Competition between *Rhizobium trifolii* Strains for Nodulation, during Growth in a Fermenter, and in Soil-based Inoculants, Studied by ELISA

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An improved ELISA technique using a new type of enzyme-conjugate – the antibody being linked to β -galactosidase by using a heterobifunctional reagent, *N*-succinimidyl-3-(2-pyridyldithio)propionate (SPDP), containing two reactive groups directed towards different functional sites – has been further improved by fine purification of the antibodies. The ELISA was applied in studies of competition among strains of *Rhizobium trifolii* in three environments: a rhizosphere, a liquid medium and in soil. The strain origin of nodules formed by strain mixtures of different relative composition was investigated, including a study of double strain occupancy. Strain 7612 was found to compete successfully with strain 285 for nodulation. No double strain occupancy occurred. Strain 7612 also competed successfully with strain 285 in soil, but strain 285 dominated when grown together with strain 7612 in a liquid medium in a fermenter.

INTRODUCTION

Production of *Rhizobium* inoculants for agricultural purposes has a long history, especially in Sweden, where it dates back to the early 1900s (Barthel & Rhodin, 1914). Introduction of inoculants to fields devoid of native *Rhizobium* may produce a substantial increase in the yield of inoculated legume crops (Nutman, 1976). Sometimes, however, the inoculation fails and no beneficial effects are noted. This lack of response to inoculation may be explained by several factors. Unfavourable conditions in the field, e.g. high/low pH (Holding & King, 1963), high/low nutrient status (Barber, 1980), or desiccation (Bushby & Marshall, 1977), are some of the soil physical and chemical factors that may strongly influence the survival of the inoculated strains.

Biological factors that may influence the success of inoculation include root exudates – those of legume as well as non-legume origin influence the chemotaxis of *Rhizobium* (Gaworzewska & Carlile, 1982) – and nematodes grazing on *Rhizobium*, which are known to influence the population density (Ramirez & Alexander, 1980). However, the possible influence of competition between strains of *Rhizobium* remains unclear. At the field site, well-adapted 'wild' strains, with low nitrogen-fixing ability and/or high nodulation ability, may dominate the highly efficient, laboratory-tested inoculated strains. A reliable method of studying these phenomena, based on immunological differences between bacterial strains, has been presented (Mårtensson *et al.*, 1984). In this work, the technique has been further improved and applied to study competition between *Rhizobium* strains in forming root nodules, during growth in a fermenter, and during growth in soil serving as a carrier for a commercial inoculant. An investigation of double strain occupancy of nodules was also included.

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METHODS

Immunization. Immunization procedures for production of antibodies were as previously described (Mårtensson *et al.*, 1984), except that intact cells of *Rhizobium trifolii*, rather than *R. meliloti*, were used.

Antibody purification. Serum samples, stored at -70°C , were thawed for 2 h at -20°C and for 4 h at 5°C . The serum was then centrifuged (20000 *g*) for 25 min at 5°C and the supernatant diluted with 3 vols 0.02 M-sodium phosphate/0.02 M-NaCl buffer, pH 6.7. Then 50% (w/w) polyethylene glycol, mol. wt 6000, in distilled water, was added dropwise, with gentle stirring, to give a concentration of 15% (w/w). The solution was allowed to stand for 16 h at 5°C , and then centrifuged (3000 *g*) at room temperature for 20 min. The precipitate was dissolved in a volume of 0.02 M-potassium phosphate/0.02 M-KCl buffer, pH 6.7, equal to 2/3 of the original serum volume. The solution was then applied to a Cibachron Blue F3G-A-Sepharose CL-6B (Pharmacia) column ($V_i = 10$ ml) equilibrated with 0.02 M-potassium phosphate/0.02 M-KCl buffer, pH 6.7. The activities of the eluted fractions were investigated by immunoelectrophoresis. Fractions showing immunoprecipitate lines were combined and diluted with 6 vols 0.02 M-sodium phosphate/0.02 M-NaCl, pH 6.7, and chromatographed on a DEAE-Sephadex A-50 (Pharmacia) column ($V_i = 15$ ml) equilibrated with 0.02 M-sodium phosphate/0.02 M-NaCl, pH 6.7. Bound material was eluted with a linear gradient (0.02–0.2 M) of NaCl in equilibration buffer. The eluted fractions were investigated by immunoelectrophoresis. Finally, fractions containing active IgG were concentrated to a final concentration of 15 mg active IgG ml^{-1} by ultrafiltration using an Amicon ultrafilter cell equipped with a PM30 membrane, and divided into samples of 2.5 mg IgG, which were stored at -70°C prior to enzyme conjugation.

Immunoelectrophoresis. Immunoelectrophoresis of eluates was done by using gel slabs (80 $\times$ 80 mm) covered with 10 ml 1% Agarose B (Pharmacia) in 0.09 M-5,5-diethylbarbituric acid/0.18 M-Tris/5 mM-calcium lactate, pH 8.6, $I = 0.050$. The slabs were prepared by cutting two rows (3 mm apart) of seven holes each in the agar; each hole had a volume of 5 μl . Samples of antigen – the supernatant of a bacterial suspension (10^{10} cells ml^{-1}) heated at 60°C for 20 min and centrifuged (2000 *g*) for 15 min – were placed in the holes facing the anode and the eluates from the chromatography were placed opposite. Electrophoresis was performed at 300 V, 100 mA for 10 min, using a flat-bed electrophoresis apparatus with very efficient cooling (Pharmacia). Then the slabs were left in 0.85% NaCl for 2–4 h, stained in 0.2% Coomassie Blue R-250 in methanol/acetic acid/distilled water (3:1:6, by vol.) for 10 min, and destained in methanol/acetic acid/distilled water (3:1:6, by vol.) for some hours.

Preparation of enzyme-conjugates. The preparation of enzyme-conjugates using a hetero-bifunctional reagent, *N*-succinimidyl-3-(2-pyridyldithio)propionate (SPDP), was done as reported earlier (Mårtensson *et al.*, 1984). Three enzyme-conjugates, against *R. trifolii* strains 285, 828 and 7612, respectively, were prepared.

Preparation of ELISA samples. Test samples, normally consisting of bacterial cells, were centrifuged (2000 *g*) at room temperature for 10 min. The bacterial cells were then resuspended in 0.02 M-sodium phosphate/0.02 M-NaCl/2 mM-MgCl₂/0.1% Tween 20, pH 7.1. The washing procedure was repeated three times.

ELISA test procedure. Polystyrene test-tubes (vol. 4 ml) were washed once with 3.5 ml washing buffer consisting of 0.9% NaCl/0.02 M-sodium phosphate/0.25% Tween 20/2 mM-MgCl₂, pH 7.1. Test samples, normally 200 μl , were added to each test-tube. Then 100 μl enzyme-conjugate, diluted in buffer [0.9% NaCl/0.02 M-sodium phosphate/0.3% dextran T40 (Pharmacia)/0.5% Tween 20/2 mM-MgCl₂/0.2% human serum albumin (Sigma), pH 7.1] to give 2 μg IgG ml^{-1} , was added and incubated for 4 h, shaken at 225 r.p.m. at room temperature. Washing buffer (2 ml) was added, and the mixture was allowed to stand for 10 min, and then centrifuged (2000 *g*) for 10 min at room temperature, after which 1.7 ml was withdrawn. This procedure was repeated three times. Then 200 μl substrate, consisting of *o*-nitrophenyl β -D-galactopyranoside (Sigma, 3.5 mg ml^{-1} in 0.2 M-sodium phosphate/2 mM-MgCl₂/20%, v/v, methanol, pH 7.1) was added and the test-tubes were incubated for 1 h at room temperature. The enzyme reaction was stopped by adding 0.7 ml 0.7 M-Na₂CO₃. The test-tubes were finally centrifuged (2000 *g*) at room temperature for 10 min and the absorbance (A_{418}) of the supernatant was recorded by using a Beckman spectrophotometer equipped with a quartz micro-cuvette of 1 cm light path.

Bacterial strains. *R. trifolii* strains 285, 828 and 7612, obtained from the Legume Laboratory, Swedish University of Agricultural Sciences, were used; they were grown and maintained on BIII-medium (Bishop *et al.*, 1976).

Reproducibility of ELISA. Strains 285 and 7612 were grown on BIII-medium in shaken flasks, 28°C , to early stationary phase. The cells were harvested and routinely washed as described above, prior to ELISA. The cell concentration of each strain was adjusted to 10^4 cells ml^{-1} by adding 0.02 M-sodium phosphate/0.02 M-NaCl/2 mM-MgCl₂/0.1% Tween 20 buffer, pH 7.1, if necessary. The strains were mixed in the following proportions: 0:1, 1:1 and 1:0. The day-to-day reproducibility of ELISA was investigated by repeating the experiment on three subsequent days, each with new batches of bacteria.

ELISA of strain mixtures. The ELISA absorbance values were recorded using several combinations of mixtures of strains 828 and 7612, prepared as described above.

Double strain occupancy of nodules studied by ELISA. Four-to five-day-old sterile red clover (*Trifolium pratense*) seedlings, cv. Britta, planted on agar slopes in test-tubes (Vincent, 1970), were inoculated with mixtures of strains 828 and 7612. After 6 weeks' growth, each nodule was carefully cut from its root system, momentarily rinsed in 70% (v/v) ethanol followed by running tap water and macerated with a glass rod in 200 μl ELISA test-tube

Table 1. *Reproducibility of ELISA*

An enzyme-conjugate against *R. trifolii* 285, cell concentration 10^4 cells ml⁻¹, was used for three replicates of each sample. The control was cell washing buffer.

Strain mixture 285:7612	A_{418} (SD in parentheses)			<i>P</i> value*
	Expt 1	Expt 2	Expt 3	
1:0	0.793 (0.153)	0.714 (0.061)	0.772 (0.087)	> 0.05
1:1	0.558 (0.014)	0.527 (0.115)	0.605 (0.065)	> 0.05
0:1	0.248 (0.023)	0.232 (0.102)	0.248 (0.014)	> 0.05
Control	0.231 (0.004)	0.215 (0.010)	0.208 (0.017)	> 0.05

* For each strain mixture, the results of the three experiments were compared with each other by the ANOVA test (Wonnacott & Wonnacott, 1972). Since the *P*-values exceeded 0.05 in all cases, the observed differences in means can be attributed to chance fluctuations.

washing buffer. Each nodule suspension was divided into two test-tubes and tested separately with enzyme-conjugates against strains 828 and 7612.

Competition between rhizobial strains. (i) *In root nodules.* Four- to five-day-old sterile red clover seedlings, cv. Britta, planted as described above, were inoculated with mixtures of strains 285 and 7612. After 6 weeks' growth, the nodules were prepared for ELISA by washing and maceration as described above.

(ii) *During growth in a fermenter.* Suspensions of strains 285 and 7612 (200 ml, OD₅₄₀ = 0.1) were used to inoculate a 4-litre fermenter, containing 3.5 litres BIII-medium, operated at 28 °C with the pH automatically adjusted to 7.4 with 40% (w/v) KOH. The OD₅₄₀ of the cell suspension was recorded and cell samples were withdrawn at intervals for ELISA; all these samples were adjusted to the same cell density.

(iii) *In soil-based commercial inoculants.* Autoclaved inoculant tins filled with soil (wet weight 125 g, pH 7), intended for routine commercial production of *Rhizobium* inocula at the Legume Laboratory, Swedish University of Agricultural Sciences (Ljunggren & Mårtensson, 1984), were inoculated with mixtures of strains 285 and 7612. After 2 weeks' incubation at room temperature, 1 g (wet weight) soil was withdrawn and mixed with 10 ml 0.02 M-sodium phosphate/0.02 M-NaCl/2 mM-MgCl₂/0.1% Tween 20 buffer, pH 7.1, in a whirlimixer for 1 min. The soil suspension was then put aside for 10 min before being decanted and filtered through filter paper (Munktell no. 3). ELISA was performed on 200 µl of the filtrate.

RESULTS AND DISCUSSION

A drawback in studying bacterial strains by serological techniques is that the antibodies used may be highly cross-reactive, and thus may fail to distinguish between strains. However, serological methods have many advantages over other identification techniques. For instance, uncertainty about the stability of antibiotic resistance markers (Stein *et al.*, 1982), and fluctuations in plasmid composition (Beynon *et al.*, 1980) and in enzyme and protein patterns (Mytton *et al.*, 1978; Roberts *et al.*, 1980) make these other techniques questionable. The cross-reactivity, when it occurs, is probably caused by inadequate purification of the antibodies. In this study we evaluated a purification system, including gentle precipitation of the IgG by polyethylene glycol, followed by affinity chromatography to remove impurities such as ferritin. The active eluates, which were eluted after 1.5 *V*_t, were combined and placed on an anion-exchange column and the active IgG was then eluted at 0.12 M-NaCl by a NaCl gradient. This technique enabled us to obtain highly specific and reactive IgG. A further advantage is that the purification steps are easily followed by a developed immunoelectrophoresis method. The derived antibodies were then linked to β -galactosidase by a hetero-bifunctional reagent, thus avoiding enzyme-enzyme and/or antibody-antibody linkages.

In evaluating different methods, reproducibility is very important. The ELISA approach used was studied for reproducibility on three separate occasions, and the results showed that the reproducibility was good (Table 1). To assess the applicability of the ELISA to quantitative studies of unknown cell samples, we investigated whether an increase in the proportion of a strain within a mixture caused an increase in ELISA absorbance values. As shown in Fig. 1, the ELISA absorbance values of a mixture of two *R. trifolii* strains increased linearly with increases in the number of bacteria of the strain which reacted with the enzyme-conjugate used. We conclude that the ELISA method is a useful quantitative assay.

Fig. 1. ELISA of mixtures of *R. trifolii* strains 828 and 7612, using enzyme-conjugate against strain 7612. The bars indicate SD ($n = 4$).

Table 2. Tests by ELISA for the occurrence of double strain occupancy of red clover root nodules by *R. trifolii* strains

Nodule no.	A_{418} using:		Does the nodule show a significant ($P < 0.05$) relationship with either of the studied strains?			
	Enzyme-conjugate vs. 828	Enzyme-conjugate vs. 7612	Enzyme-conjugate vs. 828*		Enzyme-conjugate vs. 7612†	
			Strain 828	Strain 7612	Strain 828	Strain 7612
1	0.325	0.137	yes	no	yes	no
2	0.285	0.127	yes	no	yes	no
3	0.419	0.090	yes	no	yes	no
4	0.362	0.140	yes	no	yes	no
5	0.171	0.355	no	yes	no	yes
6	0.115	0.332	no	yes	no	yes
7	0.082	0.316	no	yes	no	yes
8	0.138	0.290	no	yes	no	yes

* A_{418} with strain 828: 0.358 (SD 0.0778, $n = 10$). A_{418} with strain 7612: 0.171 (SD 0.0438, $n = 10$).

† A_{418} with strain 828: 0.119 (SD 0.0378, $n = 10$). A_{418} with strain 7612: 0.366 (SD 0.0931, $n = 10$).

Table 3. Strain origin of red clover nodules inoculated with mixtures of *R. trifolii* strains, studied by ELISA

Each nodule was tested with enzyme-conjugate against strain 285 and against strain 7612, and classified as the respective strain at the 1% significance level.

Inoculum strain mixture 285:7612	No. of nodules of strain 285	No. of nodules of strain 7612	No. of nodules tested
100:0	7	0	7
90:10	3	4	8*
75:25	1	7	8
67:33	0	10	10
50:50	0	8	8
33:67	0	7	7
25:75	0	8	8
10:90	0	6	6
0:100	0	8	8

* One nodule could not be identified.

Fig. 2

Fig. 2. Numbers of bacterial cells in a mixed culture of *R. trifolii* strains 285 and 7612 during growth in a fermenter, determined by ELISA using enzyme-conjugate against the respective strains. The cell concentration of each sample for ELISA was adjusted to 10^4 cells ml^{-1} . The results are means of three replicates. ○, A_{418} representing the amount of strain 7612; ●, A_{418} representing the amount of strain 285; □, OD_{540} representing the total amount of cells of the mixed population, determined by direct spectrophotometric measurement of cell suspensions.

Fig. 3

Fig. 3. Occurrence of *R. trifolii* strains 285 and 7612 in a commercial soil inoculant that had been inoculated with different strain mixtures 2 weeks previously, determined by ELISA using enzyme-conjugate against strain 7612. The A_{418} with strain 7612 (0.604) was taken as 100%, and the A_{418} with strain 285 (0.286) as 0%. The results are means of three replicates.

We examined whether double strain occupancy of nodules occurred by dividing nodule suspensions into two parts, and testing these separately with enzyme-conjugates against each of the inoculated strains. The results in Table 2 show that double strain occupancy did not occur. Low frequencies of double infections were reported by Brockwell *et al.* (1976) using antigenic and streptomycin resistance markers for *Rhizobium* strains in clover, and by Diatloff & Brockwell (1976) using antigenic markers for strains in soyabeans. In contrast, Johnston & Beringer (1975), using genetic markers, reported that mixed strain occupancy of nodules could exceed 20% of the pea nodules that they investigated. The present investigation has the advantage that by using enzyme-conjugates against both of the inoculated strains to investigate single nodules, we were able to double check our results. The ELISA approach also avoids the time- and space-consuming procedure conventionally used to investigate double strain infection, which involves the cultivation of nodule suspensions on agar plates. On these plates, a strain which dominates in the nodule may be outcompeted by another when growing on agar, thus giving rise to incorrect conclusions.

Having concluded that double strain occupancy does not occur, we investigated competition for nodule sites by two strains of *R. trifolii* (Table 3). Strain 7612 successfully competed with strain 285 in nodulation: even if strain 285 formed 90% of the inoculation mixture, it was recovered in only three out of eight nodules. However, during growth in liquid medium in a fermenter, the strains responded in the opposite way, and strain 285 finally dominated (Fig. 2). The possibility that the derived ELISA values might differ during growth in the fermenter, due to changes in antigenic properties, was excluded by a control experiment in which strain 7612 was grown in the fermenter alone, and tested by ELISA occasionally during growth. It did not show any differences in ELISA absorbance values during growth (the cell density of the test samples was kept constant). This contrasts with results obtained by Hrabak *et al.* (1981), who studied the antigenic properties of the root-hair linking sites of the cell wall, but these are probably not the antigenic sites of the cell wall used by us. We also investigated strain competition in soil, using commercial inoculant tins (see Methods). The results in Fig. 3 show

that as in the nodulation tests, strain 7612 dominated over strain 285, even when strain 285 constituted 90% of the initial inoculation mixture. The use of strain mixtures in commercial inoculant production, as reported by Ljunggren & Mårtensson (1984), is therefore questionable, since the present investigation demonstrates that one strain may dominate the population.

In conclusion, by means of the ELISA approach we were able to demonstrate the occurrence of competition between strains of *R. trifolii*. Strain 7612 was a successful competitor for nodulation and for growth in soil, whereas in a liquid medium strain 285 dominated. The underlying reasons for these differences in competitive ability remain to be answered.

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REFERENCES

- BARBER, L. E. (1980). Enumeration, effectiveness and pH resistance of *Rhizobium meliloti* populations in Oregon soils. *Soil Science Society of America Journal* **44**, 537–539.
- BARTHEL, C. & RHODIN, S. (1914). Experiments with cultures of leguminous bacteria for blue lupin and blue lucerne. (In Swedish) Meddelande no. 95, Centralanstalten för försöksväsendet på jordbruksområdet, Bakteriologiska laboratoriet no. 10. Stockholm: Haggström.
- BISHOP, P. E., GUEVARA, J. G., ENGELKE, J. A. & EVANS, H. J. (1976). Relation between glutamine synthetase and nitrogenase activities in the symbiotic association between *Rhizobium japonicum* and *Glycine max*. *Plant Physiology* **57**, 542–546.
- BEYNON, J. L., BERINGER, J. E. & JOHNSTON, A. W. B. (1980). Plasmids and host-range in *Rhizobium leguminosarum* and *Rhizobium phaseoli*. *Journal of General Microbiology* **120**, 421–429.
- BROCKWELL, J., SCHWINGHAMER, E. A. & GAULT, R. R. (1976). Studies of root nodule bacteria introduced into field environments. V. A critical examination of the stability of antigenic and streptomycin-resistance markers for identification of strains of *Rhizobium trifolii*. *Soil Biology and Biochemistry* **9**, 19–24.
- BUSHBY, H. V. A. & MARSHALL, K. C. (1977). Some factors affecting the survival of root-nodule bacteria on desiccation. *Soil Biology and Biochemistry* **9**, 143–147.
- DIATLOFF, A. & BROCKWELL, J. (1976). Ecological studies of root-nodule bacteria introduced into field environments. 4. Symbiotic properties of *Rhizobium japonicum* and competitive success in nodulation of two *Glycine max* cultivars by effective and ineffective strains. *Australian Journal of Agriculture and Animal Husbandry* **16**, 514–521.
- GAWORZEWSKA, E. T. & CARLILE, M. J. (1982). Positive chemotaxis of *Rhizobium leguminosarum* and other bacteria towards root exudates from legumes and other plants. *Journal of General Microbiology* **128**, 1179–1188.
- HOLDING, A. J. & KING, J. (1963). The effectiveness of indigenous populations of *Rhizobium trifolii* in relation to soil factors. *Plant and Soil* **18**, 191–198.
- HRABAK, E. M., URBANO, M. R. & DAZZO, F. D. (1981). Growth-phase-dependent immunodeterminants of *Rhizobium trifolii* which bind trifolii A, a white clover lectin. *Journal of Bacteriology* **148**, 697–711.
- JOHNSTON, A. W. B. & BERINGER, J. E. (1975). Identification of *Rhizobium* strains in pea root nodules using genetic markers. *Journal of General Microbiology* **87**, 343–350.
- LJUNGGREN, H. & MÅRTENSSON, A. M. (1984). Legume-inoculant production in Sweden. *Swedish Journal of Agricultural Research* **14**, 177–181.
- MYTTON, L. R., MCADAM, N. J. & PORTLOCK, P. (1978). Enzyme polymorphism as an aid to identification of *Rhizobium* strains. *Soil Biology and Biochemistry* **10**, 79–80.
- MÅRTENSSON, A. M., GUSTAFSSON, J.-G. & LJUNGGREN, H. D. (1984). A modified, highly sensitive enzyme-linked immunosorbent assay (ELISA) for *Rhizobium meliloti* strain identification. *Journal of General Microbiology* **130**, 247–253.
- NUTMAN, P. S. (1976). IBP field experiments on nitrogen fixation by nodulated legumes. In *Symbiotic Nitrogen Fixation in Plants (IBP Handbook no. 7)*, pp. 211–237. Edited by P. S. Nutman. Cambridge: Cambridge University Press.
- RAMIREZ, C. & ALEXANDER, M. (1980). Evidence suggesting protozoan predation on *Rhizobium* associated with germinating seeds and in the rhizosphere of beans (*Phaseolus vulgaris* L.). *Applied and Environmental Microbiology* **40**, 492–499.
- ROBERTS, G. P., LEPS, W. T., SILVER, L. E. & BRILL, W. J. (1980). Use of two-dimensional polyacrylamide gel electrophoresis to identify and classify *Rhizobium* strains. *Applied and Environmental Microbiology* **39**, 412–422.
- STEIN, M., BROMFIELD, E. S. P. & DYE, M. (1982). An assessment of a method based on intrinsic antibiotic resistance for identifying *Rhizobium* strains. *Annals of Applied Biology* **101**, 261–267.
- VINCENT, J. M. (1970). *A Manual for the Practical Study of the Root Nodule Bacteria (IBP Handbook no. 15)*. Oxford: Blackwell Scientific Publications.
- WONNACOTT, T. H. & WONNACOTT, R. J. (1972). *Introductory Statistics*. New York: John Wiley.