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Symbiotic Characteristics of *Rhizobium leguminosarum* bv. trifolii Isolates Which Represent Major and Minor Nodule-Occupying Chromosomal Types of Field-Grown Subclover (*Trifolium subterraneum* L.)†

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The symbiotic effectiveness and nodulation competitiveness of *Rhizobium leguminosarum* bv. trifolii soil isolates were evaluated under nonsoil greenhouse conditions. The isolates which we used represented both major and minor nodule-occupying chromosomal types (electrophoretic types [ETs]) recovered from field-grown subclover (*Trifolium subterraneum* L.). Isolates representing four ETs (ETs 2, 3, 7, and 8) that were highly successful field nodule occupants fixed between 2- and 10-fold less nitrogen and produced lower herbage dry weights and first-harvest herbage protein concentrations than isolates that were minor nodule occupants of field-grown plants. Despite their equivalent levels of abundance in nodules on field-grown subclover plants, ET 2 and 3 isolates exhibited different competitive nodulation potentials under nonsoil greenhouse conditions. ET 3 isolates generally occupied more subclover nodules than isolates belonging to other ETs when the isolates were mixed in 1:1 inoculant ratios and inoculated onto seedlings. In contrast, ET 2 isolates were less successful at nodulating under these conditions. In many cases, ET 2 isolates required a numerical advantage of at least 6:1 to 11:1 to occupy significantly more nodules than their competitors. We identified highly effective isolates that were as competitive as the ET 3 isolates despite representing serotypes that were rarely recovered from nodules of field-grown plants. When one of the suboptimally effective isolates (ET2-1) competed with an effective and competitive isolate (ET31-5) at several different inoculant ratios, the percentages of nodules occupied by the former increased as its numerical advantage increased. Although subclover yields declined as nodule occupancy by ET2-1 increased, surprisingly, this occurred at inoculant ratios at which large percentages of nodules were still occupied by ET31-5.

Over a period of 50 years numerous studies have shown that some rhizobial strains can occupy a greater percentage of nodules than other strains when the organisms are inoculated onto legumes as a mixture of strains (15, 38, 50). Two hypotheses have been formulated to explain this interstrain variation in nodulating ability. First, there may be interstrain variation at the level of the plant-microbe interactions which occur during nodule initiation and development (50). Support for this hypothesis comes from situations where specific strains or subpopulations dominate nodules regardless of their relative contributions to an inoculant mixture or to a soil population (37, 42). In contrast, several studies have shown that nodulation success by specific strains is influenced by the relative numbers of these strains in a mixture of strains (2, 4, 20–22, 33). In these cases, relative nodule occupancy can be changed if the ratio of strains is changed (2, 14, 21, 22). The situation that occurs when legumes grow in soil containing heterogeneous rhizobial populations has not been explained. Although it has been determined that subtypes in heterogeneous soil rhizobial populations occupy the majority of root nodules on soil-grown legumes (13, 17, 28, 29, 34, 36, 37), it has not been established that nodule-dominant types possess superior competitive traits compared with minor occupants. Indeed, there is evidence to the contrary. For example, minor nodule-occupying types of *Rhizobium meliloti* and *Rhizobium leguminosarum* bv. viciae were as competitive as nodule-dominant types when the organisms were evaluated under nonsoil conditions (12, 36).

In an accompanying paper we describe serotype AS6 of *Rhizobium leguminosarum* bv. trifolii which consistently dominates nodules of field-grown subclover (32b). In another study, the majority of the AS6 isolates were found to possess specific chromosomal types (electrophoretic types [ETs]) and to belong to a genetically distinct group of isolates in the soil population (32a). The objectives of this study were to characterize the symbiotic and competitive abilities of isolates belonging to the nodule-dominant ETs and to compare these isolates with isolates representing minor nodule-occupying ETs.

MATERIALS AND METHODS

Seed source and germination. Seeds of cultivar Nangeela subclover were obtained from G. Evers, Texas A&M University Agricultural Experiment Station, Angleton. Ten grams of seeds was sieved through a 2-mm sieve, and abnormally small and large seeds and seeds with seed coat damage were discarded. The seeds were surface-disinfested by standard procedures (52), incubated on water agar plates at 4°C for 24 h, and germinated overnight at room temperature.

Evaluation of symbiotic effectiveness of soil isolates of *R. leguminosarum* bv. *trifolii* on subclover. (i) Effectiveness test: experiment 1. The isolates used represented the major nodule-occupying serotype (AS6) and a spectrum of the numerous

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minor nodule-occupying serotypes recovered from subclover growing at a field site in western Oregon (32b). We chose seven isolates to represent several of the different chromosomal types (ETs) which comprise serotype AS6 (32a); these isolates were strains ET2-1, ET3-3, ET3-8, ET3-18, ET7-3, ET7-14, and ET8-2. We chose five isolates to represent different chromosomal types of serotypes that are infrequent nodule occupants of subclover at the Oregon site; these isolates were serotype AS27 strains ET30-1, ET30-3, and ET31-5, serotype AG4 strain ET17-1, and serotype AS21 strain ET15-2. The effectiveness potentials of these isolates were compared with the effectiveness potential of an inoculant quality strain 162X95 (= ET82-1), which was provided by R. S. Smith, LiphaTech Co., Milwaukee, Wis. The isolates were grown in yeast extract-mannitol broth for 3 days at 28°C before seedlings were inoculated.

(ii) Effectiveness test: experiment 2. After initiating this study, we discovered that serotype AS6 is composed of several subtypes and that subtype AS6-a is restricted to ET 2 and subtype AS6-b is restricted almost exclusively to ET 3 (32a, 32b). Since subtype AS6-a (ET 2) isolates were poorly represented in the first experiment, a follow-up study was conducted with subtype AS6-a isolates (strains ET2-1, ET2-2, and ET2-3) and additional representatives of subtype AS6-b (strains ET3-1, ET3-2, and ET3-3). Serotype AR23 isolate ET23-1 was used as a highly effective strain for yield comparisons.

Plant growth conditions. Seedlings were transplanted into Leonard jar assemblies (52). Each unit was composed of a bottomless 1-quart (0.946-liter) beer bottle sitting neck down in a wide-mouth 800-ml mason jar. Approximately 70 g of a perlite-vermiculite mixture (1:1 vol/vol) was dispensed into each beer bottle, and a cheesecloth wick was used to transport nutrient solution from the mason jar to the perlite-vermiculite mixture. A 400-ml portion of an N-free mineral nutrient solution was dispensed into the perlite-vermiculite mixture, and a 600-ml portion was added directly to the mason jar. The N-free mineral nutrient solution (pH 6.5) was composed of the following ingredients: 5.98 mM $CaSO_4 \cdot 2H_2O$, 0.14 mM $CaCl_2 \cdot 2H_2O$, 1.99 mM $MgSO_4 \cdot 7H_2O$, 4.99 mM K_2SO_4, 1.25 mM K_2HPO_4, 0.8 mM KH_2PO_4, 10 mg of ferric citrate per liter, and 10 ml of a micronutrient solution (18). The tops of the assemblies were covered with aluminum foil, and brown paper bags were placed around the junctions between the bottles and the jars. The units were autoclaved for 60 min and allowed to cool to room temperature for at least 24 h before planting.

After four seedlings were transplanted into each assembly, the preparations were inoculated with individual isolates of *R. leguminosarum* bv. trifolii which had been grown in yeast extract-mannitol broth for 3 days at 28°C. The assemblies were covered with petri dish lids and incubated in a growth chamber for about 5 days. The seedlings were thinned to one seedling per assembly, and sterile paraffinized sand was applied to the surface of each jar to a depth of 2 cm. The plants were transferred to a greenhouse and arranged in a randomized complete block design. Shoots were excised 54 days after planting, and three fully expanded leaves were left attached to each crown. In experiment 1, regrowth was allowed to take place for 28 days, and then the herbage was harvested and dried for 14 days at 55°C and the dry weights of the plant material from this second harvest were determined.

Total-nitrogen analysis. Dried herbage was ground in a Wiley mill and digested with a combination of Kjeldahl catalyst and concentrated H_2SO_4. The ammonium in the resulting digests was analyzed by using standard procedures (7).

Statistical analyses. An analysis of variance and separation of means were performed on the data by using the general linear model of the SAS software (Statistical Analysis System Institute, Inc.).

Competitive nodulation studies. Three ET 2 isolates (ET2-1, ET2-2, ET2-3) and three ET 3 isolates (ET3-1, ET3-2, and ET3-3) were used to examine competitive nodulation potentials compared with the competitive nodulation potentials of representatives of other ETs. The ET 2 and 3 isolates were used at population ratios of approximately 1:1 with the following isolates: ET8-1 and ET12-1, representing the most common serotype AS6-c and serotype AS21 ETs, respectively; and ET33-2, ET23-1, and ET31-5, representing minor nodule-occupying serotypes AP17, AR23, and AS27, respectively.

(i) Preparation of peat inoculants. To minimize the risk of changes that might occur in the ratio of inoculant strains during the time that the bacteria were harvested, mixed, and inoculated onto plants, the inoculants were prepared in sterile peat (LiphaTech Co.). Each isolate was grown in 30 ml of defined glutamate-mannitol nutrient broth at 28°C for 3 days. Portions (24 ml) of each culture were transferred aseptically to sterile 160-ml milk dilution bottles containing 30-g portions of sterile peat. The peat-culture mixtures were incubated at 27°C for 2 days, thoroughly mixed, and then incubated for another 12 days. Samples were taken from each peat inoculant to determine the rhizobial population density by a plate count assay. Portions (0.5 g) of each peat culture were transferred to 500-ml volumes of a sterile mineral salt solution (pH 7) containing 3.67 mM K_2HPO_4, 0.81 mM $MgSO_4 \cdot 7H_2O$, and 1.71 mM NaCl. The suspensions were diluted through a 10-fold dilution series to a final dilution of 10^{-7}. Portions (0.1 ml) of the higher dilutions were plated onto yeast extract-mannitol agar plates and incubated at 27°C. The population densities of the peat inoculants ranged between 1.7×10^9 and 4.4×10^9 cells per g. Each peat inoculant culture was subdivided into six sterile 20-ml scintillation vials and refrigerated until it was used.

(ii) Preparation of inoculant mixtures and plant growth conditions. Cultivar Nangeela subclover seeds were surface disinfested, germinated, and transplanted into Leonard jar assemblies as described above. A 0.5-g portion of each peat inoculant was diluted 10^3-fold into 500 ml of a cold (4°C) sterile mineral salt solution to achieve a final cell concentration of approximately 10^6 cells per ml. The peat suspensions were shaken vigorously for 10 min and then were allowed to settle for 5 min. A 20-ml portion of supernatant representing either an ET 2 isolate or an ET 3 isolate was mixed with an equal volume of supernatant of a competitor, and the mixture was kept in an ice bath until the seedlings were ready for inoculation. For the 10:1 ratios, 2 ml of the 10^{-3} suspension was mixed with 18 ml of cold sterile mineral salt solution and then with a 20-ml portion of the 10^{-3}-diluted peat inoculant of the competitor before inoculation onto subclover seedlings. Each seedling was inoculated with 1 ml of the appropriate inoculant mixture. Four replicate assemblies were prepared for each inoculant treatment. The controls included seedlings inoculated with each of the individual strains and uninoculated seedlings supplemented or not supplemented with 8 mM KNO_3. The different paired strain combinations used are described in the Results. The actual inoculant strain ratios were determined later by staining samples with fluorescein-labeled immunoglobulin conjugates (FAs) specific to each member of the mixture. Each assembly was covered with a sterile petri dish lid and transferred to a growth chamber that was programed for a 14-h light–10-h dark cycle. After 5 days the petri dish lids were removed, the seedlings were thinned to two seedlings per assembly, and the perlite-vermiculite mixture

was covered with sterile paraffinized sand (depth, approximately 2 cm) to prevent aerial contamination and reduce surface evaporation. The assemblies were transferred to a greenhouse and arranged in a randomized complete block fashion, and the blocks were rotated at 7-day intervals. Daylight illumination was supplemented with high-intensity lamps by using a 14-h light–10-h dark cycle, and the temperature was maintained at 25 to 27°C. The assemblies were replenished with mineral nutrient solution as needed. The plants were harvested 6 weeks after inoculation, and the root nodules were collected for nodule occupancy analysis.

(iii) Nodule analysis. The Leonard jar assemblies were dismantled, and the perlite-vermiculite mixture was washed away from the roots. Regardless of the strain combination, between 100 and 140 nodules were recovered from the combined root systems of the two plants in each assembly. From this pool 20 nodules were picked at random, surface disinfested, and crushed in 50 to 200 μl of sterile deionized water, and smears were prepared on microscope slides. The smears were air dried, heat fixed, prestained with 1 drop of rhodamine-gelatin conjugate (5), and analyzed by immunofluorescence with the appropriate FAs. In the treatments in which ET 3 representatives competed against ET2-1, duplicate smears were examined with FA-AS6 adsorbed with a combination of ET2-1 and ET8-1 and with FA-AS20 adsorbed with ET3-3. ET 2 smears reacted only with adsorbed FA-AS20, whereas only ET 3 isolates reacted positively with adsorbed FA-AS6. Nodules occupied by ET 3 isolates were distinguished from nodules containing ET8-1 since only nodules occupied by the ET 3 isolates reacted with FA-AS6 adsorbed with isolate ET8-1; nodules containing ET8-1 were identified by their strong reactions with FA-AS21 (32b).

(iv) Indirect immunofluorescence procedure for identifying representatives of serotype AS27. Although strains ET30-3 and ET31-5 reacted with antiserum AS27 in immunodiffusion tests, they did not react with the fluorescein-labeled conjugate prepared to the same antiserum. Presumably, the cross-reactive antibody was lost during preparation of the FA. An indirect FA method involving fluorescein-labeled goat anti-rabbit immunoglobulin G (Sigma Chemical Co., St. Louis, Mo.) combined with whole AS27 antiserum was used to detect strains ET30-3 and ET31-5 in nodule smears (46). Nodule smears were stained with rhodamine-gelatin conjugate and then incubated for 15 min with AS27 antiserum (diluted 1:100 and adsorbed three times with isolate AS6-1 to remove antibodies that were cross-reactive with members of serotype AS6). Excess antiserum was washed off the smears with 0.02 M sodium phosphate buffer (pH 7.2), and the smears were restained with the manufacturer's recommended dilution (1:80) of the fluorescein-labeled goat anti-rabbit immunoglobulin G (in 0.02 M sodium phosphate buffer, pH 7.2). After incubation for 15 min, the smears were rinsed, air dried, and examined by performing immunofluorescence tests as described above.

RESULTS

Subclover grew vigorously in the Leonard jar assemblies under greenhouse conditions. A ninefold difference (4.92 to 0.56 g) in herbage dry matter accumulation was observed between the most and least effective plant-isolate combinations over the two herbage harvests (Table 1). In general, isolates that represented minor nodule-occupying ETs on field-grown plants were significantly more effective than any of the eight isolates that represented the ETs that dominate nodules of subclover growing under field conditions. Indeed, two isolates, ET30-1 and ET30-3, were significantly more effective than isolate 162X95 (a commercial subclover inoculant strain) under our growth conditions.

TABLE 1. Symbiotic effectiveness potential (shoot dry matter yield) of *R. leguminosarum* bv. trifolii isolates which represent major and minor nodule occupants of field-grown subclover

Isolate	Dry matter yield (g plant^{-1})			Significance[a]
	Harvest 1	Harvest 2	Harvest 1 + harvest 2	
Minor field nodule-occupying ETs				
ET30-1	1.54	3.38	4.92[b]	S
ET30-3	1.53	3.35	4.88[b]	S
ET82-1[c]	1.24	2.46	3.69[d]	S
ET15-2	1.17	2.77	3.94[d]	S
ET31-5	1.16	2.59	3.75[d]	S
ET17-1	1.02	2.62	3.64[d]	S
Major field nodule-occupying ETs				
ET3-8	0.76	1.52	2.28[e]	S
ET8-2	0.60	0.53	1.13[f]	NS
ET8-1	0.59	0.90	1.49[f]	NS
ET3-18	0.58	0.97	1.56[f]	S
ET3-3	0.53	1.62	2.15[e]	S
ET7-3	0.40	0.53	0.93[g]	NS
ET2-1	0.25	0.30	0.56[g]	NS
ET7-14	0.21	0.37	0.57[g]	S
$LSD_{0.05}$[h]	0.31	0.32	0.52	

[a] S, second-harvest yields were significantly greater ($P \leq 0.05$) than first-harvest yields, as determined by a paired t test; NS, first- and second-harvest yields were not significantly different.

[b,d,e,f,g] Values which are not followed by the same letter are significantly different ($P \leq 0.05$) as determined by Duncan's multiple-range test.

[c] Strain ET82-1 (= 162X95) is an inoculant quality strain.

[h] $LSD_{0.05}$, least significant difference ($P \leq 0.05$).

While most of the effective plant-isolate combinations produced at least twofold more dry matter during the second growth period than during the first growth period, the yields of four of the six least effective plant-isolate combinations did not increase significantly between the first harvest and the second harvest. Indeed, only one isolate, ET3-3, produced more than twofold more herbage dry weight. Although the overall effectiveness rankings of most isolates did not change between harvests, there was one notable exception. The yield of 162X95 was equivalent to the yields of the two superior strains (ET30-1 and ET30-3) at the first harvest, but this strain declined in its overall ranking during the second growth interval.

The total N_2 fixed by the plant-isolate combinations followed the trends observed with dry weights (Table 2). In addition, the N concentrations in the subclover herbage produced by ET 2, 3, and 7 isolates were substantially lower (26.0 to 32.2 mg of N per g) than the N concentrations in the herbage produced by isolates representing the minor nodule-occupying ETs (35.4 to 38.6 mg of N per g). Nevertheless, the amount of N_2 fixed by some plant-isolate combinations during the interval between the first harvest and the second harvest was up to threefold greater than the amount of N_2 fixed before the first harvest. As a result, the concentrations of N in second-harvest herbage of plants nodulated by ET 3, 7, and 8 isolates were not significantly less than the concentrations in high-yielding plant-isolate combinations.

Since isolates belonging to one of the major nodule-occupying lineages, ET 2, were not adequately represented in the

TABLE 2. Symbiotic effectiveness potential (amount of N_2 fixed and herbage N concentration) of *R. leguminosarum* bv. trifolii isolates which represent major and minor nodule occupants of field-grown subclover

Isolate	Kjeldahl digestible nitrogen in herbage				Total N_2 fixed (mg plant^{-1})
	Harvest 1		Harvest 2		
	mg plant^{-1}	mg g^{-1}	mg plant^{-1}	mg g^{-1}	
Minor field nodule-occupying ETs					
ET30-1	60	38.6	153[a]	45.2	213[b]
ET30-3	54	35.4	149[a]	44.6	203[b]
ET82-1	44	36.3	114[a]	46.2	158[c]
ET15-2	43	36.4	128[a]	46.2	171[c]
ET31-5	44	37.3	117[a]	45.3	161[c]
ET17-1	38	38.0	118[a]	45.0	156[c]
Major field nodule-occupying ETs					
ET3-8	22	28.6	67[a]	43.2	89[d]
ET8-2	21	34.2	23[e]	43.0	44[fg]
ET3-18	18	31.6	42[a]	42.7	60[f]
ET3-3	17	31.3	71[a]	43.6	88[d]
ET7-3	13	32.2	21[e]	42.1	34[g]
ET2-1	8	30.0	10[e]	33.0	18[g]
ET7-14	5	26.0	14[a]	37.7	19[g]
$LSD_{0.05}$[h]	13	3.6	15	2.7	25

[a] Second-harvest values for the amount of nitrogen per plant were significantly greater ($P \leq 0.05$) than first-harvest values, as determined by a paired t test.
[b,c,d,f,g] Values which are not followed by the same letter are significantly different ($P \leq 0.05$) as determined by Duncan's multiple-range test.
[e] First- and second-harvest values for the amount of nitrogen per plant were not significantly different.
[h] $LSD_{0.05}$, least significant difference ($P \leq 0.05$).

two-harvest experiment, another less extensive experiment was performed to assess the symbiotic potential of these organisms (Table 3). The data obtained confirmed that both ET 2 and ET 3 isolates are suboptimally effective on subclover despite their high occupancy of nodules on field-grown plants.

Competition experiments were performed in Leonard jar assemblies which gave the plants an opportunity to grow vigorously. Despite the fact that the three ET 3 isolates were not significantly different in symbiotic effectiveness, these organisms were different in competitive ability (Table 4). Although isolates ET3-1 and ET3-2 occupied significantly more nodules than all of their competitors except ET23-1, the results obtained with isolate ET3-3 were less consistent. Although ET23-1 was recovered from the field on only one occasion, it was equally competitive with each of the three ET 3 isolates under nonsoil conditions.

TABLE 3. Herbage dry matter yields of subclover plants nodulated by several ET 2 and 3 isolates, yield of plants nodulated by a strain that exhibited superior effectiveness (ET23-1), and yield of plants that were nitrate fed

Isolate or prepn	Herbage dry wt (g plant^{-1})
Nitrate fed	1.08[a]
ET23-1	0.91[b]
ET3-1	0.66[c]
ET3-3	0.64[c]
ET3-2	0.55[c,d]
ET2-1	0.45[c,d]
ET2-2	0.47[c,d]
ET2-3	0.44[c,d]
Uninoculated	0.03
$LSD_{0.05}$[e]	0.16

[a,b,c,d] Values which are not followed by the same letter are significantly different ($P \leq 0.05$) as determined by the Duncan's multiple-range test.
[e] $LSD_{0.05}$, least significant difference ($P \leq 0.05$).

A somewhat different situation was observed with three ET 2 isolates (Table 5). Although ET 2 isolates occupied a high proportion of nodules when they were mixed 1:1 with some competitors (ET33-2 and ET12-1), they could not prevent the

TABLE 4. Competitive nodulating abilities of three ET 3 isolates when they were tested with representatives of other ETs on cultivar Nangeela subclover

Paired strain combination[a]	% of nodules occupied by[b]:		Significance[c]
	ET 3 isolate	Competitor	
ET3-1 + ET2-1	81 (67–91)	25 (14–38)	S
ET3-2 + ET2-1	95 (95)	32 (19–43)	S
ET3-3 + ET2-1	58 (48–67)	42 (33–52)	NS
ET3-1 + ET8-1	80 (71–86)	20 (9–14)	S
ET3-2 + ET8-1	98 (95–100)	2 (0–5)	S
ET3-3 + ET8-1	86 (71–90)	24 (15–29)	S
ET3-1 + ET33-2	81 (67–91)	19 (10–33)	S
ET3-2 + ET33-2	84 (81–86)	17 (14–19)	S
ET3-3 + ET33-2	79 (57–95)	25 (14–43)	S
ET3-1 + ET12-1	84 (74–91)	16 (10–29)	S
ET3-2 + ET12-1	86 (81–91)	14 (10–19)	S
ET3-3 + ET12-1	62 (43–81)	38 (19–57)	NS
ET3-1 + ET23-1	38 (24–57)	62 (16–43)	NS
ET3-2 + ET23-1	36 (24–43)	68 (57–81)	NS
ET3-3 + ET23-1	30 (19–48)	70 (52–81)	NS

[a] The inoculant ratios ranged from 0.98:1 to 1:1.3.
[b] The nodule occupancy values are the means of four replicates and include the values for both singly occupied nodules and cooccupied nodules. The values in parentheses are the ranges of occupancy values.
[c] S, nodule occupancy value of the ET 3 isolate was significantly ($P \leq 0.05$) greater than nodule occupancy value of the competitor as determined by a paired t test; NS, values were not significantly different.

TABLE 5. Competitive nodulating abilities of three ET 2 isolates when they were tested with representatives of other ETs on cultivar Nangeela subclover

Paired strain combination[a]	% of nodules occupied by[b]:		Significance
	ET 2 isolate	Competitor	
ET2-1 + ET33-2	68 (48–95)	44 (33–52)	NS[c]
ET2-1 + ET33-2[d]	95 (91–100)	5 (0–10)	—[e]
ET2-2 + ET33-2	86 (76–91)	26 (24–29)	—[e]
ET2-3 + ET33-2	66 (57–76)	39 (24–48)	NS
ET2-1 + ET12-1	64 (57–71)	40 (29–48)	NS
ET2-1 + ET12-1[d]	94 (86–100)	6 (0–14)	—[e]
ET2-2 + ET12-1	63 (52–71)	37 (29–48)	NS
ET2-3 + ET12-1	71 (62–81)	29 (19–38)	—[e]
ET2-1 + ET23-1	12 (0–24)	93 (81–100)	—[f]
ET2-1 + ET23-1[d]	55 (38–76)	49 (24–62)	NS
ET2-1 + ET31-5	26 (14–31)	80 (76–90)	—[f]
ET2-1 + ET30-3	6 (5–11)	95 (90–100)	—[f]

[a] Inoculant ratios were approximately 1:1 except as indicated below.
[b] The nodule occupancy values are the means of four replicates and include the values for both singly occupied nodules and cooccupied nodules. The values in parentheses are the ranges of occupancy values.
[c] NS, no significant difference between the percentage of nodules occupied by ET2-1 and the percentage of nodules occupied by the competitor.
[d] Inoculant ratios were approximately 10:1 in favor of ET2-1.
[e] The ET 2 isolate occupied significantly more nodules than the competitor ($P \leq 0.05$).
[f] The competitor occupied significantly more nodules than the ET 2 isolate ($P \leq 0.05$).

competitors from also occupying large proportions of root nodules. When ET2-1 was given a modest (6:1 to 10:1) numerical advantage over isolates ET33-2 and ET12-1, it made significant gains in occupancy at the expense of the two competitors. In contrast, although occupancy by isolate ET2-1 increased when this organism was given a numerical advantage over ET23-1, the latter isolate still occupied a large percentage of nodules.

Although ET2-1 could not outcompete either of the more symbiotically effective competitors (ET23-1 and ET31-5), we observed a suppression of plant yield despite the large percentages of nodules occupied by the highly effective isolates. This yield-suppressing effect was examined in a more detailed experiment in which we used different inoculant ratios of ET2-1 and ET31-5 (Tables 6 and 7). When the inoculant ratio reached 10:1 in favor of ET2-1, the plant yield and amount of

TABLE 6. Influence of different inoculum ratios on nodule occupancy by isolates ET2-1 and ET31-5

Ratio of ET2-1 to ET31-5	% of nodules occupied by[a]:			Significance[b]
	ET2-1	ET31-5	Both isolates	
25:1	73 (58–86)	43 (20–68)	20	NS
10:1	32 (20–45)	78 (60–95)	13	S
1:1	10 (0–20)	100	10	S
1:10	0	100	0	S

[a] Nodule occupancy values for ET2-1 and ET31-5 are the means of four replicates and include the values for both singly occupied nodules and cooccupied nodules. The values in parentheses are the ranges of occupancy values.
[b] ET31-5 occupied significantly more nodules than ET2-1 ($P \leq 0.05$), as determined by a paired t test. NS, occupancy values were not significantly different.

TABLE 7. Influence of nodule occupancy by isolates ET2-1 and ET31-5 of *R. leguminosarum* bv. trifolii on subclover yield parameters

Ratio of ET2-1 to ET31-5	Herbage yield parameters		Total N (mg plant^{-1})
	Dry wt (g plant^{-1})	N concn[a] (mg g^{-1})	
ET2-1	0.12	21.1	2.5
25:1	0.22	32.0	7.0
10:1	0.61	37.0	22.6
1:1	0.74	37.5	27.8
1:10	0.95	34.9	33.2
ET31-5	0.98	40.0	39.2
$LSD_{0.05}$[b]	0.15		

[a] N concentrations were determined by using a composite sample from each treatment.
[b] $LSD_{0.05}$, least significant difference ($P \leq 0.05$).

N_2 fixed values were between 30 and 50% less than the values observed with plants nodulated by ET31-5 alone, despite the fact that only 20 to 45% of the nodules were occupied by ET2-1. With an inoculant ratio of 25:1, both the yield and the amount of N_2 fixed values were similar to values for plants nodulated by ET2-1 alone, even though 43% of the nodules were occupied by ET31-5.

DISCUSSION

Our findings have bearing on a number of unresolved issues concerning nitrogen fixation in subclover in particular and interstrain competition for nodulation in general. Despite the importance of subclover to the productivity of a large area of pastures on hill and range lands in the far western United States, there have been several previous reports which have shown that field-grown subclover in this region is nodulated primarily by rhizobia that exhibit suboptimal symbiotic effectiveness (13, 25, 27, 30). Furthermore, agronomists have reported that the N content of subclover herbage grown throughout Oregon and California is erratic and can range from 2 to 4.5% (1, 16, 31, 40, 45). Our observations that the nodule-dominant isolates on the Abiqua soil site fix 2- to 10-fold less N_2 on subclover than minor occupants fix and produce first-harvest herbage that has suboptimal protein concentrations complement the observations described above. Since suboptimally effective nodule-dominant isolates from another site (13) also belong to the same chromosomal lineage as the isolates described in this study (32a), it will be important to determine how widespread the occurrence of subclover nodulated primarily by strains belonging to this group is.

Despite the fact that there is an extensive literature on the subject of competitive nodulation, we have little understanding of the factors which influence this phenomenon under soil conditions (6, 24). One major problem has been the lack of laboratory studies performed with isolates that have been proven to be nodule-dominant types on field-grown plants. In this study we showed that such isolates (ET 2 and 3 isolates) are not necessarily more competitive than isolates which are minor nodule occupants on field-grown plants at the same location. Our findings agree with those of other workers who have shown that strains which dominate field nodules are not necessarily more competitive than minor nodule occupants when the organisms are evaluated under environmentally controlled nonsoil conditions (12, 36). Nevertheless, we cannot exclude the possibility that enhanced competitiveness is an

unstable trait which is easily lost during subculturing after isolation from nodules. It is well documented that symbiotic effectiveness is unstable in some rhizobial strains (23), and both genomic instability and plasmid instability have been observed in *R. leguminosarum* bv. phaseoli (10, 19, 43). In addition, since plasmids play a role in nodulation competitiveness (8, 9, 11, 35) and plasmid diversity occurs in *R. leguminosarum* isolates having the same chromosomal type (32, 44, 55), the possibility that there is intra-ET variation in competitiveness seems likely.

It is intriguing to speculate about reasons why there are isolates which exhibit enhanced competitiveness and yet are minor occupants of field-grown plants. It is possible that field conditions suppress the inherent competitive potential of these bacteria. Several reports have shown that soil temperature and soil pH can modify the nodulating success of strains of various *Rhizobium* species on soil-grown plants (17, 24, 26, 41, 47, 51, 53, 54). Equally plausible, however, is the possibility that the highly effective competitive strains are nonrandomly distributed in the soil at a site because they are recent immigrants or because they represent the remnants of a previously well-distributed population that is now in decline. Alternatively, these strains may represent rare intrasite recombinants that have not had the opportunity to disperse. In an accompanying paper (32b), Leung et al. show that some serotypes were rare nodule occupants in most areas of a field but occupied substantially more nodules in one or two localized sites within the field. More work is needed to discriminate between soil factors and nonrandom distribution as explanations for the lack of competitive types in field nodules.

Finally, our observations on yield suppression by isolate ET2-1 could provide insight into how suboptimally effective strains might exert a negative effect on legume growth in the field. Previous studies have described antagonistic effects on clover growth when plant nodules were occupied by more than one rhizobial strain (3, 14, 30). Demezas and Bottomley (14) noted that subclover plants grew suboptimally when their nodules were occupied by suboptimally effective strain WS2-01 despite the fact that 50% of the nodules on the same plants were occupied by strain WS1-01 which exhibited superior effectiveness. Additional studies will be needed to determine whether the lower yield obtained was simply the result of the fact that a high proportion of nodules were occupied by a strain with poor N_2-fixing ability or whether it was due to an antagonistic property of ET2-1 directed either at plant metabolism or at the metabolism of the other nodule-occupying strain. There have been reports of strains of *Bradyrhizobium japonicum* (48) and *Rhizobium tropici* (39) that have negative effects on legume growth, as well as a report of a strain of *R. leguminosarum* bv. trifolii that inhibits the growth and nodulating ability of other strains of the same biovar (49).

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