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Competition among Strains of *Rhizobium leguminosarum* biovar *trifolii* and Use of a Diallel Analysis in Assessing Competition†

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Competition between indigenous *Rhizobium leguminosarum* biovar *trifolii* strains and inoculant strains or between mixtures of inoculant strains was assessed in field and growth-room studies. Strain effectiveness under competition was compared with strain performance in the absence of competition. Field inoculation trials were conducted at Elora, Ontario, Canada, with soil containing indigenous *R. leguminosarum* biovar *trifolii*. The indirect fluorescent-antibody technique was used for the identification of nodule occupants. Treatments consisted of 10 pure strains, a commercial peat inoculant containing a mixture of strains, and an uninoculated control. Inoculant strains occupied 17.5 to 85% of nodules and resulted in increased dry weight and nitrogen content, as compared with the uninoculated control. None of the strains was capable of completely overcoming resident rhizobia, which occupied, on average, 50% of the total nodules tested. In growth-room studies single commercial strains were mixed in all possible two-way combinations and assessed in a diallel mating design. Significant differences in plant dry weight of red clover were observed among strain combinations. Specific combining ability effects were significant at the 10% level, suggesting that the effectiveness of strain mixtures depended on the specific strain combinations. Strains possessing superior effectiveness and competitive abilities were identified by field and growth-room studies. No relationship was detected between strain effectiveness and competitive ability or between strain recovery and host cultivar. The concentration of indigenous populations was not considered to be a limiting factor in the recovery of introduced strains at this site.

Rhizobium leguminosarum biovar *trifolii* has colonized large areas of soil in central and southern Ontario following the production of red clover (*Trifolium pratense* L.). Of 616 isolates selected from 10 sites in Ontario, only 35 were considered to be highly effective (N. Ames-Gottfred, Ph.D. thesis, University of Guelph, Guelph, Ontario, Canada, 1988). Most of these 35 isolates compared favorably to commercial strains in terms of plant dry weight and nodulation. However, these isolates represent a very small proportion of the total indigenous populations present in Ontario soils.

The introduction and establishment of more effective strains in soils containing indigenous populations is often unsuccessful, owing to interstrain competition (1, 4, 5, 15). Successful establishment of an introduced strain in soil containing an indigenous population has been attributed to inoculum concentration (10), strain effectiveness (17), soil factors (21), host genotype (2, 12), and competition with other rhizosphere organisms (8, 13).

Successful introduction of effective *R. leguminosarum* biovar *trifolii* strains into soils containing indigenous rhizobia has resulted in improved red clover production in Mississippi (14). Similar results have been reported for other *Trifolium* species (7; L. A. Materon, Diss. Abstr. Int. B **43**:2073). At present, no data exist on strain survival, recovery, or effectiveness in Ontario soils containing indigenous populations of *R. leguminosarum* biovar *trifolii*.

In this study, the effectiveness and competitive ability of several strains of *R. leguminosarum* biovar *trifolii* were studied in field and growth-room experiments with one or two cultivars of red clover. In the field, native populations of *R. leguminosarum* biovar *trifolii* provided competition for inoculant strains. In the growth-room, field soil was sterilized and either used to provide a noncompetitive environment or inoculated with mixed strains to simulate competition. The latter experiment was treated as a diallel mating system to determine if general combining ability (GCA) and specific combining ability (SCA) effects would provide additional information on interstrain competition.

MATERIALS AND METHODS

Field experiment. Competition between indigenous and introduced strains of *R. leguminosarum* biovar *trifolii* was studied in a field trial at the Elora Research Station (latitude, 43° 39'N; elevation, 376 m). The soil was a London loam series Grey Brown Luvisol (Typic Hapludalf). The numbers of indigenous *R. leguminosarum* biovar *trifolii* were determined by using the most-probable-number technique (20). Two red clover cultivars, Altaswede (single cut) and Arlington (double cut), were seeded on 25 May 1985 at the equivalent of 7 kg/ha. Each plot consisted of a single row 3 m long and spaced 1 m apart. Each plot was inoculated with 1 of 10 pure strains of *R. leguminosarum* biovar *trifolii*, a commercial peat inoculant, or no inoculant. The experimental design consisted of split plots arranged in a randomized complete block design with four replications. Main plots were strains, and subplots were cultivars.

The 10 pure strains tested were supplied by Nitragin Co. Inc., Milwaukee, Wis., and were designated CC1, E7, K13, P28, P17a, P30d, P30e, S31, P44, and P45. Strains were inoculated into flasks of yeast mannitol broth (20) and incubated at 28°C until cultures contained approximately 10^9 cells per ml (approximately 4 days). Liquid inoculum was transported to the field in coolers containing ice and applied to the sown seed by using sterile pipettes. Broth culture (50 ml) was spread along each seed row, and seeds were covered by hand. Commercial peat inoculum containing a mixture of

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seven *R. leguminosarum* biovar *trifolii* strains, including P30e, P28, and E7, was supplied by Nitragin Co. and applied as a liquid culture as described by Somasegaran and Hoben (19). A control treatment with no inoculum was included to compare the effectiveness of indigenous strains at this site.

On 15 August 1985 four randomly selected plants from each plot were sampled for tops and roots to a depth of 15 cm. Roots were separated from tops just below the crown and placed in plastic bags with surrounding soil. Tops from each plot were dried and weighed individually and then bulked for nitrogen analysis. Total ammoniacal nitrogen determinations were performed at Agri-Food Laboratories, Guelph, Ontario, Canada, by automated macro-Kjeldahl analysis (3). Roots and surrounding soil were placed on wire mesh and washed with a light stream of water. Nodulation was rated visually on a scale of 0 to 5 (0: no nodules or small ineffective nodules; 5: prolific nodulation, more than 25 small or 15 large effective nodules) based on nodule size, number, and color. From each plant 10 randomly selected nodules were taken for strain identification by the indirect fluorescent-antibody technique (19).

For analysis of variance, nodulation scores were transformed $(x + 1)^{1/2}$, and treatment means were compared by the Student-Neuman-Keuls test.

Strain effectiveness in sterile field soil. In the fall of 1985 the field experiment was repeated with sterilized field soil in the growth chamber to assess strain effectiveness in the absence of competition. The experiment was modified to include two controls, strain P47 (Nitragin Co.) and strain TA1 (provided by D. C. Jordan, University of Guelph). The two controls were not inoculated, and one received no nitrogen, while the other received nitrogen. The commercial peat inoculum mixture was not included in this experiment. Field soil was collected from a site adjacent to that of the field experiment and mixed with peat (2:1). The soil mixture was placed in plastic pots, covered with aluminum foil, and autoclaved at 15 lb/in^2 for 30 min. Red clover seeds (cultivars Arlington and Altaswede) were surface sterilized in 0.1% mercuric chloride for 10 min, rinsed with sterile distilled water, and seeded at a rate of four seeds per pot. *Rhizobium* cultures were prepared in yeast mannitol broth as previously described and inoculated onto seeds at concentrations of 1 ml per seed. Pots were placed in a growth cabinet and separated by using a grid system of transparent plastic sheeting (Crystaplex Plastics Ltd., Mississauga, Ontario, Canada) to prevent cross-contamination. The plants were grown for 16 h each day, at day and night temperatures of 22 and 18°C, respectively, and with illumination at the top of the pots of 242 microeinsteins per m^2 per s. All pots were watered daily with tap water and twice per week with N-free nutrient solution (only one control received nitrogen).

The pots were arranged in split plots in a randomized complete block design with four replications. Inoculant strains were whole plots, and cultivars were subplots. Eight weeks after emergence, the plants were harvested and evaluated for top dry weight, nitrogen content, and nodulation score.

Strain diallel study. Thirteen *R. leguminosarum* biovar *trifolii* strains were evaluated alone and in all possible two-strain combinations on Arlington red clover in a randomized complete block experiment with four replications. The study was conducted in a controlled-environment chamber with sterile field soil from Elora mixed with peat (2:1). The red clover seeds were surface sterilized and seeded into pots containing the soil mixture. Yeast mannitol broth inoculum was prepared for each strain and incubated at 28°C until each culture contained approximately 10^9 cells per ml. For strain combinations, equal quantities of yeast mannitol broth cultures were mixed under aseptic conditions just prior to seedling inoculation.

After germination, each seedling was inoculated with 2 ml of broth culture. Pots were separated by using transparent plastic to eliminate cross-contamination. The plants were grown for 16 h each day, at day and night temperatures of 22 and 18°C, respectively, and with illumination of 237 microeinsteins per m^2 per s at the pot level. The plants were watered daily with tap water and twice per week with N-free nutrient solution.

After 8 weeks of growth, the tops were harvested and measured for dry weight and nitrogen content. The data were treated as a diallel and analyzed according to Method 2, Model 1 (6).

TABLE 1. Mean plant dry weight, nitrogen content, and nodulation rating of two red clover cultivars grown with various inoculant strains in Elora in 1985[a]

Inoculant strain	Plant dry wt (g/plant)	Nitrogen content (%)	Total N (g, 10^{-1})	Nodulation rating[b]
P45	2.26 a	3.09 ab	0.698	2.49 ab
P30d	2.12 ab	2.83 abc	0.600	2.58 ab
P30e	1.90 abc	3.03 ab	0.576	2.09 bcd
K13	1.95 abc	2.71 c	0.528	2.03 bcd
Commercial peat	1.79 abcd	2.79 bc	0.499	1.88 d
P17a	1.53 bcde	2.80 bc	0.428	2.36 abc
P44	1.46 bcde	3.12 a	0.455	1.71 d
CC1	1.46 bcde	3.12 a	0.455	2.66 ab
E7	1.28 cde	2.89 abc	0.369	2.92 a
P28	1.25 cde	3.07 ab	0.383	1.67 d
S31	0.94 e	3.07 abc	0.288	2.43 abc
Control	0.94 e	2.90 abc	0.273	2.27 abc
Mean	1.53	2.95		2.24
Coefficient of variation (%)	59.9	6.10		12.96
F	3.55[c]	3.30[c]		2.14[d]

[a] Means followed by the same letter were not significantly different, as determined by the Student-Neuman-Keuls test, at $P \leq 0.05$.
[b] Based on the number, size, and color of nodules (5 = maximum nodulation, 0 = no nodulation). Values shown represent retransformed means. Analysis was performed on transformed means $(x + 1)^{1/2}$.
[c] Highly significant ($P \leq 0.01$).
[d] Significant ($P \leq 0.05$).

RESULTS

Field experiment. The field soil contained substantial amounts of indigenous *R. leguminosarum* biovar *trifolii* (2.8×10^4 cells per g). These provided inocula for the uninoculated controls and would compete for nodule sites with the inoculant strains. Plants inoculated with the different strains exhibited significant differences in dry weight, nitrogen content, and nodulation scores (Table 1).

Five of the strains produced plant dry weights significantly greater than those of the control. The control treatment with indigenous rhizobia resulted in the lowest plant dry weight; however, nodulation scores and nitrogen content were above average (Table 1). Although the rankings for these three criteria were not consistent, three strains, P30d, P30e, and P45, were among those with the highest values in all cases. The large coefficient of variation for plant dry weight (59.9) was based on single plant data and was due, in part, to the genetic heterogeneity within each cultivar.

TABLE 2. Recovery of inoculant strains in plots of 10-week-old Arlington and Altaswede red clover inoculated with 10 *R. leguminosarum* biovar *trifolii* strains and grown in Elora

Inoculant strain	Total no. of nodules tested	Recovery of inoculant strain from (%):		Mean recovery (%)
		Arlington	Altaswede	
P45	16	85	85	85.0
P30e	16	85	80	82.5
S31	20	80	85	82.5
CC1	20	67	50	58.5
P44	18	50	50	50.0
P17a	18	50	40	45.0
E7	20	50	30	40.0
P30d	20	10	30	20.0
K13	16	20	15	17.5
P28	20	25	10	17.5
Mean	18	52	48	

The nodulation scores for the two cultivars were not significantly different, but Arlington had a significantly higher plant dry weight than did Altaswede (1.69 versus 1.38 g per plant). Altaswede had a significantly higher nitrogen content (3.00 versus 2.90%).

Differences in dry weight and nitrogen content (Table 1) could not be attributed entirely to the inoculant strain because the percent recovery from the nodules was inconsistent (Table 2). Inoculant strains were recovered from approximately half of the nodules sampled at the Elora field site. Strains P45, P30e, S31, and CC1 competed best against the indigenous strains. Strains K13, P30d, and P28 were poor competitors, occupying only 20% of the nodules. Of these, K13 and P30d had previously been shown to be less effective and P28 had previously been shown to be moderately effective. In this trial K13 and P30d were more effective than P28 (Table 1), but consideration of the recovery data indicated that the response was due to indigenous strains and not inoculant strains (Table 2). Strains S31 and CC1 were competitive against native strains, but plant performance was similar to that of the control, indicating that competitiveness and effectiveness were not necessarily related characteristics.

Strain effectiveness in sterile field soil. Strains tested in the field plus additional strains were evaluated for effectiveness under controlled conditions with sterilized Elora field soil.

There were no significant differences in plant dry weight or in nitrogen content as a result of inoculation (Table 3). Strain P30e was the only strain resulting in high dry matter in the field and above-average dry matter indoors.

Some strains, such as P17a, P28, and E7, produced high yields in this trial (i.e., were effective) but low yields in the field. They were apparently unable to compete with the indigenous population in the field (Tables 1, 2, and 3).

Significant differences observed in the field were not detected under controlled conditions, in which competition from indigenous strains was eliminated by the use of sterile soil. It appears that growth-room studies do not reflect results obtained in situ, which may indicate that some form of competition is beneficial to strains which are both effective and competitive relative to native populations.

Strain diallel study. The plant dry weights were significantly different for the 91 treatments (Table 4) (13 *Rhizobium* strains alone and 78 paired combinations). The GCA, or average performance alone in all combinations, of the strains was not significant. This result confirmed the results of the previous experiment which indicated that in sterile field soil the different strains had no significant effect on plant dry weight. The SCA, which indicates any deviation from what would be expected on the basis of GCA, was significant (10% level). SCA effects indicated superior strain combinations in terms of plant dry weight (Table 5).

The plant dry weights ranged from 0.70 g (P30d plus E7) to 1.98 g (P47 plus E7) (Table 6). These data suggested that many strain combinations performed better or worse than either strain alone.

The percent nitrogen content of the plants did not differ significantly among treatments, nor was the GCA effect or the SCA effect significant. The mean nitrogen content was 3.18%.

TABLE 3. Evaluation of *R. leguminosarum* biovar *trifolii* strains with two cultivars of red clover in sterile field soil under controlled conditions

Strain	Plant dry wt (g/plant)	Nitrogen content (%)
CC1	1.26	3.50
17a	1.24	3.21
P30e	1.22	3.27
P28	1.11	3.23
P44	1.09	3.16
E7	1.08	3.24
P45	0.97	3.24
P47	0.96	3.58
S31	0.91	2.95
TA1	0.87	3.42
P30d	0.84	3.18
K13	0.75	3.39
Control (with N)	1.42	3.21
Control (without N)	0.95	3.38
Mean	1.05	3.28
F	0.75[a]	1.15[a]

[a] Not significant.

DISCUSSION

In the field experiment, with one exception, inoculation with one of the introduced strains of *R. leguminosarum* biovar *trifolii* resulted in increased dry weight of the red clover plants. For 4 of the 10 strains, the increase was significant. For nodulation ratings and nitrogen content, the means for the indigenous population were close to the mean for the experiment. Considering these results, one is tempted to conclude that the inoculant strains were more competitive than the indigenous population. However, for both red

TABLE 4. Griffing[a] analysis of variance for dry weight in the nonreciprocal diallel strain combination system

Source	df	Mean square
Blocks	3	4.23[b]
Strains	90	0.35[c]
GCA	12	0.09[d]
SCA	78	0.09[e]
Error (combining ability)	270	0.07
Error	270	0.28

[a] See reference 6.
[b] Highly significant ($P \leq 0.01$).
[c] Significant ($P \leq 0.05$).
[d] Not significant.
[e] Significant ($P \leq 0.10$).

TABLE 5. SCA effects of single strains and strain mixtures on dry weight

Strain	SCA effect of strain:												
	K13	P30d	P17a	P28	P30e	P44	P45	P46	P47	E7	CC1	S31	TA1
K13	−0.33	−0.25	−0.17	0.39	0.21	0.28	0.38	−0.29	0.10	0.15	−0.31	−0.14	0.30
P30d		−0.08	−0.03	0.16	−0.22	0.05	−0.14	0.65	0.27	−0.41	0.18	0.11	−0.20
P17a			0.12	0.08	0.15	−0.15	0.13	0.22	−0.65	0.09	0.04	−0.24	0.29
P28				−0.22	−0.18	0.61	−0.12	−0.33	0.12	−0.04	0.04	−0.03	−0.26
P30e					−0.01	0.02	−0.09	0.03	0.11	0.29	0.16	−0.24	0.23
P44						−0.02	0.11	−0.24	−0.33	−0.31	0.22	0.14	−0.37
P45							−0.34	−0.21	0.26	−0.27	0.23	−0.35	0.76
P46								−0.24	−0.01	0.24	0.42	0.10	−0.11
P47									−0.30	0.64	−0.38	0.52	−0.06
E7										−0.05	−0.43	0.07	0.09
CC1											−0.09	0.11	−0.10
S31												−0.27	0.50
TA1													−0.32

clover cultivars, only about 50% of the nodules tested contained the particular inoculant strain. Nodule occupancy varied from 17.5 to 85%, depending upon the strain. Some strains were able to compete successfully with the native population. Some strains, such as P45, were competitive (85% nodule occupancy) and effective (dry weight, 2.26 g). Others, such as S31, were competitive (82.5% nodule occupancy) but less effective (dry weight, 0.94 g). Thus, competitiveness and effectiveness were distinct characteristics.

Robinson (17) found that effective strains of *R. leguminosarum* biovar *trifolii* consistently occupied the majority of nodules when used in mixed inoculations with ineffective strains. He suggested that the host plant could distinguish between effective and ineffective strains. The two red clover cultivars used in these studies did not preferentially select strains with superior effectiveness. Other studies have shown that effective strains are not necessarily more competitive than ineffective strains (11, 12).

When red clover was grown indoors in sterilized field soil, single-strain inoculations resulted in no significant differences in plant dry weight or in nitrogen content. Similarly, in the diallel study, there were no differences among strain means (GCA) for either trait. In the absence of competition, these strains appeared to be equally effective or ineffective.

In the diallel study, the different combinations of strains significantly affected plant dry weight but not nitrogen content. Some combinations performed more or less as expected on the basis of the performance of the pure strains. For example, with pure cultures, plant dry weights for 17a and K13 were 1.5 and 0.8 g, respectively. The mixture of these two strains gave a dry weight of 1.1 g. In other mixtures, antagonistic effects were observed (e.g., E7 plus CC1 and P47 plus P17a, with which dry weights were lower than with either strain alone). Antagonistic effects of strain mixtures have been reported for *R. meliloti* (16). On the other hand, some combinations, such as E7 plus S31, resulted in much higher dry weights than either strain alone.

The results from the growth-room study did not always provide an explanation for field performance. Strain P28 had the lowest nodule occupancy (17.5%), indicating a lack of competitive ability, but its GCA was higher than those of most strains. Specific combinations involving P28 performed much better or much worse than expected; this result suggests that P28 could be competitive but does not express this characteristic under all conditions. On the other hand, P45 was very competitive and effective under field conditions. In the diallel study, plant dry weights were greater in every mixture with P45 than with this strain alone. It is possible that in the field the indigenous population in the remaining 15% of the nodules was highly effective and able to overcome the negative impact of P45. Another explanation is that P45 needs a "helper" strain to express its effectiveness. Johnston and Beringer (11) found that a mixture of effective and ineffective *R. leguminosarum* strains resulted in increased effectiveness. This could explain why strains such as K13 and P30d had low nodule occupancy but still increased plant dry weight relative to the control.

The competitive environment which exists in the field

TABLE 6. Mean plant dry weights resulting from treatment with single strains and strain mixtures

Strain	Mean plant dry wt (g) after treatment with strain:													Mean
	K13	P30d	P17a	P28	P30e	P44	P45	P46	P47	E7	CC1	S31	TA1	
K13	0.79	0.81	1.08	1.68	1.3	1.39	1.50	0.91	1.40	1.32	0.93	1.07	1.45	1.20
P30d		0.92	1.17	1.40	0.81	1.10	0.93	1.79	1.51	0.70	1.35	1.26	0.89	1.13
P17a			1.50	1.50	1.38	1.09	1.39	1.55	0.78	1.39	1.41	1.10	1.58	1.30
P28				1.25	1.09	1.89	1.18	1.04	1.60	1.31	1.45	1.35	1.08	1.37
P30e					1.05	1.09	1.01	1.21	1.38	1.44	1.37	0.95	0.90	1.15
P44						1.08	1.22	0.95	0.95	0.85	1.44	1.33	0.78	1.17
P45							0.79	1.00	1.56	0.90	1.47	0.86	1.92	1.21
P46								1.04	1.37	1.49	1.73	1.38	1.13	1.28
P47									1.17	1.98	1.03	1.90	1.28	1.38
E7										1.17	0.85	1.33	1.30	1.23
CC1											1.26	1.43	1.17	1.30
S31												1.02	1.74	1.29
TA1													0.875	1.24

differs from simulated competition since it is made up of a number of heterogeneous strains. These strains may vary in effectiveness and competitive ability and may even be ineffective. Winarno and Lie (22) reported the occurrence of a native non-nodulating *R. leguminosarum* strain which competed with effective strains and suppressed nodulation completely.

One of the most difficult questions to address in competition studies is the importance of inoculum concentration relative to the numbers of indigenous strains. While literature on this subject is numerous the results have been conflicting. Ireland and Vincent (10) showed that successful introduction of rhizobia could be achieved by increasing the concentration of the inoculant strain relative to the numbers of indigenous strains. Similar associations between inoculum concentrations and competitive ability have been reported by others (1, 2). In another study, an inoculant strain which formed the majority of nodules at one location failed to become established at a second location containing equivalent numbers of indigenous strains (15). Roughley et al. (18) found greater recovery of an *R. leguminosarum* biovar *trifolii* strain at sites with more numerous indigenous populations.

The application of large concentrations of an inoculant strain may result in a successful recovery in the nodules but may not reflect the characteristic competitive ability of that strain. In the present study the inoculum applied was 8×10^7 rhizobium cells per seed, numerically equivalent to 8×10^4 cells per g of soil (to a 10-cm depth), as compared with soil populations of approximately 2.8×10^4 cells per g of soil. Therefore, the inoculant strains were at a slight advantage in terms of numbers and strategic placement near the host, while the indigenous strains enjoyed the benefit of being adapted to the site. With these factors considered, both indigenous strains and inoculant strains were believed to have an equal opportunity to infect the host, so that a valid assessment of competitive ability could be made.

Only two cultivars were used in these studies. When both were used in the same experiment, there was no evidence of a strain-cultivar interaction, but the possibility of host selection must be considered. Red clover cultivars are relatively heterogeneous populations, with a great deal of genotypic variability within a cultivar. Therefore, individual plants within a cultivar may exhibit differential selection of strains, as has been suggested for *T. repens* (12).

Combining ability analysis has been used in studies of plant competition (9). This analysis (9) used yielding ability (equivalent to GCA) and competitive influence to explain interactions among maize hybrids. This type of analysis can be used to separate the effects of large numbers of combinations into meaningful statistics and can be valuable for assessing *Rhizobium* competition. The GCA component provides an estimate of the overall combining or competitive ability of a strain. The SCA component indicates the deviation in performance when strains are mixed. Performance as a pure strain indicates effectiveness. GCA and SCA effects are indications of average and specific competitive abilities.

These results illustrate the importance of assessing both qualitative and quantitative aspects of the indigenous population when determining competitiveness. Heterogeneous soil populations most likely have various competitive abilities, and even a small population of highly competitive strains will represent an imposing competitive barrier to an inoculant strain.

To form the majority of nodules an introduced strain must be more competitive than the indigenous or competing strains. Although effectiveness and competitiveness are not related, potential inoculant strains for Ontario must possess both traits. Further studies should involve testing selected competitive strains in a variety of environmental conditions. Failure to detect clear host selection effects warrants testing of additional red clover cultivars.

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