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Studies on the Inoculation and Competitiveness of a *Rhizobium leguminosarum* Strain in Soils Containing Indigenous Rhizobia

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The competitiveness of a *Rhizobium leguminosarum* strain was investigated at two separate locations in field inoculation studies on commercially grown peas. The soil at each location (sites I and II) contained an indigenous *R. leguminosarum* population of ca. 3×10^4 rhizobia per g of soil. At site I it was necessary to use an inoculum concentration as large as 4×10^7 CFU ml^{-1} (2×10^6 bacteria $seed^{-1}$) to establish the inoculum strain in the majority of nodules (73%). However, at site II the inoculum strain formed only 33% of nodules when applied at this (10^7 CFU ml^{-1}) level. Establishment could not be further improved by increasing the inoculum concentration even as high as 10^9 CFU ml^{-1} (9.6×10^7 bacteria $seed^{-1}$). The inoculum strain could be detected at both sites 19 months after inoculation. Analysis by intrinsic antibiotic resistance patterns and plasmid DNA profiles indicated that a dominant strain(s) and plasmid pool existed among the indigenous population at site II. Competition experiments were carried out under laboratory conditions between a dominant indigenous isolate and the inoculum strain. Both strains were shown to be equally competitive.

Nitrogen fixation by the legume-*Rhizobium* symbiotic partnership represents an inexpensive alternative to the use of chemical nitrogen fertilizers in the production of food protein and oil. The process requires that the host crop be adequately nodulated by *Rhizobium* bacteria effective in nitrogen fixation. Inoculation of grain and forage legumes with suitable rhizobial strains is carried out in many countries to ensure effective nodulation (40). Inoculation can improve crop yields in cases where (i) appropriate rhizobia are not present in the soil or (ii) the soil contains a significant proportion of nonnodulating or ineffective nitrogen-fixing strains (35). The successful use of rhizobial inoculants in the latter situation requires knowledge of the indigenous rhizobial population in terms of numbers, effectiveness, and competitive ability (18, 36, 37). Symbiotic effectiveness can be very variable within a given area (14), and in this situation, inoculation serves to standardize nitrogen fixation rates and thus enhance crop yields. The numbers of rhizobia present in the soil before inoculation will influence the concentration of inoculant required. A study of soybean inoculation indicated that an inoculation rate 1,000 times the size of the indigenous population was needed before inoculant rhizobia became successfully established (41). One reason for such a situation presumably was the competitiveness of the indigenous population (10, 19, 24, 41). Soil rhizobia, including ineffective strains, may be highly competitive for nodule formation, partly because they have adapted to local environmental conditions (23, 42). Thorough testing of inoculant strains, especially in field conditions, is essential to consistently benefit from inoculation.

In this study, the inoculation and competitiveness of a *Rhizobium leguminosarum* strain was investigated in soils which contained indigenous rhizobia.

MATERIALS AND METHODS

Strain. *R. leguminosarum* C53 was obtained originally from D. Phillips, University of California, Davis, Calif. A spontaneous mutant resistant to 100 μg of streptomycin per ml (CL1 Str^r) was the inoculum strain used in this study.

Growth conditions. Cultures were grown at 30°C on mannitol-salts-yeast extract medium (MSY), described previously (32). Cultures were stored on MSY agar slants at 4°C or as liquid suspensions containing glycerol (40%) at −20°C. Cultures for field inoculation were grown aerobically in an industrial fermentor (200 liters). The medium contained mannitol (0.5%), yeast extract (0.1%, industrial grade), and MSY basal salts. The pH of all media was adjusted to 6.8.

Field experiments. Field inoculations of commercially grown peas were carried out at two locations. Site I in Tallow, County Waterford, was identified as soil association 4 on the General Soil Map of Ireland (13). The soil in this area is a podzol type. The soil pH at this site was 6.5. Site II, in Killeagh, Co. Cork, was identified as soil association 7, (13); The soil at site II is an Acid Brown Earth. The soil pH was 6.8 to 7.0. Soil fertilizer was not applied.

Areas of ca. 1,100 m^2 were inoculated with *R. leguminosarum* CL1 (501) by using a liquid inoculation system. A manifold attached to a seed drill allowed simultaneous implantation of captan-treated seed (*Pisum sativum*; 12 seeds m row^{-1}) and liquid inoculum in the soil. At site I, two areas were inoculated at a concentration of 4×10^8 rhizobia ml^{-1} (2.3×10^7 rhizobia $seed^{-1}$; 2.73×10^7 cm row^{-1}) and one area each with 4×10^7 and 1×10^6 rhizobia ml^{-1}. Areas of ca. 1,100 m^2 were left uninoculated between trial areas. At site II, trial areas were inoculated with 1.7×10^9 (9.6×10^7 per seed; 1.1×10^8 cm row^{-1}), 2.3×10^8, 5×10^7, and 2.2×10^6 rhizobia ml^{-1} and separated by uninoculated areas of ca. 550 m^2. Inoculation dates were 16 April 1982 (site II) and 3 May 1982 (site I). To determine strain establishment, nodules were picked from 15 to 20 plants at two selected points within each inoculated area. These were randomly chosen within each inoculated area to ensure that representative sampling was being achieved.

Identification of inoculant strain. At least 50 nodules from each inoculated sampling point and a total of 200 nodules from uninoculated areas in each field were processed for strain identification. Nodules were washed and surface sterilized by exposure to 95% ethanol for 3 min and acidified $HgCl_2$ (0.1%) for 3 min. The nodules were then washed in six changes of sterile distilled water, crushed, and streaked on

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MSY agar. After incubation at 30°C, colonies were streaked on MSY agar containing streptomycin (100 μg ml^{-1}) and incubated at 30°C. Establishment of the inoculum strain was then estimated as the percentage of isolates which were resistant to streptomycin.

Persistence of inoculated rhizobia. Soil samples from each site were collected 19 months after inoculation (12 December 1983). Peas were cultivated in pots (500 ml) containing test soil. Individual pots contained three seeds and eight replicates for each soil type that was used. Seeds were germinated in the dark in a growth room and then transferred to a glass house which was maintained at 22°C. Nodules were collected from mature plants. The percentage of streptomycin-resistant *R. leguminosarum* nodule isolates was determined for each soil as described above.

Enumeration of rhizobia. The indigenous population of *R. leguminosarum* in soil from each site was estimated by the plant infection method (7). Pea seedlings inoculated with soil dilutions were cultivated in pots of sand and perlite mixture. Numbers of rhizobia in field inocula were determined by plate count on MSY agar containing streptomycin (100 μg ml^{-1}).

Antibiotic sensitivity testing. Nodule isolates were typed by their intrinsic resistance to low levels of antibiotics as described by Josey et al. (29). To ensure that suspensions containing uniform numbers of active cells were used, bacteria were inoculated into MSY broth from frozen stocks and grown to late log phase. Dilutions were added to the wells of a multi-inoculator so that each loop transferred either 5×10^3 or 5×10^4 bacteria. Antibiotic plates were incubated at 30°C for 3 days, and growth was compared with growth on MSY control plates.

Plasmid screening. Plasmid DNA was isolated by the method of Eckhardt (12) with cultures grown in TY medium (4). Prewashing of cells with sodium dodecyl sulfate (0.1%) was carried out before loading into wells. *R. leguminosarum* CL1 was used as a standard.

Competition experiment. Competition studies between the inoculum strain, CL1, and an indigenous isolate, GC65 Sp^r (marked by selecting a spontaneous spectinomycin-resistant mutant), were carried out based on the procedure outlined by Amarger and Lobreau for *R. meliloti* strains (1). Cell concentrations of ca. 5×10^7 and 5×10^8 CFU ml^{-1} were mixed in different ratios and applied to seeds. Exact inoculum numbers were determined by the plate count method (see Table 3). Seeds of *P. sativum* were surface sterilized and germinated in pots (two seeds per pot) containing an autoclaved mixture of sand and perlite (ratio, 1:1 by weight). The plant medium composition was as follows (grams $liter^{-1}$): KCl, 0.125; KH_2PO_4, 0.05; $MgSO_4 \cdot 7H_2O$, 0.05; $CaSO_4 \cdot 2H_2O$, 0.05; and trace elements as for MSY (pH 7.0). Cell suspensions (2 ml, 10^8 CFU ml^{-1}) were added to pots around the seedlings. There were 12 replicate pots for each treatment. Pots were placed under artificial light (14 h of illumination, 10 h of darkness) and watered with sterile plant medium. After 7 weeks, nodule isolates were identified by testing for growth on MSY agar containing streptomycin (100 μg ml^{-1}) or spectinomycin (100 μg ml^{-1}) coupled with plasmid DNA analysis. Detached nodules from all the plants in each treatment were pooled, and 50 randomly chosen nodules were analyzed for occupancy.

RESULTS

Inoculum establishment in pea nodules. The competitiveness of an inoculum strain (*R. leguminosarum* CL1) was assessed in soils containing indigenous populations of *R. leguminosarum*. Inoculation was carried out on field-grown peas at two sites in a commercial growing region. The soil at each site contained significant numbers of *R. leguminosarum* (3.6×10^4 rhizobia g^{-1}) as determined by the plant infection technique. The object of these experiments was to determine the minimum concentration of inoculum required for strain CL1 to become established in the majority of nodules in the presence of the indigenous rhizobia. At site I, the minimum inoculum concentration required for successful establishment (73%) of the inoculum strain was 10^7 CFU ml^{-1} (Table 1). Reducing the inoculum concentration by 10-fold resulted in poor establishment. In contrast, at site II, the inoculum strain did not form the majority of nodules even when the inoculum level was increased by ca. 100-fold (10^9 CFU ml^{-1}). The ability of the inoculum strain to persist in both soils was investigated after the pea crop was harvested. *R. leguminosarum* CL1 was detected at both sites after a period of 19 months. At site I, CL1 formed a significant proportion of the nodules; in contrast, at site II, the presence of CL1 in nodules was considerably lower. The failure of the inoculum strain to become established at site II may have been due to soil environmental factors or to the nature of the indigenous microbial population.

Identification of dominant indigenous strains. The failure of the introduced strain to establish as the major nodulating strain at site II may have been due to the presence in the soil of a competitive *Rhizobium* strain which was dominant among the indigenous population. To investigate this possibility, the native strains were characterized by using intrinsic antibiotic resistance patterns and plasmid profile analysis. When 62 nodule isolates from uninoculated control areas at site II were screened for intrinsic antibiotic resistance patterns, 12 distinct patterns were detected (Table 2). When isolates were grouped on the basis of similar intrinsic antibiotic resistance patterns, it was found that almost half of the total number tested (31) belonged to groups I and II. These groups differed from one another by only one antibiotic resistance (neomycin; 1.25 mg $liter^{-1}$). Similarly, groups III and IV differed by only one resistance compared with group I. Resistance patterns varied by one or two resistances in repeat experiments carried out under standardized conditions. A reference strain was used in all experiments of this nature. Reports of similar variation in resistance patterns have also been previously highlighted (29, 39).

When 52 of the isolates were also compared on the basis of their plasmid profiles, three different plasmid profiles were

TABLE 1. Establishment and persistence of *R. leguminosarum* CL1 in two separate field experiments

Site of inoculation	Inoculum level (CFU ml^{-1})[a]	% Recovery of introduced strain	
		After inoculation	19 mo later
Site I	4.0×10^8	76.2 ± 9.9	43 ± 11.0
	4.0×10^7	73.2 ± 10.4	—[b]
	1.0×10^6	22.0 ± 8.3	—
	0	0	—
Site II	1.7×10^9	31.0 ± 8.3	10.2 ± 3.2
	2.3×10^8	23 ± 2.8	—
	5.0×10^7	33 ± 12.0	—
	2.2×10^6	26.4 ± 12.6	—
	0	0	—

[a] The concentration of cells in inocula and inoculum recovery rates were determined as described in the text.
[b] —, Recovery rate not determined.

TABLE 2. Antibiotic resistance patterns of indigenous isolates of *R. leguminosarum*

Group	No. of isolates	Response to the following antibiotic (concn in mg liter^{-1})[a]:													
		Kan		Rif		Nal		Neo		Str		Pol		Ery	
		4	10	1	2	5	10	1.25	2.50	2.5	10	5	10	2.5	5.0
I	15	−	−	−	−	−	−	+	−	−	−	−	−	+	−
II	15	−	−	−	−	−	−	−	−	−	−	−	−	+	−
III	7	−	−	+	−	−	−	+	−	−	−	−	−	+	−
IV	5	−	−	−	−	−	−	+	−	−	−	−	−	−	−
V	8	−	−	−	−	−	−	−	−	−	−	−	−	−	−
VI	5	−	−	+	−	−	−	−	−	−	−	−	−	+	−
VII	2	−	−	+	−	+	−	+	−	−	−	−	−	+	−
VIII	1	−	−	−	−	+	−	+	−	−	−	−	−	+	−
IX	1	+	−	−	−	−	−	+	−	−	−	−	−	+	−
X	1	−	−	+	−	−	−	+	−	−	−	−	−	−	−
XI	1	−	−	−	−	+	−	−	−	−	−	−	−	−	−
XII	1	−	−	−	−	−	−	+	−	+	+	+	−	−	−

[a] Intrinsic antibiotic resistance patterns were determined as outlined in the text. Antibiotics: kanamycin (Kan), rifampicin (Rif), nalidixic acid (Nal), neomycin (Neo), streptomycin (Str), polymyxin (Pol), and erythromycin (Ery). + indicates growth (resistance); − indicates no growth (sensitivity).

detected (Fig. 1). One plasmid band seemed to be common to all the isolates tested. This was a large plasmid similar in size to the large plasmid of *R. leguminosarum* CL1 (230 megadaltons). The other plasmids detected were smaller in size. Plasmid profile C (Fig. 1), which had three plasmid bands, was the most common profile among the isolates. It was detected in 32 of the 52 isolates. Profile D (two plasmid bands) was found in 18 isolates, and profile B (three plasmid bands) was detected in two isolates. A comparison of intrinsic antibiotic resistance patterns and plasmid profiles indicated that plasmid profile C was present in 66% of the isolates that composed antibiotic resistance groups I and II. Collectively, these results indicated that the majority of nodules were occupied by isolates of similar characteristics.

Competitiveness of a dominant indigenous isolate. An experiment was carried out to assess the competitive ability of the dominant indigenous isolate *R. leguminosarum* GC65 compared with the inoculum strain, *R. leguminosarum* CL1. Strain GC65 belonged to antibiotic resistance group I and had the dominant plasmid profile (profile C). A spectinomycin-resistant derivative of this strain was tested against the inoculum strain, *R. leguminosarum* CL1, in a competition experiment under laboratory conditions. The results (Table 3) demonstrated that plants inoculated with equal numbers of strains CL1 and GC65 were nodulated by both strains in almost equal proportions. When unequal ratios of the two strains were used, the strain present in higher numbers formed the majority of nodules. Thus, strains GC65 and CL1 were equally competitive against one another under the experimental conditions employed. It was interesting to note that when the available data were plotted by the formula of Amarger and Lobreau (1), $\log (N_a/N_b) = K \log (1a/1b) + \log Cab$, a straight line passing through the origin was obtained. The existence of such a relationship between the logarithm of the ratio of nodules formed by two strains A and B (N_a/N_b) and the logarithm of the ratio of the numbers of each strain in the inoculum ($1a/1b$) indicates that both strains were equally competitive (1).

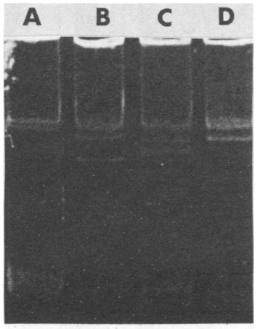

FIG. 1. Agarose gel electrophoresis of plasmid DNA in indigenous isolates of *R. leguminosarum*. Strains were grown in TY medium, and plasmid DNA isolation and electrophoresis were carried out as described in the text. The three plasmids profiles detected in isolates are labeled B, C, and D. Lane A contains *R. leguminosarum* CL1 as a control.

DISCUSSION

A key property of an inoculum strain is that it must be capable of outcompeting the indigenous soil rhizobia for nodule formation. Often, this can be achieved by using a sufficiently large concentration of rhizobial cells in an inoculum (21, 41). However, it has been noted that factors other than the numbers of the inoculum strain relative to the indigenous population are also important (37). In this study, an inoculum strain which became established in the majority of nodules at one location failed to become established at a second location, containing similar numbers of indigenous *R. leguminosarum*. It is possible that the superior competitive ability of soil strains of *R. leguminosarum* at site II was responsible for the poor establishment of the inoculum strain, as has been reported for inoculation of soybeans (10, 19, 24).

Antibiotic "fingerprinting" of nodule isolates from uninoculated control areas of site II indicated that a heterogeneous population existed in the soil. However, certain strains (particularly groups I and II) dominated the nodulating population. This method only screens that portion of the soil rhizobial flora capable of forming nodules on the host plant

TABLE 3. Results of a competition experiment with the inoculant strain CL1 and an indigenous soil isolate, GC65

Inoculum ratio (CFU ml^{-1}) of CL1/GC65	Establishment ratio (%) of CL1/GC65
$6.5 \times 10^7/4.4 \times 10^8$	29/71
$6.5 \times 10^7/4.4 \times 10^7$	58/42
$6.5 \times 10^8/4.4 \times 10^7$	81/19

(5). Thus, the frequency with which dominant intrinsic antibiotic resistance subgroups occur among indigenous isolates may be a reliable index of competitive ability.

Plasmid profile analysis has proved useful in identifying indigenous strains of *R. japonicum* showing potential as inoculants (16). All isolates tested in this study shared a common large plasmid, and 61.5% of the isolates had three plasmids in common. The finding that large plasmids in *Rhizobium* spp. are self-transmissible and can harbor key genetic determinants for symbiotic activity (2, 3, 6, 11, 20, 26, 27) may explain the existence of dominant plasmid pools at site II. These plasmids may be maintained because they confer selective advantages on host strains.

The results of a competition study between the inoculum strain, CL1, and a dominant indigenous isolate, GC65 (Table 3), do not explain the inability of CL1 to become established under field conditions at site II. Both strains were genetically marked for accuracy in determining nodule occupancy (25, 38). It has been reported that antibiotic resistance mutants may be symbiotically inferior to wild-type strains (9, 28, 33, 34, 43). However, antibiotic resistance markers have been successfully used in competition studies (22) and field trials (8, 17, 30, 31). In this study, both the inoculum strain CL1 and the indigenous strain GC65 were equally competitive in a laboratory experiment. However, results obtained under defined laboratory conditions must be interpreted with caution, as different results may be obtained in test soils and media (22). Thus, when strains are strongly competitive, environmental factors may decide which strains predominate in nodules. Environmental parameters of possible significance in field situations include host genotype, microbial antagonism, soil factors, and climatic conditions (15, 18). Thus, under field conditions, the inability of CL1 to establish at site II may be due to the fact that the inoculation technique and conditions did not establish at the root level a number of inoculum bacteria equal to or greater than the soil rhizobia. Competition by indigenous *Rhizobium* strains unable to nodulate the host legume (and not detected by the isolation method used in this study) may also be a factor (42). The poor persistence of the inoculum strain at site II compared with that at site I may indicate that this strain was not adapted to the local condition(s).

The results presented in this study indicate that the competitive ability of potential inoculant strains must be rigorously tested in a wide variety of environmental situations and especially under field conditions. Therefore, in the context of inoculation, it would appear desirable to use environmentally adapted indigenous strains as inoculants for soils containing indigenous rhizobial populations.

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