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STORAGE EFFECTS ON IDENTIFICATION OF RHIZOBIA IN SOYBEAN NODULES BY ELISA[1]

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Abstract

Serological analyses of rhizobia in soybean [*Glycine max* (L.) Merr.] nodules are time-consuming and often must be delayed for extended periods. The objective of this study was to assess the impact of drying and long-term storage on identification of rhizobia in soybean nodules by indirect enzyme-linked immunosorbent assay (ELISA). Nodules formed on 'Coker 338' by *Bradyrhizobium japonicum* strains USDA 3I1b110, NC1004, and B587 were either air-dried at 40°C or freeze-dried, and then stored for 1 month at either ambient temperature, 5, −5 or −60°C. Rhizobia within nodules from all drying and storage combinations were suitable for identification by ELISA. Results of this study indicate that large numbers of nodules can be collected and analyzed for serological identification of rhizobia at a later date, without loss of accuracy.

Additional Index Words: **drying, serology, *Bradyrhizobium japonicum, Rhizobium japonicum, Glycine max.***

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DINITROGEN FIXATION by *Bradyrhizobium japonicum* is an important facet of soybean [*Glycine max* (L.) Merr.] production on the sandy, low N soils of the southeastern USA (Matheny and Hunt, 1983). Many investigators have examined the effects of crop management systems and environmental conditions on the ability of rhizobia to fix N_2 (Lindemann and Ham, 1979; Hunt et al., 1981, 1983; Matheny and Hunt, 1983). Generally, these types of studies require serological identification of rhizobia occupying soybean nodules. Commonly-used serological analyses—agglutination, immunodiffusion, immuno-fluorescence, and indirect enzyme-linked immunosorbent assay (ELISA)—are time-consuming and often must be delayed until a convenient time arises to perform them. These delays may necessitate the drying and/or storage of nodules for extended periods.

A major concern with drying and storing nodules is the effect it may have on serological analysis. Somasegaran et al. (1983) reported that oven-dried nodules were as suitable for strain identification by immunofluorescence and agglutination as fresh, desiccated, or frozen nodules. However, Skrdleta and Mareckova (1971) reported that similarly treated nodules may not be suitable for identification by immunodiffusion. The objective of this study was to assess the suitability of air- and freeze-dried soybean nodules stored at various temperatures for use in strain identification by the indirect enzyme-linked immunosorbent assay.

Materials and Methods

Treatments

Soybean (cv. Coker 338) plants were grown in horticultural-grade vermiculite in a greenhouse. Seeds were inoculated with *Bradyrhizobium japonicum* (strains USDA3I1b110, NC1004, or B587) at planting. All plants were watered with a modified Ahmed and Evans nutrient solution (Speidel and Wollum, 1980) as needed for the duration of the growing period. Five-week-old plants were harvested, and nodules were removed from the roots and washed with distilled water. Harvested nodules were divided into four groups and received the following primary (drying) treatments:

1. Group I nodules were air-dried at 40°C for 2 d in a Blue M laboratory oven;
2. Group II nodules were quick frozen overnight at −60°C in a RHEEM ultralow freezer and then freeze-dried for 7 d in a VIRTIS Freezemobile-24;
3. Group III nodules (nondried) were sealed in sterile containers and immediately placed in storage; and
4. Group IV nodules (control) were serologically assayed by indirect ELISA within 24 h after removal from the roots.

Following the primary treatment (drying vs. nondrying), Group I, II, and III nodules were subdivided and received a secondary (storage) treatment for 1 month. Group I nodules (air-dried) were either stored on a laboratory shelf at ambient temperature, stored in a laboratory refrigerator at 5°C, or stored in a commercial freezer at −5°C. Group II nodules (freeze-dried) were stored at either ambient temperature, 5°C, −5°C, or in an ultralow freezer at −60°C. Group III nodules (nondried) were stored at either ambient temperature, −5°C, or −60°C. Upon conclusion of the secondary treatment, nodules were serologically assayed by indirect ELISA.

Elisa

Twenty nodules were randomly selected from each of the 11 treatment combinations. Each nodule was placed in an individual culture tube, macerated in 1 mL of a saline solution containing 14.5 m*M* NaCl, and incubated for 1 h at ambient temperature. The turbid nodule suspension was then heated for 1 h in a water bath at 100°C and diluted to an optical density of 0.5 at 600 nm with 0.05 *M* carbonate buffer (pH 9.6). The indirect ELISA was performed as follows: (i) 100 μL of the nodule suspension was added to the wells of microelisa plates (Dynatech Immunlon 1 "U" bottom)[3] and incubated overnight in a humid environment at 5°C; (ii) wells were emptied and washed three times by flooding for 3 min each time with phosphate buffer NaCl (pH 7.3) containing 0.5 mL L^{-1} polyoxyethylene sorbitan monolaurate (TWEEN-20); (iii) 100 μL of the specific rhizobia rabbit antiserum[4] (diluted with phosphate buffered NaCl containing 0.2 g L^{-1} ovalbumin and 0.1 m*M* polyvinylpyrrolidone) was added to the test wells and incubated for 1 h at ambient temperature; (iv) Step 2 was repeated; (v) 100 μL of anti-rabbit IgG Alkaline phosphatase conjugated serum (Sigma A7778) was added to the test wells and incubated for 1.5 h in a humid environment at 35°C; (vi) Step 2 was repeated; (vii) 100 μL of enzyme substrate (0.5 mg mL^{-1} paranitrophenylphosphate in 0.92 *M* diethanolamine buffer) was added to each test well and incubated at ambient temperature for 1 h; and (viii) test wells were measured for degree of color development by a Dynatech Minireader II at an absorbance of 410 nm. Negative reactions were determined on nodules formed by nonreacting strains. Each antiserum was tested for specificity on all treatment combinations. Data were analyzed by analysis of variance, least significant difference

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[3] Mention of trademark, proprietary product, or vendor does not constitute a guarantee or warranty of the product by the USDA and does not imply its approval to the exclusion of other products or vendors that may also be suitable.
[4] Antiserum and titers were provided by P. Musclewhite, North Carolina State University, Raleigh.

Table 1. Storage and drying effects on identification of rhizobia in soybean nodules by ELISA.

Storage temperature	Drying method			$LSD_{0.05}$
	Nondried	Air-dried	Freeze-dried	
	ELISA units†			
	Strain 110			
Control‡	52 ± 15	--	--	
Ambient	62 ± 11	42 ± 9	44 ± 5	
5°C	--	31 ± 14	52 ± 11	
−5°C	49 ± 17	50 ± 12	54 ± 12	
−60°C	37 ± 13	--	46 ± 13	10
	Strain 1004			
Control	48 ± 12	--	--	
Ambient	64 ± 15	37 ± 17	39 ± 16	
5°C	--	35 ± 12	47 ± 16	
−5°C	65 ± 13	36 ± 17	53 ± 18	
−60°C	39 ± 17	--	29 ± 16	13
	Strain 587			
Control	35 ± 13	--	--	
Ambient	75 ± 14	47 ± 11	32 ± 10	
5°C	--	47 ± 10	35 ± 7	
−5°C	50 ± 7	53 ± 10	38 ± 8	
−60°C	37 ± 8	--	25 ± 3	8

† Absorbance values at 410 nm × 100.
‡ Controls were nodules which were serologically analyzed within 24 h after removal from the roots.

(LSD), and standard deviation as outlined by Steel and Torrie (1960).

Results and Discussion

Enzyme-linked immunosorbent assay (ELISA) is a colorimetric technique for identification of rhizobia. Positive ELISA reactions are characterized by intense color development, whereas, negative reactions develop color slowly. Negative reactions (resulting from the natural degradation of the enyzyme substrate) had readings of 10±4 ELISA units. Data in Table 1 represent the quantitative measurement of color development associated with the various treatments.

Neither of the drying methods nor storage temperatures inhibited color development associated with positive reactions (Table 1). Color development for all treatment combinations was three- to six-fold greater than negative reactions. The nondried nodules stored at ambient temperature were generally more reactive than the other treatment combinations, however, this was probably a result of fungal contamination. In this study, nondried nodules stored at ambient temperature were more susceptible to fungal growth than the other treatment combinations. Means for the freeze-dried nodules stored at 5°C or −5°C were consistently similar to the mean of the control. However, since the other treatment combinations provided material suitable for ELISA, the similarity between freeze-dried nodules and the controls would not justify the expense of purchasing freeze-drying equipment. Additionally, air-dried and freeze-dried nodules lost pliability and required rehydration for maceration. Suspending nodules in saline solution for about 1 h restored pliability and provided material suitable for ELISA.

Conclusions

Identification of rhizobia in soybean nodules by ELISA was not inhibited by drying or by storage at the various temperatures for an extended period. For efficient resource management, we prefer freeze-dried nodules stored in sealed containers at −5°C. However, one or more of the alternative procedures may be more practical in some laboratories, depending on the number of samples collected and the time, personnel, and analytical facilities available to conduct serological analyses.

References

Hunt, P.G., A.G. Wollum, II, and T.A. Matheny. 1981. Effects of soil water on *Rhizobium japonicum* infection, nitrogen accumulation, and yield of Bragg soybean. Agron. J. 73:501–505.

Hunt, P.G., T.A. Matheny, A.G. Wollum, II, D.C. Reicosky, R.E. Sojka, and R.B. Campbell. 1983. Effect of irrigation and *Rhizobium japonicum* strain 110 upon yield and nitrogen accumulation and distribution of determinant soybeans. Commun. Soil Sci. Plant Anal. 14:223–238.

Lindemann, W.C., and C.W. Ham. 1979. Soybean plant growth, nodulation, and nitrogen fixation as affected by root temperature. Soil Sci. Soc. Am. Proc. 43:1134–1137.

Matheny, T.A., and P.G. Hunt. 1983. Effects of irrigation on accumulation of soil and symbiotically-fixed N by soybean grown on a Norfolk loamy sand. Agron. J. 75:719–722.

Skrdleta, V., and H. Mareckova. 1971. Serotyping of fresh and/or dried soybean nodules. Zentral-blatt fur Bakteriologie Parasitenkunde, Infektionskrankheiten und Hygiene Abs. II 7:656–658.

Somasegaran, P., R. Woolfenden, and J. Halliday. 1983. Suitability of oven-dried root nodules for *Rhizobium* strain identification by immunofluorescence and agglutination. J. Appl. Bacteriol. 55:253–261.

Speidel, K.L., and A.G. Wollum, II. 1980. Evaluation of leguminous inoculant quality: A manual. N.C. State Univ. Tech. Bull. 266.

Steel, R.G.D., and J.H. Torrie. 1960. Principles and procedures of statistics. McGraw-Hill Book Co., Inc., New York.