to 7 nodules were placed in a 5 ml culture tube and bacteroid antigen prepared as described by Means *et al.* (1964). Unheated and heat-treated (30 min in boiling water) suspensions were prepared and standardized to *ca* 1×10^9 cells/ml. Antisera were prepared in two-fold dilutions. Standard agglutination tests were performed by reacting equal volumes (5 drops) of antigen and antibody in Dreyer tubes. Following incubation in a water bath (Vincent 1970) tubes were read after overnight storage at 4°C.

VERIFICATION OF SIMILARITY IN AGGLUTINATION BETWEEN FRESH AND DRIED NODULES

Fresh and oven-dried nodules of two *R. japonicum* strains (TAL 102, TAL 379) were investigated by reacting bacteroid antigen against the homologous antibody. Fresh nodules were packed in small plastic bags and immersed in boiling water for heat treatment (Somasegaran 1977). Nodules were transferred to U-plates (one nodule per well plus 6 drops of saline) and bacteroids pressed out with a round-ended glass rod without homogenising the nodule. Dried nodules were imbibed in water and processed similarly but without heat treatment. Agglutination was performed in U-plates using 2 drops of antiserum (1 : 25 dilution) and 2 drops of antigen per well. Serum and antigen controls were included. Wells were sealed with cellophane tape and plates incubated at 37°C (oven) for 2 h followed by overnight refrigeration. Agglutinations were read using a darkfield Quebec colony counter (American Optical, Buffalo, NY, USA).

RELIABILITY OF OVEN-DRIED NODULES FOR STRAIN IDENTIFICATION

To verify that oven dried nodules produce the same strain identification information as fresh nodules, bacteroids in fresh nodules were initially identified by IF. The same nodules were then oven dried and identified again by IF and by agglutination. Toothpicks were used for smear preparation. To avoid bias, immunofluorescence and agglutination were done by different workers. By maintaining the nodules in their specific locations in the U-plate, each nodule was examined when fresh and later after drying. Agglutination was performed in the same well previously occupied by the nodule. Two drops of antigen and 2 drops of antiserum (1 : 25 dilution) were used. Plates were incubated and read as before.

Results

REHYDRATION OF NODULES AND BACTEROID STAINING

Dried nodules were easier to squash when allowed to imbibe following overnight storage in a refrigerator than at room temperature for 1 h.

The amount of nodular material required to produce a thin smear was difficult to control when squashed nodules were used. Thick smears produced from excessive application of nodular material interfered with the staining and therefore with grading of the fluorescence. Regulating nodular material for smear preparation was, however, greatly facilitated by the use of toothpicks. Size, thinness, and precise placement (spotting) of smears on the slide were easily controlled by this method. Though there was definite restriction on the amount of material that could be removed with toothpicks, this did not interfere with the obtaining of sufficient material for smear preparation.

The immunofluorescence staining characteristics of bacteroids of each species of *Rhizobium* to show a 4+ fluorescence with FA of different dilutions was variable depending on the nodule treatment (Table 2). Generally, bacteroid smears dried at 70°C had higher staining titres than other treatments. Staining titres for bacteroids from nodules dried at room temperature (28°C) and from desiccated nodules were similar.

STABILITY OF BACTEROID ANTIGEN

The retention of specificity of bacteroid antigens from oven-dried cowpea nodules is shown in Table 3. No cross-reactions or loss of ability of bacteroids to stain with their homologous FA were detected amongst the six strains of rhizobia. Long term storage did not change the specificity of the bacteroids.

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Table 2. Immunofluorescent staining of bacteroids in treated nodules as affected by dilution of the fluorescent antibody

Nodules of	Nodule treatment			
	Frozen	28°C for 48 h	70°C for 48 h	Dessicated over silica-gel
R. phaseoli (TAL 182)	2*	4	4	4
R. meliloti (TAL 380)	8	16	16	16
R. japonicum (TAL 379)	16	32	64	16
Rhizobium sp. (TAL 620)	8	8	16	8
R. leguminosarum (TAL 634)	4	8	16	8
R. leguminosarum (TAL 638)	4	NT	4	NT
Rhizobium sp. (TAL 1148)	1	2	4	4

* Numbers indicate dilution of the fluorescent antibody giving a 4 + reaction.
NT, not tested.

Table 3. Test for stability of bacteroid antigens from ovendried nodules by immunofluorescence staining. Nodules of the various strains were produced on cowpea (*Vigna unguiculata*).

Antigen		Fluorescent antibody of					
		TAL 102	TAL* 169	TAL 209	TAL* 309	TAL 379	TAL 1371
R. japonicum TAL 102	B^1	4+	–	–	–	–	–
	C	4+	–	–	–	–	–
	B^2	4+	–	–	–	–	–
Rhizobium sp. TAL 169	B^1	–	4+	–	4+	–	–
	C	–	4+	–	4+	–	–
	B^2	–	4+	–	4+	–	–
Rhizobium sp. TAL 209	B^1	–	–	4+	–	–	–
	C	–	–	4+	–	–	–
	B^2	–	–	4+	–	–	–
Rhizobium sp. TAL 309	B^1	–	3+	–	4+	–	–
	C	–	4+	–	4+	–	–
	B^2	–	4+	–	4+	–	–
R. japonicum TAL 379	B^1	–	–	–	–	4+	–
	C	–	–	–	–	4+	–
	B^2	–	–	–	–	4+	–
Rhizobium sp. TAL 1371	B^1	–	–	–	–	–	3+
	C	–	–	–	–	–	4+
	B^2	–	–	–	–	–	3+

B^1 Bacteroids from nodules dried at 70°C for 48 h.
B^2 Bacteroids from nodules dried at 70°C for 48 h and stored for 12 months in vials without desiccant.
C Laboratory culture cells.
– No fluorescence; 3 + bright yellow green; 4 + brilliant yellow-green.
* Known cross-reacting strains.

Table 4. Immunofluorescent staining of bacteroids from dried and dessicated nodules previously kept under storage for 12 months

Nodules of	FA Titre	Nodule treatment & immunofluorescence* 28°C	70°C	Dessicated over silica-gel	Cultured cell-control
R. phaseoli (TAL 182)	8	2+–4+†	4+	NT	4+
R. meliloti (TAL 380)	4	4+	4+	4+	4+
R. japonicum (TAL 379)	10	4+	NT	4+	4+
Rhizobium sp. (TAL 620)	10	4+	NT	4+	4+
R. leguminosarum (TAL 634)	10	4+	4+	4+	4+
R. leguminosarum (TAL 638)	4	4+	4+	2+–4+	4+
Rhizobium sp. (TAL 1148)	4	4+	4+	4+	4+

* Twelve nodules were examined for each treatment; fluorescence grading: 1 + dull, 2 + yellow-green, 3 + bright yellow-green, 4 + brilliant yellow-green.
† Variable staining reaction resulting from some thick smears.
NT not tested.

Table 5. Immunofluorescent staining and agglutination reactions of bacteroids from oven-dried (70°C) root nodules previously stored under various conditions

Previous storage*	Nodules of	Immunoflourescence (IF)† Cultured cells	Bacteroids	Agglutination titres with homologous antiserum‡¶ Cultured cells	Bacteroids§	Bacteroids‖
1	*R. japonicum* (TAL 379)	4+	4+	400	400	400
2	*R. japonicum* (TAL 379)	4+	4+	400	400	400
3	*R. japonicum* (TAL 379)	4+	4+	400	400	400
3	*R. japonicum* (TAL 102)	4+	4+	800	400	400
1	*R. japonicum* (TAL 102)	4+	4+	800	200	200
3	*Rhizobium* sp. (TAL 209)	4+	2+–4+	6400	1600	800–1600
3	*Rhizobium* sp. (TAL 620)	4+	4+	800	NT	200–400
3	*Rhizobium* sp. (TAL 1145)	4+	2+–3+	1600	800	800
3	*Rhizobium* sp. (TAL 82)	4+	4+	3200	200	200
4	*R. phaseoli* (TAL 182)	4+	2+–4+	800	NT	800

* 1, nodules dried at 70°C for 48 h; 2, nodules dried at 70°C for 48 h and stored in open vials for 9 months at room temperature (28°C); 3, kept frozen for 1–2 years then dried at 70°C for 48 h; 4, refrigerated for 1 year then dried at 70°C for 48 h.
† IF with 1 : 8 diluted FA; fluorescent grading: 1 + dull, 2 + yellow-green, 3 + bright-yellow-green, 4 + brilliant yellow-green.
‡ Antiserum prepared against laboratory cultured cells. Low titre antisera used here were not the same as those used for conjugating with FITC.
§ Steamed bacteroid antigen;
‖ unsteamed bacteroid antigen.
¶ Numbers refer to 1 in 400, 1 in 800 etc.
NT Not tested.

Fig. 1. No agglutination (A) and positive agglutination (B) with bacteroid antigens (from oven dried nodules) in wells of U-plates. Note the central compact button of sedimented cells in A and the uniform pellicle film in B.

STABILITY OF BACTEROID ANTIGEN DURING STORAGE

Drying nodules at room or oven temperatures and then keeping them stored under room temperature conditions in loosely capped or open vials for 12 months did not impair the ability of the bacteroids to react with its fluorescent antibody (Table 4). Desiccation over silica gel did not confer any additional advantage over storage without the desiccant. A significant observation on all smears that were prepared from the dried nodules was the absence of non-fluorescing bacterial contaminants indicating that other microbial forms did not develop during storage.

The ability of bacteroid antigens to withstand some physical stresses without noticeable changes in their reactions towards the homologous FA is indicated in Table 5. Fluorescent titres of bacteroids of TAL 209 and TAL 182 differed from that of the cultured bacterial genotype and these observations were true even on oven-dried nodules which were previously frozen for 1–2 years.

Agglutination titre comparisons (Table 5) between cultured cells and bacteroids, performed with antiserum developed against cultured cells, produced identical or lower titres for the bacteroids with strain differences. Bacteroid agglutination titres were 2–16 fold lower than those of cultured cells.

STRAIN IDENTIFICATION BY IF AND AGGLUTINATION

It was verified that oven-dried nodules of soybean can be confidently used for nodule identification, as bacteroids from fresh and dried nodules showed similar abilities to agglutinate. Typical positive and negative agglutinations with bacteroid antigens from oven dried nodules are shown in Fig. 1. Agglutinations were of equal intensity in all cases where different final serum dilutions (1 : 33·3 and 1 : 50) were established in the U-plates by varying the antiserum, antigen and saline volumes.

The suitability and reliability of oven-dried nodules was further investigated by identifying

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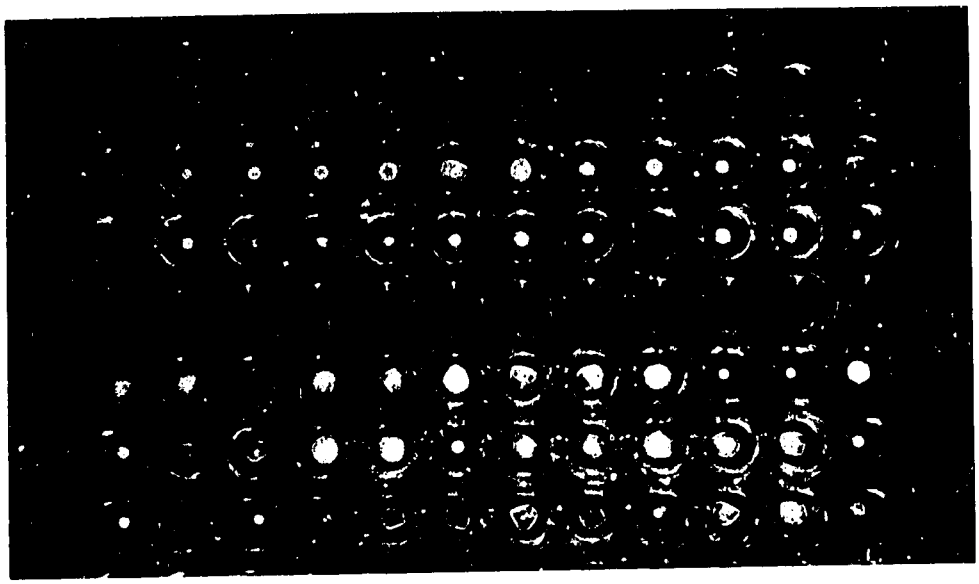

Fig. 2. Identification of two *R. japonicum* strains using bacteroid antigen from oven dried nodules. Nodules were from soybean plants inoculated with a mixture of TAL 102 and TAL 379. Horizontal well series A, B, and C contain anti-TAL 102 and horizontal well series E, F, and G contain anti-TAL 379.

soybean nodules formed by a mixed inoculum using agglutination and immunofluorescence. The results are shown in Fig. 2 and Table 6. The dried nodules, regardless of the method, produced the same information in the identification of the two *R. japonicum* strains occupying the nodules. The IF identification data of the fresh nodules matched exactly with the dried nodule data.

Discussion

The insoluble somatic antigens on the surface of *Rhizobium* bacteroids in legume nodules have been shown to be highly specific towards their homologous antisera in strain identification by immunofluorescence (Trinick 1969) and agglutination (Means *et al.* 1964; Parker & Grove 1970). These properties were shown when using nodules which were either fresh or preserved by methods which did not involve prior heat treatment of the nodules

This work demonstrated in a series of experiments that root nodules dried in the oven and stored under room temperature conditions were as suitable and reliable as fresh, desiccated, or frozen nodules for strain identification by immunofluorescence and agglutination. Similarly treated nodules, however, may not be satisfactory for identification by immunodiffusion due to inhibition in the precipitin band formation (Skrdleta & Mareckova 1971).

Remarkable thermostability of the somatic antigen has been shown by steam-heated saline suspensions of the cultured cells and nodule homogenates (Means & Johnson 1968). Oven-drying of nodules, however, is somewhat different since a highly dehydrated nodule (complete removal of moisture takes place at 60–70°C) is produced, compared with the usual process of heat treating saline suspensions of the antigen where the moisture level thoughout the heating is very much unchanged. The information pre-

Table 6. Summary of data for the identification of two strains of *R. japonicum* (TAL 379 and TAL 102), applied as a mixed inoculum, in fresh and oven dried nodules

	Nodule Analysis		
Strain	Fresh (IF)*	Dried (IF)	Dried (aggin)
TAL 379	26	26	26
TAL 102	8	8	8
Mixed	2	2	2
Total	36	36	36

(IF) immunofluorescence
(aggln) agglutination
Mixed nodules containing both strains

sented in this work suggests that, in spite of the progressive loss of moisture during the drying, the antigen still retained its specificity and reactivity upon rehydration. This would be an additional property of the protein-polysaccharide-lipid complexes described for O-type antigens (Salton 1960) found in rhizobia (Means & Johnson 1968).

Bacteroids of *R. japonicum* (TAL 102) gave two different sets of agglutination titres depending on the host on which the nodules were formed. Bacteroid antigens from nodules produced on soybean variety Clarke 63 had titres which were two-fold lower than that of the cultured cells. Antigen of the same strain but from nodules produced on cowpea were four-fold lower than that of the cultured cells There were no major significant titre differences between heated (steamed) and unheated bacteroid antigens. Agglutination titre end points were sometimes difficult to determine with the steamed bacteroid antigens of two strains (TAL 209 and TAL 620) because of persistent turbidity in the supernatant liquid.

The differences in the agglutination titres between cultured cells and their bacteroids shown by some of the strains in this study emphasize that the reaction of the bacteroids with the anti-cultured cell serum can be different and therefore needs to be determined for each strain. This information will be useful in determining the optimal and economical serum dilution, to obtain positive agglutination in strain identification. The need to know the reactions of the bacteroids before embarking on strain identification has been emphasized since bacteroids can completely fail to agglutinate with the anti-cultured cell serum or show wider cross agglutinations than the cultured cells (Means *et al.* 1964). Even with immunofluorescence the staining titre of the bacteroids must be evaluated since bacteroids can show a lower titre than the cultured cells (Trinick 1969).

The fact that previously frozen nodules can be removed from deep-freeze, oven-dried, and identified by immunofluorescence or agglutination could be advantageous in locations where electricity supply is interrupted for long periods. In such instances, nodules may be removed from deep freeze and oven-dried for storage.

Though U-plates are convenient for performing agglutinations with bacteroids or cultured cells, prior familiarization in reading agglutinations with this method is important. This is mainly because the agglutinations in the wells of the U-plate have a different appearance from agglutinations in tubes. Furthermore, optimal proportions of the diluted antiserum and antigen need to be determined before typing large numbers of nodules.

The evidence presented in this work indicates that the insoluble somatic antigens of the bacteroids are well preserved in the dried nodules. Its significance lies in the fact that nodules, once dried, may be preserved for at least a year without the use of freezer-space or desiccant. Even though the dried nodules were stored under room temperature conditions without any special precautions, fungal and bacterial contaminations were not evident. In environments of high humidity, however, nodules should be stored in tightly capped vials.

The resulting lightness and volume reduction of root nodules upon drying would permit convenient transportation requiring low postage costs to distant laboratories where serological typing facilities may be offered.

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