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Short communication

Strain-specific antisera to identify Thai *Bradyrhizobium japonicum* strains in preserved soybean nodules

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Summary

Strain-specific antisera were produced against six Bradyrhizobium japonicum strains using two immunization procedures. These specific antisera were used for detection of bradyrhizobia in preserved soybean nodules. Antisera specific for two of these strains were either conjugated with a fluorescent dye or used with a fluorescent secondary antibody for identification of bradyrhizobia in soybean nodules that were preserved in four different storage conditions. Results show that soybean nodules dried in the oven, stored under room temperature, or at −20 °C are as suitable as fresh nodules for strain identification using fluorescent antisera.

Introduction

Serological methods have been extensively used for reliable identification of *Rhizobium* strains forming nodules on leguminous plants. Most serological studies involve the use of agglutination, immunodiffusion and immunofluorescence tests (Bohlool & Schmidt 1973; Dudman 1977) and ELISA (Renwick & Jones 1985). Antibodies specific for a particular *Rhizobium* strain can be easily produced and manipulated. However, it is not a single procedure of animal immunization that guarantees an ideal product for all requirements. During the past decade, the production procedures for antibodies have been intensively studied to achieve the high yield and affinity of the antibodies and to ascertain better safety procedure for the animals used for immunization. In this report we used two immunization procedures to enhance antisera production. The objective was to obtain high titres and reduce the time required for antibody production.

The specific antisera obtained were further used for determination of the appropriate conditions for detection of bradyrhizobia by fluorescent antibody techniques. Since the bacteroids in the nodules share common antigenic properties with *Rhizobium* grown in culture (Zipfel 1912), fresh nodules have been used directly for nodule identification. Furthermore, the best specimen used for analysis by serological technique should be fresh to avoid denaturing or conformational change in the sample. It is difficult to assay a large number of nodules to identify bradyrhizobia on a single day. Therefore, the nodules need to be preserved so that they can be assayed at a convenient time. In this experiment we aimed at determining suitable storage conditions to detect bradyrhizobia in nodules using fluorescent antibody.

Materials and methods

Specific antisera for *B. japonicum* strains THA7, SEMIA5019, UN8, NA1, NA8 and NA17 were produced in healthy-six month old rabbits by two immunization scheme. Scheme I: on the first day of immunization, injections were performed by the intravenous route with 0.5 ml, intramuscularly with 1.0 ml and subcutaneously with 1.0 ml with Freund's Complete Adjuvant. On days 2, 3, 7, 8, 9, the rabbits were injected intravenously by 1.0, 1.5, 1.5, 2.0 and 2.0 ml, respectively. In scheme II, the antigen was injected intravenously with 0.5 ml and the volume increased by 0.5 ml every day up to 5 days. The agglutination titre test was done at day 12 by the tube agglutination technique using cultured cells as the source of antigen (Somasegaran & Hoben 1994). The second immunization was performed with 2.0 ml of concentrated suspension with Freund's Complete Adjuvant subcutaneously. After the titre had reached an acceptable level, bleeding by

cardiac puncture to obtain 30–50 ml of blood was performed.

Antigens were prepared by culturing *Rhizobium* aerobically (agitation at 250 rev/min) on YEM medium (0.05% (w/v) K_2HPO_4, 0.02% (w/v) $MgSO_4 \cdot 7H_2O$, 0.01% (w/v) NaCl, 1% (w/v) mannitol and 0.05% (w/v) yeast extract) at 28 °C for 5–7 days. Cell cultures were then harvested, washed and resuspended in filter-sterilized saline to achieve a cell concentration of 10^{10} cells/ml for using as concentrated suspension. One half of the concentrated suspension was diluted to 10^9 cells/ml for using as working suspension by using McFarland standards.

The soybean cultivar used in this experiment was SJ5 obtained from the Department of Agriculture, Bangkok, Thailand. The germinated seeds were planted in Leonard's jars and inoculated with 1 ml of 10^8 cells/ml of *Bradyrhizobium* strains SEMIA5019 and THA7. Plants were supplemented with N-free medium and held at 25 °C in a room equipped with a light source, which provided a flux density of light of about $450/\mu Es^{-1}\ m^{-2}$ with a 12–12 h light–dark regime. Plants were harvested at four weeks after inoculation. Nodule typing was done by fluorescent antibody assay (FA) using antisera conjugated with either a fluorescent dye FITC for detection (direct FA) or using a fluorescent secondary antibody (indirect FA). Fluorescent antisera specific to SEMIA-5019 and THA7 were obtained according to Somasegaran & Hoben (1994). Bradyrhizobia were assayed in nodules, prepared or preserved in four different ways: (i) fresh nodule (examined immediately), (ii) frozen nodules (kept at −20 °C for 2 weeks), (iii) air-dried nodules (left at room temperature until completely dried) and (iv) oven-dried nodules (dried at 80 °C for 12 h).

Results and discussion

Specific antisera of *Bradyrhizobium* strains SEMIA5019, THA7 and UN8 produced by Scheme I immunization developed a low titre at first immunization (Figure 1). After the second immunization, the titre increased sharply by 4, 16 and 32 times for strains THA7, UN8, and SEMIA5019, respectively. Scheme II immunization of strains specific to NA1, NA8 and NA17 showed titres of 50, 400 and 800, respectively. After the second immunization, the titre remained unchanged for strains NA8 and NA17, and increased 32-fold for NA1 (Figure 1). These results show that Scheme I immunization is the more effective procedure to stimulate immune response, because the titre development was four times higher than through Scheme II immunization. Small releases of antigens by several injection routes; intravenous, intramuscular and subcutaneous, especially, subcutaneous with Freund's Complete Adjuvant can activate various cells involved in the immune response, particularly macrophages. The activated macrophages in turn help activation of T and B cells, thus increasing the final titres (Rudbach 1988). Scheme II immunization exhibited a high titre within a short stimulation time. Injection via the intravenous route could directly deliver and quickly release antigens into the lymphatic circulation, causing a rapid immune response. However, antibodies produced through intravenous immunization may not be sustained over a long period of time (Kaeberle 1986). Nevertheless, Scheme II immunization is appropriate for production of antiserum within a short time without going for a second immunization.

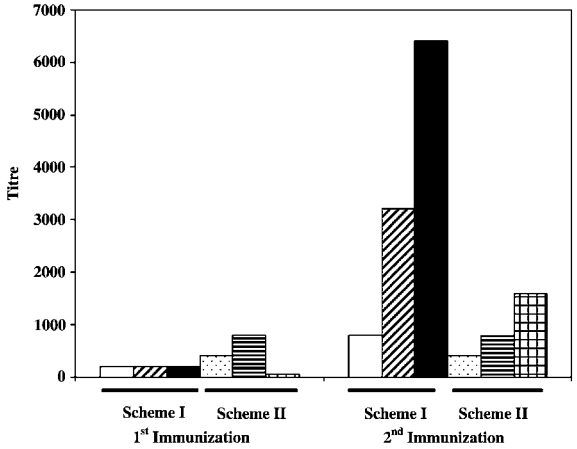

Figure 1. Agglutination titres of antisera for strain THA7 (□), UN8 (▨), SEMIA5019 (■), NA8 (⊡), NA17 (⊟) and NA1 (⊞) after the first and second immunization using the Schemes I and II of antigen delivery.

Table 1. Percentages of nodules showing bacteroids with bright green (4+) and light green (2+) fluorescence after staining with specific fluorescent antisera.

Strain	Condition	Total-nodules	Direct FA		Indirect FA	
			4+ fluorescence	2+ fluorescence	4+ fluorescence	2+ fluorescence
SEMIA 5019	Fresh nodule	50	98	2	90	10
	Frozen nodule	50	92	8	98	2
	Air dried nodule	50	98	2	98	2
	Oven dried nodule	50	86	14	98	2
THA7	Fresh nodule	50	96	4	80	20
	Frozen nodule	50	96	4	88	12
	Air dried nodule	50	100	0	100	0
	Oven dried nodule	50	100	0	96	4

Soybean nodules preserved under different storage conditions were used for determination of *Bradyrhizobium* occupancy using specific fluorescent antisera, which were produced according to Scheme I. Results show that the four preserved conditions; fresh nodule, frozen nodule, air-dried nodule and oven-dried nodule did not affect the detection procedure using fluorescent antibody. Fluorescent antiserum specific to SEMIA5019 detected bright green fluorescence in more than 85% nodules in both direct and indirect FA, while antiserum specific to THA7 expressed bright green fluorescence in more than 95% nodules in direct examination and in more than 80% of nodules in indirect examination (Table 1). When both the bright green (4+) and the light green (2+) fluorescences of the bacteroid were taken together, 100% of nodules were detected with these antibodies using either the fluorescent dye or the secondary antibody for detection. Therefore, root nodules dried in the oven, stored under room temperature and frozen were as suitable and reliable as fresh nodules for strain identification by immunofluorescence. In spite of the progressive loss of moisture during the drying, the antigen still retained its specificity and reactivity upon rehydration. When the fluorescent antisera specific for SEMIA5019 were used against free-living cells for bacteroids in the nodules formed by THA7, no fluorescence was detected. Similarly, the antisera specific for THA7 did not cross-react with SEMIA5019. Therefore, these fluorescent antisera can be used as a detection tool in nodule-typing in competitive experiments.

Acknowledgements

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