Interstrain Competition between Representatives of Indigenous Serotypes of *Rhizobium trifolii*†

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The symbiotic characteristics of *Rhizobium trifolii* strains 1-01 and 2-01 were evaluated both individually and in various combinations on two cultivars (Mt. Barker and Woogenellup) of subterranean clover (*Trifolium subterraneum* L.). Nodules were observed on day 8 independent of cultivar or strain. Cultivar differences were measured in nodulating efficiency by 1-01 since 54% of the primary nodules were formed on cv. Mt. Barker and only 15% were formed on cv. Woogenellup in the zone above, or 1 cm below, the root tip location at the time of inoculation. The percentage of nodules formed in this zone by 2-01 was similar on both cultivars (31 to 32%). When mixtures of strains 1-01 and 2-01 (230:1 and 1:20) were used to inoculate plants, >90% of the nodules on both cultivars were occupied by the more abundant strain in the inoculum regardless of sampling date (4 or 8 weeks). In contrast, large percentages of nodules on 4-week-old plants of both cultivars exposed to a 5:1 inoculum mixture were doubly occupied (64 and 74%). By week 8 these values had decreased significantly ($P \leq 0.01$) and were accompanied by large increases in the percentage of nodules occupied by either strain 1-01 alone (1 to 65%) on cv. Mt. Barker or 2-01 alone (4 to 49%) on cv. Woogenellup. The superior (cv. Mt. Barker) and inferior (cv. Woogenellup) symbiotic performance of plants inoculated with the 5:1 mixture correlated more closely with the 8-week than the 4-week nodule occupancy data. Primary nodule occupancy by 1-01 and 2-01 was significantly influenced by changes in the inoculum ratios of 1-01/2-01 from 5.7:1 to 0.67:1 on cv. Mt. Barker and from 1.9:1 to 0.67:1 on cv. Woogenellup. Despite evidence for extensive proliferation of the inoculant strains on the rhizoplanes, no evidence was obtained for either interstrain antagonism or selective proliferation as a valid reason to explain the outcome of primary nodulation.

Interstrain competition for nodulation between members of indigenous soil populations of *Rhizobium* spp. is a poorly understood aspect of *Rhizobium* ecology. Evidence has been summarized which correlated the nodulating success of particular indigenous serogroups of *Bradyrhizobium japonicum* with abiotic soil factors (14). Little information, however, has been forthcoming on the mechanism(s) which confers the competitive attribute to those highly successful serogroups. Studies carried out under controlled conditions in an attempt to elucidate the mechanism(s) which allows serogroup 123 to be a particularly dominant nodule occupant of soybeans have been equivocal. The serotype strain of *B. japonicum* serogroup 123, USDA 123, has failed to be as competitive under defined conditions as members of serogroup 123 are in the soil (17). Since serogroup 123 is known to be heterogeneous (13), the "type" strain might not be representative of the serogroup when competitive attributes are being evaluated.

We have previously identified two serotypes of *Rhizobium trifolii*, namely, 1-01 and 2-01, both of which occupied large percentages of nodules on field-grown subclover cv. Mt. Barker. Characterization of members within each of these two serotypes showed them to be homogeneous in several aspects (8). Representatives from these serotypes were selected for use under controlled growth conditions to evaluate the following questions raised as a result of soil studies described elsewhere (9). First, the population of serotype

2-01 in the majority of rhizosphere soil samples outnumbered that of 1-01 by as much as 8:1. Samples were found, albeit less frequently, where serotype 1-01 outnumbered 2-01 by 2:1. It was of interest to know whether such small differences between the numbers of the two serotypes could have been a factor influencing the outcome of primary nodulation. Second, the soil study had shown that nodule occupancy by serotype 1-01 on cv. Mt. Barker increased with plant age along with the percentage of nodules coinhabited by either serotype 1-01 or 2-01 and nonidentifiable strains. Experiments were carried out under controlled growth conditions with the following objectives: (i) to determine if the high frequency of coinhabited nodules and their dynamic nature could be detected by two different methods of strain identification (immunofluorescence and intrinsic antibiotic resistance) and on plants grown in non-soil (vermiculite and growth pouch)-rooting environments; and (ii) to determine if there might be an influence of coinhabited nodules on the symbiotic performance of the plants since members of serotype 1-01 were known to be of superior effectiveness to those of serotype 2-01 (8).

MATERIALS AND METHODS

***Rhizobium* strains.** The isolates of *R. trifolii* used throughout this study, namely 1-01 and 2-01, are representatives of two serotypes which have been previously described. Members within each of serotypes 1-01 and 2-01 have been shown to be very homogeneous with respect to growth and serological characteristics, sodium dodecyl sulfate-polyacrylamide gel electrophoresis protein profiles, and symbiotic effectiveness on cultivars of subterranean clover (8).

Evaluation of nodulating ability of strains 1-01 and 2-01 on subclover cv. Mt. Barker and Woogenellup. (i) Inoculum

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production. To obtain cultures of 1-01 and 2-01 in a similar physiological state and growth phase, cells were grown from slants in defined medium (10) at a temperature (25°C) at which both strains maintain suboptimum but identical generation times (4.5 h). Small portions (30 µl) of these cultures were back-transferred to 50 ml of fresh broth and grown for 54 h to the late exponential phase of growth. The cells were harvested by centrifugation, washed in sterile 0.15 M phosphate buffer (pH 7.2), and suspended to an optical density of 0.5 at 660 nm in a 1-cm-path length cuvette. These suspensions, containing $1 \pm 0.06 \times 10^9$ cells per ml, were serially diluted in ice-cold, one-half-strength plant growth solution, pH 5 ± 0.1 (10), to a final density of approximately 10^4 cells per ml and maintained in an ice bath to minimize further cell proliferation.

(ii) Plant inoculation and growth conditions. Surface-sterilized seeds of subclover cv. Mt. Barker and Woogenellup were germinated by incubation at 4°C for 48 h, followed by 24 h at room temperature (4). Thirty steam-sterilized growth pouches (Northrup King, St. Paul, Minn.), previously wetted with 9 ml of one-half-strength plant growth solution, were each sown with four seedlings of either cv. Mt. Barker or cv. Woogenellup. Seedlings were chosen based on uniformity of radicle length (1.5 cm). Approximately 1,000 cells of either 1-01 or 2-01 were inoculated onto the radicle of each plant in 15 pouches of each cultivar. The inoculum size was derived from knowing the weight of rhizosphere soil associated with soil-grown seedlings and the population densities of the indigenous serotypes in rhizosphere soil (9). The positions of the root tips were marked on the outside of the pouches at this time and were used as reference points for scoring nodulation at a later date (3). The pouches were divided into three replicates each consisting of five pouches per cultivar per inoculant strain, placed in restraining sleeves constructed of screen gauze, and suspended in water baths at $25 \pm 0.2^\circ\text{C}$ such that the root radicles were below the water level. Noninoculated pouches of seedlings of each cultivar were also included as contamination controls. Shoot temperature was maintained between 22 and 25°C for the duration of the experiment. A light intensity of approximately $200 \mu\text{Einstein m}^{-2} \text{ s}^{-1}$ was supplied from a combination of fluorescent (F30T12-D-RS; Sylvania, Danvers, Mass.) and incandescent (25-W Soft lite; General Electric Co., Cleveland, Ohio) bulbs suspended 30 cm above the pouches during a 14-h light/10-h dark cycle. Sterile distilled water was added to the pouches every other day in quantities just sufficient to saturate the paper towels.

(iii) Appraisal of nodulation. Eight days after inoculation, the numbers and positions of nodules on the seedling roots were recorded.

Evaluation of nodulating and nitrogen-fixing abilities of combinations of strains 1-01 and 2-01. **(i) Preparation of inocula.** Strains 1-01 and 2-01 were grown and harvested as described above. Appropriate portions of each strain were mixed together in an attempt to obtain three ratios of 1-01 and 2-01 equal to 100:1, 1:1, and 1:100. The actual compositions of the mixtures were determined by plate counting the final dilutions of each strain prior to mixing them (see Table 1).

(ii) Plant inoculation and plant growth conditions. Portions of sterile vermiculite, adjusted to pH 5.2, were inoculated individually with the various mixed suspensions of 1-01 and 2-01 to provide a total cell density of approximately 10^5 per g of vermiculite. Fourteen sterile modified Leonard jar assemblies, complete with plant growth solution adjusted to pH 5.2 (7), were each filled with a specific vermiculite

inoculum mixture. Treatments consisting of vermiculite inoculated with either strain 1-01 and 2-01 alone and noninoculated controls also were included.

Seeds of subclover cv. Mt. Barker and Woogenellup were surface sterilized and germinated as described above. Seven Leonard jars per inoculum mixture were each sown with three seedlings of cv. Mt. Barker and another seven jars were each sown with three seedlings of cv. Woogenellup. The seeds were covered with 1.5 cm of sterile perlite, and the jars were arranged in a completely randomized design in a greenhouse under daylight illumination. Daytime temperatures ranged from a high of 30°C to a lower limit of 15°C at night. Three and four of the seven replicates were harvested after 4 and 8 weeks, respectively. Shoot dry weights were obtained after drying at 60°C for 5 days, and total shoot nitrogen was determined after Kjeldahl digestion and ammonia distillation.

(iii) Nodule occupant analysis. Nodules were surface sterilized by immersing the intact root systems of plants for 30 s into an aqueous solution (0.2%, vol/vol) of Tween 20 (Sigma Chemical Co., St. Louis, Mo.), followed by 95% (vol/vol) ethanol for 10 min and then by five successive rinses in sterile distilled water. Ten nodules were selected at random from each plant at the week 4 and 8 harvests, and occupancy was evaluated by immunofluorescence as described elsewhere (9).

Intrinsic antibiotic resistance characteristics of strains 1-01 and 2-01. Cultures of strains 1-01 and 2-01 were challenged against a broad spectrum of antibiotics to determine their intrinsic antibiotic resistance characteristics. The antibiotics used were as follows (milligrams per liter): ampicillin (2.5, 5), kanamycin sulfate (2.5, 5), nalidixic acid (5, 10) novobiocin sodium salt (1, 2.5), rifampin (1, 3), spectinomycin dihydrochloride (1, 2.5), streptomycin sulfate (1, 2.5), vancomycin hydrochloride (1, 2.5), and erythromycin (2.5, 5) (Sigma Chemical Co.). Stock solutions were made in sterile distilled water, with the exception of erythromycin and rifampin, which were both dissolved in small volumes of 95% ethanol, and nalidixic acid, which was dissolved in a small volume of 1 M NaOH. All of the stock solutions were filter sterilized (0.4-µm pore size; Nuclepore Corp., Pleasanton, Calif.), and appropriate volumes were added to 100-ml portions of sterile yeast extract-mannitol agar (26) held at 49°C to produce the desired final concentration. Procedures for analysis of antibiotic resistance were as described previously (16).

Influence of "rhizosphere-like" ratios of strains 1-01 and 2-01 on the outcome of competitive nodulation of subclover cultivars. **(i) Inoculum production.** Cultures of strains 1-01 and 2-01 were grown, harvested, and suspended as described above to a final density of approximately 10^5 cells per ml. An attempt was made to produce cell suspensions containing the following ratios of 1-01 to 2-01: 10:1, 5:1, 1:1, 1:5, and 1:10. Enumeration of each strain was first carried out microscopically with a hemacytometer. Appropriate volumes of the individual cell suspensions were mixed, and additional plate counts were obtained both on the original cell suspensions prior to mixing and on all mixed inocula, using yeast extract-mannitol agar supplemented with antibiotics. The actual ratios of the five inoculum mixtures are reported in Table 3.

(ii) Plant inoculation and growth conditions. Forty-five steam-sterilized growth pouches were each sown with four surface-sterilized seedlings of either cv. Mt. Barker or cv. Woogenellup. Each plant within nine pouches of each cultivar was inoculated with 10 µl of a particular inoculum

suspension of 1-01 and 2-01 containing between 1,000 and 1,400 total cells. The positions of the root tips of the seedlings were marked as described previously. The pouches were divided into three replicates each consisting of three pouches per cultivar per inoculum treatment and incubated under the water bath growth conditions described above. Pouches containing noninoculated seedlings of each cultivar were included in each replicate.

(iii) **Nodule occupant determination.** Three weeks after inoculation, the nodules were harvested, surface sterilized, and squashed. Identification of occupants was determined by transferring small portions of the homogenates to yeast extract-mannitol agar plates containing streptomycin (2.5 mg/liter), rifampin (1 mg/liter), or no antibiotic and incubating at 25°C.

(iv) **Final rhizoplane populations of strains 1-01 and 2-01.** Duplicate samples of nodule-free root segments (10 to 50 mg, dry weight) were placed into bottles containing 50 ml of 0.15 M phosphate buffer (pH 7.2), 0.5% (vol/vol) phenol, and 5 g of glass beads (3-mm diameter). These were shaken for 15 min on a wrist-action shaker, and the numbers of strains 1-01 and 2-01 were determined by immunofluorescence as described elsewhere (9).

TABLE 1. Effect of varying inoculum ratios on symbiotic performance of cv. Mt. Barker and Woogenellup grown in vermiculite

Cultivar	Inoculum composition (1-01/2-01)	Shoot dry wt (g per plant) ^a	Kjeldahl-N (mg per plant) ^b
Mt. Barker	1-01 alone	0.98 ± 0.13	38.1
	230:1	0.82 ± 0.07	28.9
	5:1	0.80 ± 0.10	30.1
	1:20	0.35 ± 0.05	11.7
	2-01 alone	0.27 ± 0.06	9.4
Woogenellup	1-01 alone	0.63 ± 0.24	23.8
	230:1	0.78 ± 0.09	29.0
	5:1	0.24 ± 0.04	8.5
	1:20	0.20 ± 0.08	7.5
	2-01 alone	0.17 ± 0.03	6.0

^a Shoot dry weight was determined for 8-week-old plants after drying for 5 days at 60°C.

^b Kjeldahl-N was determined for shoots of 8-week-old plants.

RESULTS

The results of experiments to determine the nodulating behavior of the two representative serotype isolates, 1-01 and 2-01, provided no evidence for differences in time of nodule appearance by either strain on either cv. Mt. Barker or cv. Woogenellup. Nodules could be observed clearly 8 days after inoculation, independent of the strain or cultivar. Observations, however, on the position of nodules suggested cultivar differences with regard to the efficiency of nodulation by strain 1-01 (Fig. 1). Of the total nodules on cv. Mt. Barker and Woogenellup, 54 and 15%, respectively, were formed above or within 1 cm below the initial root tip mark by strain 1-01. In contrast, similar percentages (31 and 32%) of the total nodules on cv. Mt. Barker and Woogenellup, respectively, were formed in the same region by strain 2-01. There was no significant cultivar or strain influence on the number of nodules per plant, the average of which ranged from 3.3 to 4.1.

There was no evidence for a cultivar effect when the strains were inoculated singly onto plants. Although strain 1-01 was symbiotically superior to strain 2-01 on both cultivars (Table 1), differences in symbiotic effectiveness were observed when a mixture containing a specific proportion of 1-01 to 2-01 (5:1) was used as the inoculant. Plants of both cultivars inoculated with a 230:1 excess of 1-01 over 2-01 produced shoot dry weights and nitrogen contents similar to plants of both cultivars inoculated with strain 1-01 alone. The mean shoot dry weight and nitrogen content of the opposite extreme treatment (1:20) resulted in an inferior symbiotic trait similar to that of plants of both cultivars nodulated by strain 2-01 alone. However, the symbiotic effectiveness response to the intermediate inoculum mixture of a 5:1 excess of 1-01 over 2-01 varied depending on the cultivar. In the case of cv. Mt. Barker plant yields and nitrogen content were similar to those of plants inoculated with strain 1-01 alone. On cv. Woogenellup, the 5:1 ratio resulted in a symbiotic performance equivalent to that of the symbiotically inferior strain 2-01.

Most of the nodule occupancy data are supportive of the symbiotic performance data described above (Table 2). For example, when there was an excess of either strain 1-01 (230:1) or 2-01 (20:1), the more numerous strain was the single occupant of ≥91% of the nodules independent of cultivar or sampling date. However, the occupancy data

FIG. 1. Efficiency of nodulation by *R. trifolii* strains 1-01 and 2-01 on subclover. (A and B) cv. Mt. Barker and Woogenellup, respectively, inoculated with strain 1-01; (C and D) cv. Mt. Barker and Woogenellup, respectively, inoculated with strain 2-01. Arrows indicate positions of root tips at the time of inoculation.

TABLE 2. Effect of varying inoculum ratios on nodule occupancy by strains 1-01 and 2-01 on two cultivars of subclover^a

Cultivar	Sample date (wk)	Inoculum composition (1-01/2-01)	No. of nodules analyzed	% ^b of strain:		
				1-01	2-01	M ^c
Mt. Barker	4	230:1	77	95	5	0
		5:1	82	1	35	64
		1:20	67	6	94	0
	8	230:1	76	97	3	0
		5:1	88	65***	18*	17**
		1:20	90	4	92	4
Woogenellup	4	230:1	88	99	1	0
		5:1	89	21	4	75
		1:20	79	1	99	0
	8	230:1	90	97	2	1
		5:1	65	17NS	49**	34**
		1:20	89	9	91	0

^a Plants were grown in vermiculite, and nodule occupancy was determined by immunofluorescence.

^b A positive occupancy contained ≥ 10 fluorescing cells per field of view, equivalent to a minimum of 10^5 to 10^6 per nodule.

^c M, Co-occupied nodules.

^d One-way analysis of variance carried out on the arcsine $\sqrt{\%}$ transformed data from the 4- and 8-week sample dates for each cultivar (** $P \leq 0.01$; * $P \leq 0.05$; NS, not significant).

obtained as a result of the intermediate mixture (5:1) provided further evidence of differences between the two cultivars in their interactions with the two strains. A strikingly large and similar percentage of the nodules formed on 4-week-old plants of cv. Mt. Barker (64%) and Woogenellup (75%) were occupied by both strains 1-01 and 2-01 (Table 2). In contrast these values were significantly lower by week 8 and had been accompanied by significant increases ($P \leq 0.01$) in the percentages of nodules occupied by 1-01 alone on cv. Mt. Barker (1 to 65%) and 2-01 alone (4 to 49%) on cv. Woogenellup.

The outcome of primary nodulation between mixtures of the two strains could be influenced by even smaller ratios of strains 1-01 and 2-01 which more accurately reflected the ratios of the two serotype populations actually observed in rhizosphere soil (Table 3). In this case, intrinsic antibiotic resistance provided the means for identification. Although both strains were extremely susceptible to all antibiotics tested, rifampin (1 mg/liter) and streptomycin (2.5 mg/liter) were successfully used as positive selectors for 1-01 and 2-01, respectively.

The complete dominance by strain 1-01 over 2-01 ob-

served earlier with a 230:1 mixture was also seen with a much smaller excess of 1-01 over 2-01 (11.4:1) (Table 3). However, as the ratio of 1-01/2-01 was decreased, the number of nodules occupied singly by strain 1-01 decreased, regardless of the cultivar. Significant changes ($P = 0.05$) were observed on cv. Woogenellup even when the ratio was changed from only a 1.9:1 excess of 1-01 to a 0.67:1 deficit in favor of 2-01. In this particular case single occupancy by strain 1-01 declined significantly from 61 to 17% and was replaced stoichiometrically with an equivalent number of nodules occupied by both strains. For cv. Mt. Barker, single occupancy by 1-01 declined significantly when the ratio was changed from 5.7:1 to 0.67:1. Concomitant increases were observed in the number of nodules occupied by 2-01 alone and by both organisms.

After the nodules were harvested at 21 days, the rhizoplane populations of 1-01 and 2-01 were determined on root segments devoid of nodules (Table 4). During the 3 weeks in the presence of the host rhizosphere, a considerable amount of proliferation had taken place. As much as a 600-fold increase had occurred in the density of the original individual strain populations applied to the roots, with the

TABLE 3. Effect of rhizosphere-like ratios in inoculum mixtures of strains 1-01 and 2-01 on nodule occupancy on cv. Mt. Barker and Woogenellup

Cultivar	Inoculum ratio (1-01/2-01)	No. of nodules per plant	No. of nodules analyzed	% of strain ^a :		
				1-01	2-01	M ^b
Mt. Barker	11.4	3.9	16	100a ^c	0a	0a
	5.7	3.2	32	78a	6ab	16ab
	1.9			ND	ND	ND
	0.67	3.0	54	34b	26b	40b
	0.17	2.3	32	38b	19b	43b
Woogenellup	11.4	2.9	31	97a	0a	3a
	5.7	2.7	38	76ab	11a	13a
	1.9	4.2	36	61b	3a	36b
	0.67	4.0	63	17c	3a	80c
	0.17	4.1	82	16c	7a	77c

^a Nodule occupancy was determined with rifampin (1 mg/liter) and streptomycin (2.5 mg/liter) to identify strains 1-01 and 2-01, respectively.

^b M, Co-occupied nodules.

^c Percentages within a column and not followed by the same letter differ significantly from each other ($P = 0.05$) according to Duncan's new multiple range test. Each cultivar was analyzed separately.

TABLE 4. Comparison of population densities of strains 1-01 and 2-01 in the initial inoculum and on the rhizoplanes of 21-day-old seedlings

Cultivar	Initial inoculum ratio (1-01/2-01)	Rhizoplane density (10 ⁶ cells per g of root dry wt) ^a		Final rhizoplane ratio (1-01/2-01)	P ^b
		1-01	2-01		
Mt. Barker	11.4	3.2 (0.25) ^c	3.4 (0.02)	0.94	<0.01
	5.7	110.0 (0.24)	21.0 (0.04)	5.2	NS
	1.9	4.2 (0.15)	1.9 (0.08)	2.2	NS
	0.67	20.3 (0.08)	77.0 (0.12)	0.26	NS
	0.17	9.8 (0.03)	55.0 (0.17)	0.18	NS
Woogenellup	11.4	29.0 (0.31)	6.3 (0.03)	4.6	NS
	5.7	15.0 (0.29)	8.4 (0.05)	1.8	NS
	1.9	12.0 (0.18)	6.9 (0.09)	1.7	NS
	0.67	11.0 (0.10)	41 (0.14)	0.27	NS
	0.17	4.1 (0.03)	100 (0.19)	0.04	NS

^a Rhizoplane densities were determined by immunofluorescence.

^b Probability that the final rhizoplane ratio differed from the initial inoculum ratio as determined by chi-square analysis (df = 1). NS, Not significant.

^c Density values in parentheses are those of the strains in the original inoculant mixtures.

final population sizes in all treatments ranging from 10⁶ to 10⁸ cells per g of root dry weight. In only one of the 10 inoculum mixture treatments did the final ratio of the two strains deviate significantly ($P \leq 0.01$) from the original ratio. In this particular case (11.4, cv. Mt. Barker), although there had been sufficient imbalanced increase in the numbers of strain 2-01 relative to 1-01 to result in a slight excess of the latter over 1-01, this had not influenced the outcome of nodulation since 2-01 did not occupy any nodules.

DISCUSSION

Several aspects of the data reported here support hypotheses speculated upon from the soil rhizosphere study (9). In other instances, however, the data obtained under nonsoil conditions did not complement the phenomena observed in soil. For example, although these data confirmed the increase in nodule occupancy by 1-01 on cv. Mt. Barker as the plants matured, the increase in occupancy by 2-01 as cv. Woogenellup plants aged was not observed on soil-grown plants. The data confirmed that subclover nodules could be extensively coinhabited and were not an artifact of the method of determination or the rooting matrix. Whereas co-occupancy in soil-grown plants increased with plant age only on cv. Mt. Barker, in these nonsoil studies extensive co-occupancy was observed in primary nodules of both cultivars and declined with plant age.

The only previous study reported in the literature and designed with the rationale used here involved USDA 123 as a type strain of serogroup 123 of *B. japonicum*. The latter organism was found to be inferior to strain USDA 110 at forming nodules on soybeans under defined conditions (17). These findings are in marked contrast to the outcome of nodule occupancy observed when indigenous serogroups 123 and 110 are compared in soil-grown soybeans (11, 17, 21, 24). Our findings are similar, since under nonsoil conditions 1-01 competitively nodulated against 2-01 regardless of cultivar, and, within population ratios representative of those found in the rhizosphere soil, was not excluded from primary nodules by excess of 2-01. Obviously, the nodulating behavior of 1-01 in soil is quite different from that expressed under nonsoil conditions. Moreover, our data suggest that attention should be given to competitive phenomena occurring after nodule initiation as well as to the

more traditional sites, i.e., in the rhizosphere prior to infection.

Some interesting effects were observed in our study which are worthy of discussion in light of methods commonly used for evaluating competition. Recent studies have shown that the symbiotic performances of alfalfa (1, 15) and white clover (22) were correlated with the percentage of nodules occupied by effective strains. Indeed, Amarger (1) concluded that the shoot dry weight of the plant could be used as a means to evaluate the competitive ability of effective strains mixed with ineffective strains. The large percentage of coinhabited nodules observed in our studies as a result of the intermediate inoculum mixture (Table 2), the change with time in this value, and the profound differences observed between cultivars in symbiotic performance (Table 1) should make others wary of this approach for assessing the competitiveness between strains upon different hosts. Furthermore, we second the recent opinion of Bushby (6) that a single nodule harvested is ill-advised for assessing nodulation success, especially when these findings are then related to symbiotic performance.

Others have reported that large percentages of co-occupied clover nodules are an artifact of nonsoil substrata (5, 20). We have confirmed in both vermiculite and growth pouches the high incidence of co-occupied nodules reported previously in soil-grown subclover plants (9). Our data are in agreement with recent literature where as much as 36 to 38% of the total nodules from lentils grown in either vermiculite or soil were found to be co-occupied by highly competitive strains of *R. leguminosarum* (19). When the two experiments reported in this study are taken as a whole, large percentages of co-occupied nodules were detected on plants exposed to ratios of 1-01/2-01 ranging from 5:1 (experiment 1) to 0.17:1 (experiment 2) and not when ratios were in excess of 11.4 or 1:20. These observations suggest that the frequency of coinhabited nodules observed in the soil rhizosphere study (9) may have been due to the narrow range between these two serotype populations (2:1 to 0.13:1) and undoubtedly other unidentified *R. trifolii* indigenous to Whobrey soil.

Previous experiments to ascertain the effect of inoculum or rhizosphere ratios on relative nodule occupancy often evaluated ratios of the different strains which ranged over as much as 8 to 10 log₁₀ units (2, 12, 25). In this study, we used ratios and absolute population sizes of the two organisms

which equalled those values observed in the soil. The large ranges used by previous workers may not be realistic and could be providing misleading concepts on competitive phenomena for soil-grown legumes.

Finally, it has been emphasized that rhizoplane populations of competing strains should always be evaluated since they can deviate substantially from the initial inoculum ratios and confound data interpretation (18, 23). In our studies, despite substantial proliferation, the ratios of the densities of 1-01 to 2-01 on the rhizoplanes of both cultivars after 21 days of growth were similar to the starting population ratios. No evidence was obtained from which one might invoke either growth antagonism between the strains or selective enrichment by either cultivar toward either strain as a valid reason for nodulation success or failure.

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