Competition in Soil between Effective and Ineffective Strains of *Rhizobium leguminosarum* biovar *trifolii* in the Nodulation of Red Clover, *Trifolium pratense* L., Studied with ELISA

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The ability of two strains of *Rhizobium leguminosarum* biovar *trifolii* (formerly *R. trifolii*) to compete for nodule sites in soil with indigenous *R. l.* biovar *trifolii* was studied using ELISA. In pot experiments with soil containing effective indigenous strains, one of the inoculated strains, 285, was able to form nodules on red clover (*Trifolium pratense* L.), not only on the tap root but also on later-formed roots. Under field conditions, also in a soil containing effective indigenous *R. l.* biovar *trifolii* strains, both inoculated strains were detectable in minor proportions in the nodules of the established red clover. The inoculated strains survived in the soil during the field season. As the indigenous strains were effective, no increase in dry matter production was obtained in this experiment. However, in pot experiments in a soil with ineffective indigenous red clover bacteria, the other inoculated strain, 7612, known to be an effective nitrogen-fixing strain, was less successful and was only detectable incidentally. Although the nodule occupancy of the successful competitor strain, 285, only ranged between 7% and 27% on the upper tap root and was merely occasional on the other parts of the root in the 'ineffective' soil, increases in dry matter production were obtained, indicating that the nodules on a specific plant have varying significance for the apparent nitrogen fixation.

INTRODUCTION

The successful establishment of legumes in fields which contain indigenous rhizobial strains from the correct 'cross-inoculation' group will depend upon the effectiveness, i.e. the nitrogen-fixing ability and several other characteristics, of these strains (Dowling & Broughton, 1986). To prevent unwanted nodulation by the native ineffective strains, seeds are frequently inoculated (Stacey & Upchurch, 1984). The inoculants consist of selected strains with the ability to induce high nitrogen fixation; characteristics such as growth rate, survival capacity and genetic stability are also selection criteria for such strains (Vincent, 1970). In many cases, the use of an inoculant increases the crop yield considerably (Nutman, 1976). Sometimes, however, the beneficial effects fail to occur (Roughley *et al.*, 1976; Boonkerd *et al.*, 1978). One reason for this may be that the indigenous strains are highly infective and have a high nitrogen-fixing ability. Another reason may be that the indigenous strains are highly infective but have low nitrogen-fixing ability. The former situation would result in a high crop yield, the introduced and indigenous strains being comparable in nitrogen-fixing capacity; the latter would result in a low yield, the competitive indigenous strains with a low nitrogen-fixing ability forming poor nodules. These explanations are merely hypothetical and deserve further investigation. However, it is possible that the characteristics which have been considered when selecting the inoculant strains are inappropriate (Brockwell *et al.*, 1968). For instance, strains which express a

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high nitrogen-fixing ability as shown by a high dry matter yield of plants grown axenically may not be the most suitable under field conditions. Highly effective strains of *R. leguminosarum* can vary considerably in their degree of competitiveness (May & Bohlool, 1983). In test programmes for strain selection, a number of parameters have been recognized, one of which is competitive ability with other strains for infection sites (Brockwell *et al.*, 1968). Competition under field conditions, however, is difficult to study.

In Sweden, the lack of positive effects on crop yields by inoculation has been noted on several occasions. In order to investigate the influence of the indigenous strains in such cases, we have used an ELISA technique (Mårtensson *et al.*, 1984) for identifying the inoculated strains. We now report how the inoculated strains, in competition with each other, were able to nodulate red clover in soils containing either effective indigenous strains or ineffective indigenous strains.

METHODS

Immunization. Antibodies were produced by immunization of rabbits as previously described (Mårtensson *et al.*, 1984), except that intact cells of *Rhizobium leguminosarum* biovar *trifolii* (formerly *R. trifolii*), rather than *R. meliloti*, were used.

Antibody purification. The antibodies were purified as earlier reported (Mårtensson & Gustafsson, 1985).

Enzyme conjugation. Enzyme-conjugates were prepared in accordance with our previous report (Mårtensson *et al.*, 1984). Two enzyme-conjugates, against *R. l.* biovar *trifolii* strains 285 and 7612, were made.

Preparation of ELISA samples. The nodules to be investigated were surface-sterilized in 70% (v/v) ethanol for 15 s, followed by rinsing in sterile distilled water. The nodules were crushed, one by one, in 200 µl 0.02 M-sodium phosphate/0.02 M-NaCl/2 mM-MgCl₂/0.1% Tween 20, pH 7.1. The suspension of 200 µl from each nodule was put into a polystyrene test tube (vol. 4 ml) plugged and stored at -70 °C prior to analysis.

ELISA test procedure. The ELISA tests were run as previously reported (Mårtensson *et al.*, 1984).

Competition for nodulation in soil with ineffective indigenous strains of *R. l.* biovar *trifolii*. Plastic pots were filled with 1.25 kg soil containing approx. 4×10^3 indigenous *R. l.* biovar *trifolii* (g dry wt of soil)⁻¹. The original number of bacteria was determined by the plant infection method (Vincent, 1970). On the soil surface of each pot five groups of two seeds of red clover (*Trifolium pratense* L.) were placed. The intended inoculated replicates were given a dose of 1 ml of a suspension (5×10^4 cells ml⁻¹) of strain 285 or 7612, or of a mixture of the two, per group of seeds. Uninoculated replicates received 1 ml sterile tap water per group of seeds. Finally, a thin layer (0.5–1 cm) of sterile sand was spread on the soil surface. The pots were placed on benches outside, thinned to give five plants per pot after 2 weeks and watered with 1/10-strength sterile Jensen's medium (Vincent, 1970) when necessary. After harvest, at 2.5 months of age, above-ground material was collected, dried and weighed. Roots were washed free from soil, and nodules were counted and picked from each root separately and prepared for ELISA analysis as described above. The position on the root was noted for each nodule.

Competition for nodulation in soil with effective indigenous strains of *R. l.* biovar *trifolii*. Plastic pots were filled with 1.25 kg soil containing approx. 5×10^5 indigenous bacteria (g dry wt of soil)⁻¹. Seeds were sown and inoculated as described above. The pots were kept in a greenhouse and treated as described above. Nodule positions and dry weights of plants were noted as before. The nodules were analysed by ELISA.

In a second experiment, under field conditions, a field with effective *R. l.* biovar *trifolii* strains was chosen. Inoculations with bacteria were carried out immediately after sowing a red clover/grass mixture on 13 May 1986, by sprinkling 50 litres of bacterial suspension (8×10^6 cells ml⁻¹) on to 1.0 m² square plots. Four plots were used for each inoculation. The plots were randomly distributed with at least 5 m between each plot in order to avoid bacterial cross-contamination. In October, red clover plants with intact root systems were collected and the nodules were analysed by ELISA (the position of the nodules was again noted). The number of free-living *R. l.* biovar *trifolii* in the soil was counted using the plant infection method (Vincent, 1970) at sowing and at sampling in October. Nodules on plants from this test were analysed by ELISA to determine the proportion of the inoculated strains in the total clover rhizobial population. In addition, nodules from field-grown red clover plants were investigated after plant establishment in October.

RESULTS

To determine if strain occupancy of nodules differed with respect to nodule age, the nodules from the pot experiments were divided into groups, representing nodules of decreasing age. Initially, five distinct nodule zones were defined. However, this was found to be too high a level of differentiation with respect to the differences in nodule occupancy of the different strains. It was therefore decided that three zones were sufficient; these are shown in Fig. 1.

Fig. 1. The division of root nodules into zones.

Table 1. Dry weights and strain origin of nodules of red clover inoculated with mixtures of *R. l. biovar trifolii* strains in soil with ineffective indigenous populations of *R. l. biovar trifolii*

Strain mixture 285:7612	Dry weight of shoots (g per plant, SD in parenthesis)*	P value†	Percentage of nodules containing strain 285‡		
			Root zone:		
			1	2	3
0:0	0.57 (0.06)	—	0	0	0
0:100	0.56 (0.10)	NS	0	0	0
25:75	0.65 (0.13)	NS	10	0	0
50:50	0.83 (0.11)	0.05	16	0	0
75:25	0.62 (0.09)	NS	15	0	30
100:0	0.79 (0.15)	0.05	27	7	3

* Mean values, $n = 30$.

† Observed differences in means of inoculated plants were compared with the mean of the uninoculated control. NS, Not significant.

‡ The average nodule number per plant was 59; nodules from one plant per pot were examined. The number of pots per treatment was 5. Strain 7612 was not detectable in any of the nodules in any of the treatments.

Competition for nodulation in soil with ineffective indigenous strains of R. l. biovar trifolii. Inoculation with strain 285 increased the plant dry matter yields; higher proportions of the strain caused a correspondingly higher yield (Table 1). Inoculation with strain 7612 did not

Table 2. *Dry weights and strain origin of nodules of red clover inoculated with mixtures of R. l. biovar trifolii strains in soil with effective indigenous populations of R. l. biovar trifolii*

Strain mixture 285:7612	Dry weight of shoots (g per plant, SD in parenthesis)*	P value†	Percentage of nodules containing inoculant strain‡					
			285			7612		
			Root zone:			Root zone:		
			1	2	3	1	2	3
0:0	1.05 (0.07)	—	0	0	0	0	0	0
0:100	1.14 (0.07)	0.10	0	0	0	0	0	0
25:75	ND		33	0	0	0	19	0
50:50	1.06 (0.10)	NS	38	15	0	0	0	0
75:25	1.15 (0.09)	NS	79	29	0	0	0	0
100:0	1.06 (0.11)	NS	71	24	0	0	0	0

ND, Not determined.

* Mean values, $n = 30$.

† Observed differences in means of inoculated plants were compared with the mean of the uninoculated control.

‡ The average nodule number per plant was 59; nodules from one plant per pot were examined. The number of pots per treatment was 5.

Table 3. *Effect of inoculation of red clover under field conditions with R. l. biovar trifolii strains 285 and 7612*

	13 May 1986 (inoculation date)	19 October 1986 (harvest date)
$10^{-6} \times$ No. of <i>R. l. biovar trifolii</i> (g dry wt of soil) ⁻¹ :		
Uninoculated	0.66	0.68
Strain 285	13.52	1.01
Strain 7612	14.11	1.20
Inoculated strain as percentage of total no. of <i>R. l. biovar trifolii</i> in soil:		
Plant infection method		
Strain 285	95	33
Strain 7612	95	43
ELISA method		
Strain 285	65	21
Strain 7612	68	38
Strain origin of nodules (%)*:		
Strain 285	—	12
Strain 7612	—	26
Unidentified	—	62
Shoot dry weight (g per plant):		
Uninoculated	—	1.21
Strain 285	—	1.27
Strain 7612	—	1.19

* Ten plants, and an average of 18 nodules per plant, were examined.

cause any increase in dry matter production. Strain 285 was detectable in nodules in all treatments where it was included in the inoculation mixture (Table 1). Strain 7612, however, was not detectable in any of the treatments.

Competition for nodulation in soil with effective indigenous strains of R. l. biovar trifolii. The soil chosen for the greenhouse experiment contained highly effective bacteria, as was evident in the dry matter yields obtained with uninoculated replicates (Table 2). The dry matter yields of nodulated plants from the pots inoculated with strains 285 and 7612, respectively, also indicate that the indigenous strains were just as effective as the introduced strains. Table 2 also shows the ability of inoculated strains to survive and nodulate red clover roots in soil. Strain 285 seemed to be better suited to the prevailing soil conditions, as it was still detectable, to some extent, in

nodules from zone 2. Strain 7612, however, seemed to be less adaptable to the soil conditions, as it was recovered in just one treatment (Table 2).

The total numbers of inoculated strains which were free-living under field conditions, after inoculation and five months later, are presented in Table 3. The dry matter yields were similar in inoculated and control plots, indicating that the indigenous strains were effective. Two methods were used to estimate the proportions of the inoculated *R. l. biovar trifolii* with respect to the total population of free-living bacteria. In the plant infection method, the total number of *R. l. biovar trifolii* in each treatment was determined, and the proportions of each strain were calculated, assuming that the increase in number of *Rhizobium* in the inoculated plots was caused by the inoculated bacteria. In the ELISA method, nodules on the plants used for the plant infection method were analysed by ELISA. The results are presented in Table 3.

Nodulation by the added bacteria in the field was also determined, by ELISA of bacteria in nodules from established red clover plants (Table 3).

DISCUSSION

The results show how difficult it is to predict which strain to use as an inoculant. If a choice between 285 and 7612 for use as an inoculant was strictly based on the criterion of increase in dry matter production, one might have chosen 7612, as it caused a greater dry matter increase than 285 when tested in a soil with effective indigenous *R. l. biovar trifolii*. This increase was, however, not significant (Table 2). The results obtained when inoculating plants grown in soil with ineffective indigenous *R. l. biovar trifolii* indicate that 7612 is a poor competitor. This is not only shown by the lack of increase in dry matter production (Tables 1 and 3), it is also confirmed by the poor recovery of the strain in nodules, where it occurred only incidentally. The present results contrast with those obtained in our previous study, where strain 7612 was shown to be a successful colonizer in sterile soil compared to 285 (Mårtensson & Gustafsson, 1985). In that study, strain 7612 was also shown to dominate in nodules of plants inoculated with mixtures of the two strains. In a competition experiment done by McLoughlin & Dunican (1985) similar results, i.e. strains showing high competitiveness in test-tube experiments proving less successful in soil, were obtained. It can therefore be concluded that testing inoculants by infecting sterile plants under aseptic conditions must be followed by investigations under field conditions. In a study comparing root colonization and nodulation efficiency, Moawad *et al.* (1984) obtained results indicating that the ability to colonize the rhizosphere readily was an insufficient criterion for selecting strains, since a particular serogroup, which was not superior in root colonization, formed numerous nodules.

Another interesting observation from our experiments is that establishment of a successful symbiosis does not necessarily require all the occurring nodules to be derived from the added inoculum of effective bacteria (Tables 2 and 3). An increase in dry matter production of the inoculated plants was still obtained in soil with an effective indigenous flora, even though the inoculated strains occupied only 7–27% of the nodules formed in root zones 1 and 2. In later-formed nodules, the inoculated strains only occurred occasionally. However, one should also note the tendency that increasing the inoculum of the successful inoculant, 285, gave higher dry matter production (Table 1). Studying nodule occupancy and efficiency of inoculants used for *Trifolium* spp., *Medicago* spp., *Glycine max* and *Lotus pedunculatus* in South Africa, Van Rensburg & Strijdom (1985) showed that the nodule occupancy of inoculants varied between 18 and 100% and that the effectiveness was 7–73% higher than that of unidentified bacterial isolates, which is in agreement with our observations. In that report, however, no account was taken of the position of the nodules investigated. Our data, indicating just some nodules contributing to an increase in nitrogen fixation, might be explained if one considers the physiological functions of the plant. Nodules situated near the tap root, as the first ones formed are, are supposed to be favoured in the supply of photosynthates from the shoot, and, as nitrogen fixation is relatively energy demanding, this may result in more active nitrogen-fixing nodules than those formed later. It could therefore still be worth inoculating legumes even if the inoculated strains are poor survivors in the soil, the important characteristic being ability to

initiate the first-formed nodules. Another factor to be kept in mind is that it is not known in systems with perennial legumes, like the ones we studied, if the nodules formed during the seedling state of the plant survive during the winter period, and if they resume their activity in the next season or if there is a nodule succession. If the former is the case, the longevity and maintained infectivity and nitrogen-fixation ability of the inoculated strains will be very important factors.

Many other factors at the field site may influence the success of inoculation. In a study in France, 100% of the nodules formed were derived from the introduced strain at one field site with a low pH, whereas only 11% of the investigated nodules were derived from that strain in another soil type, when initially inoculated with the same inoculant level, 5×10^7 bacteria per seed (Arsac & Cleyet-Marel, 1986). More knowledge of the underlying reasons for the success or failure of the inoculants in different environments is essential. By obtaining more data on the major influencing environmental factors, it should become possible to design highly competitive and effective inoculants by gene manipulation.

In conclusion, competitive ability may vary between strains which show equal nitrogen-fixation abilities in aseptic conditions. New methods for selection of inoculant strains must be used and tested in order to obtain reliable inoculants.

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