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Influence of Location, Host Cultivar, and Inoculation on the Composition of Naturalized Populations of *Rhizobium meliloti* in *Medicago sativa* Nodules†

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A phage typing system was used to evaluate the composition of indigenous populations of *Rhizobium meliloti* inhabiting nodules of *Medicago sativa* cultivars grown with and without inoculation at two field sites during 1983 and 1984. Soil at both locations contained established populations of *R. meliloti* at planting. Analysis of 1,920 nodule isolates revealed 55 unique phage types of indigenous *R. meliloti* at one site and 65 indigenous types at the other location. The distributions of phage types differed markedly between locations. At one site, the nodule population was dominated by two phage types; seven others occurred consistently but at lower frequency, and the remainder were encountered infrequently. No indigenous types predominated at the other location, although nine occurred more frequently than the remaining types. Indigenous *R. meliloti* predominated in nodules from inoculated plots at both sites, with inoculant recovery varying between 10 and 38% in each of two years. The frequency of occurrence of particular phage types at one location was significantly influenced by both *M. sativa* cultivar and inoculation. At this location, the interaction of cultivar and inoculation on the incidence of phage types suggests that the presence of an inoculant strain differentially affected nodule occupancy of *M. sativa* cultivars by members of the indigenous *R. meliloti* population. At both sites, the frequency of specific phage types differed between years. The data emphasize the importance of understanding the ecology and characteristics of indigenous *Rhizobium* populations as a prerequisite for elucidating problems of inoculant establishment and persistence in competitive situations.

Nodule induction at high frequency by introduced inoculant strains has been readily demonstrated in soils where indigenous rhizobia are deficient (3, 4, 31). However, most inoculated legume seed is sown into soils containing established *Rhizobium* populations, and in these situations there are reports of inoculant strains inducing the majority of nodules in the first year and of their progressive disappearance and replacement by indigenous rhizobia in succeeding years (9, 31, 35). In other instances, indigenous rhizobia have been a barrier to the successful introduction of inoculants, resulting in unacceptably low levels of establishment of the applied strains in the year of inoculation (17, 22, 26). Therefore, an understanding of the nature of indigenous populations of *Rhizobium*, of the factors that affect their distribution and dynamics, and of their role in inoculant strain competition and persistence is of considerable agricultural significance.

While serological techniques have often been used to demonstrate the heterogeneity of serogroups within nodule samples of indigenous *Rhizobium* spp. (21, 28), alternative methods of differentiation have shown that serology can underestimate the diversity of indigenous *Rhizobium* (10, 11, 13, 26). Nevertheless, serological analysis has provided information on the impact of environmental (6, 7, 15) and biotic (5) factors on the serogroup distribution of indigenous *Rhizobium japonicum* inhabiting nodules. More recently, separation of cellular proteins by gel electrophoresis has shown considerable variety within nodule populations of indigenous *R. japonicum* (26) and among isolates of indigenous *Rhizobium trifolii* (8, 10, 11).

Information on the diversity of indigenous *Rhizobium meliloti* in nodules of *Medicago sativa* is scarce. One report (18) showed heterogeneity of serogroups among 12 indigenous isolates from *Medicago* and *Melilotus* spp. grown at a single location. Another, more recent report with serology and protein profile patterns (19) showed that 32 cultures of indigenous *R. meliloti* from *M. sativa* grown at one site were heterogeneous and that two distinct groups of isolates dominated the nodule sample.

While the *M. sativa* host has been shown to influence the competitiveness of simple mixtures of strains (3, 16, 27), there are no published reports concerning the impact of the host plant on the composition of indigenous nodule populations of *R. meliloti*, the influence of field location, or the interaction and effect of introduced inoculant strains.

The objective of the present investigation was to evaluate the composition of nodule populations of indigenous *R. meliloti* as influenced by *M. sativa* cultivar, inoculation, and field site. This was facilitated by application of a phage typing system (24) which was reported to be suitable for differentiating large numbers of previously uncharacterized isolates of *R. meliloti*.

MATERIALS AND METHODS

Seed inoculation. The inoculum strain, *R. meliloti* 2011str^r, is resistant to 500 μg of streptomycin per ml and symbiotically effective on *M. sativa*; its derivation and other characteristics have been described previously (3). A culture of *R. meliloti* 2011str^r in yeast-extract mannitol (YEM) broth (3) was used to prepare inoculant in finely ground peat sterilized by gamma irradiation (30). Seeds of *M. sativa* cv. Apollo and *M. sativa* cv. Saranac, which had not been treated with pesticide, were inoculated and lime pelleted using 40% (wt/vol) gum arabic as

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adhesive (30); uninoculated seeds were coated with sterile peat without rhizobia and lime pelleted. All lime-pelleted seed was stored overnight at 4°C and then sown. The number of *R. meliloti* 2011str^r on inoculated seed at the time of sowing (5×10^4 per seed of each cultivar) was estimated from colony counts (36) on YEM agar containing streptomycin (300 μg/ml) and cycloheximide (150 μg/ml).

Site description. Field trials were carried out at two sites (A and B) approximately 2 km apart, located on the Central Experimental Farm, Ottawa, Ontario. The soil at site A was a Manotick sandy loam (pH 7.0, water) with moderately good drainage. The area had been used for horticultural purposes and had no previous history of *M. sativa* cultivation. Soil at site B was a Kars gravelly sandy loam (pH 6.1, water) with good drainage and had grown *M. sativa* on at least two occasions; at the time the trial was established, the area was being used for *M. sativa* cultivation. No records of inoculation practice had been kept, although all *M. sativa* plants sampled from the area were well nodulated. Soil nitrate and ammonium nitrogen levels, determined by specific ion electrode, were 60 and 6 (site A) and 45 and 4 (site B) mg/kg, respectively.

Trial establishment. Field trials at sites A and B were established in June 1983. A level area (14.5 by 8.5 m) at each location was selected, cleared of vegetation, ploughed, and disced. Basal applications of 20 and 40 kg of K per ha (as KCl) were applied at sites A and B, respectively; other mineral nutrients were adequate according to soil tests and fertilizer guide recommendations. Seeds of *M. sativa* cv. Apollo and *M. sativa* cv. Saranac, with and without inoculation, were hand sown at a rate of 5 kg/ha in rows 3 m long and 0.5 m apart. All planting equipment was cleaned with 70% alcohol between treatments. The experimental design at both sites was three randomized blocks of two main plots (6.5 × 1.5 m), each split between inoculated and uninoculated treatments (subplots). Each main plot consisted of four rows of one of the two *M. sativa* cultivars. Main plots and subplots were separated by 0.5-m paths and blocks were separated by 1-m paths sown with grass to minimize *R. meliloti* cross-contamination. Based on soil tests, the application of KCl was repeated in April 1984 at a rate of 40 kg of K per ha at each site.

Sampling and *R. meliloti* isolation. Nodules were collected and yield was estimated (shoot dry weight) at the onset of flowering in 1983 and 1984. Ten plants were taken at random from those dug from 1-m sections of one row free of edge effects (second row in 1983 and third row in 1984) of each replicate subplot at both sites. From each of these plants, 10 nodules were taken at random and dried over silica gel for subsequent isolation of *R. meliloti*; the shoots were removed and dried to constant weight at 80°C. All sampling operations in the experimental areas were performed with aseptic precautions. Composites of 30 soil samples, collected to a depth of 10 cm at each site, were used to estimate numbers of *R. meliloti* by the most-probable-number method (36) with *M. sativa* cv. Apollo and *M. sativa* cv. Saranac as test plants. The most-probable-number values for *R. meliloti* in each soil sample were averaged over *M. sativa* cultivars since both provided similar results. Immediately before sowing (1983) at sites A and B, most-probable-number values were, respectively, 7.8×10^3 and 84×10^3 per g of soil; at the onset of flowering (1984), these values for inoculated and uninoculated plots were, respectively, 130×10^3 and 310×10^3 (site A) and 130×10^3 and 170×10^3 (site B) per g of soil.

For isolation of *R. meliloti*, desiccated nodules were soaked in sterile water until turgid, surface sterilized (3), aseptically crushed, streaked on YEM agar, and grown at 28°C for 3 days. Forty isolates with well-separated colonies were taken at random from those representing each replicate subplot at each site and year, and a single colony from each isolate was transferred to slants of YEM agar in screw-capped bottles. Cultures from single colonies were stored at 4°C until required. All cultures tested were confirmed as *R. meliloti* by their ability to nodulate *M. sativa*.

Phage typing. Nodule isolates of *R. meliloti* were typed by the procedure of Lesley (24), using the same 16 phages at routine test dilution (RTD) × 10. This phage typing system was reported (24) to provide reproducible results with no change in phage sensitivity of *R. meliloti* after extended subculture or reisolation from nodules. Titers of stock phages, determined by standard methods (1), ranged from 4×10^8 to 2×10^{11} PFU per ml. Phage inoculation was modified from that described previously (24) by use of a multiple inoculator which simultaneously delivered samples (ca. 5 μl) of each typing phage to each plate. The multiple inoculator, mounted on a drill press stand, consisted of an assembly of 16 stainless-steel prongs (5-mm diameter) which could be lowered into corresponding wells (each containing the appropriate phage) in an aluminum block. The reaction of each phage with *R. meliloti* was scored as 0 for the absence of lysis and as 1 for discrete plaques and confluent and semiconfluent lysis. The reproducibility of phage sensitivity was confirmed by testing 100 *R. meliloti* nodule isolates at different times; each isolate provided an identical result on all occasions. Reference cultures of *R. meliloti* representing four distinct phage sensitivity patterns (phage types) were included with each batch of field isolates typed. There was no deviation in phage sensitivity of the reference cultures on any occasion.

Statistical treatment of the data. Data for the frequency of phage types of naturalized *R. meliloti* were analyzed by the weighted least-squares method of Grizzle et al. (14) as implemented in the computer program SAS PROC FUNCAT (32). The factors included in the analyses were *M. sativa* cultivar, inoculation treatment, and sample year together with their first- and second-order interactions. Since most *R. meliloti* phage types occurred at low frequency in individual replicates, only the total frequencies of the three replicates were analyzed. To examine the recovery of the inoculant strain, the frequencies of phage types of indigenous *R. meliloti* were pooled and subsets of the data were analyzed separately.

RESULTS

Phage typing revealed that 89 phage types, each with a unique phage sensitivity pattern, represented the 1,920 nodule isolates of *R. meliloti* from sites A and B in 1983 and 1984 (Table 1). Figure 1 illustrates the phage sensitivity patterns of six representative phage types of *R. meliloti*. Data for the distribution of phage types of *R. meliloti* inhabiting nodules of *M. sativa* cv. Apollo and *M. sativa* cv. Saranac grown at sites A and B are presented in Table 2. At both locations, indigenous *R. meliloti* were the predominant isolates from inoculated plots, with the inoculant (phage type 26) recovered from between 10 and 38% of the nodules sampled from both cultivars in each of 2 years. Totals of 55 and 65 indigenous phage types of *R. meliloti* were encountered at sites A and B, respectively. Although 31 of these phage types were common to both field locations, the distribution of indigenous types representing all inoculation, year, and cultivar treatment combinations at site A differed markedly

TABLE 1. Phage sensitivity patterns of *R. meliloti* from two locations[a]

Phage type	Phage sensitivity pattern	Phage type	Phage sensitivity pattern
1	1 0 1 0 1 1 1 0 1 1 0 1 0 1 0 1	46	1 0 0 0 1 1 0 0 0 1 0 0 0 1 0 0
2	1 1 1 0 1 1 1 0 1 1 0 1 0 1 0 0	47	1 0 1 0 1 0 1 0 1 0 0 1 0 1 0 1
3	0 0 1 0 0 1 1 0 1 0 0 0 0 0 0 0	48	1 0 1 0 0 1 1 0 1 1 1 1 1 0 1 0 0
4	0 0 1 1 0 1 1 0 1 1 0 1 0 0 0 0	49	0 0 1 0 0 0 1 0 1 0 0 1 0 0 0 0
5	1 0 0 1 0 0 0 0 0 0 0 0 0 1 0 0	50	0 0 0 0 0 0 0 0 0 0 0 1 0 0 0 0
6	1 0 1 0 1 0 1 0 1 0 0 1 0 1 0 0	51	0 0 0 0 0 0 1 0 0 0 0 0 0 0 0 0
7	1 0 0 0 1 0 0 0 0 0 1 0 0 1 0 0	52	0 0 0 0 0 0 0 0 0 1 0 0 0 0 0 1
8	1 0 0 0 1 0 0 0 0 0 0 1 0 1 0 0	53	1 0 1 0 1 1 1 1 1 1 0 0 0 1 0 0
9	1 0 1 0 1 0 1 0 1 0 1 1 0 1 0 0	54	0 0 1 0 0 0 1 0 0 1 0 1 0 0 0 0
10	1 0 1 0 1 0 1 0 0 1 0 0 0 1 0 1	55	1 0 1 1 1 1 1 0 1 0 0 1 0 1 0 0
11	1 0 0 1 1 0 0 0 0 0 0 0 0 1 0 0	56	0 0 0 0 0 0 0 1 0 0 0 0 0 0 0 0
12	1 0 1 1 1 0 1 0 1 0 0 0 0 1 0 0	57	1 0 0 0 1 0 0 0 0 1 0 0 0 1 0 1
13	1 0 0 0 0 0 0 0 0 0 0 0 0 1 0 0	58	0 0 0 0 0 1 0 0 1 1 0 1 0 0 0 0
14	1 0 0 0 1 0 0 0 0 0 0 1 1 0 1 0 0	59	0 0 0 0 0 0 0 0 0 1 0 0 1 0 0 0 0
15	0 0 0 0 0 0 0 0 0 1 0 1 0 0 0 0	60	1 0 1 0 1 0 0 0 1 0 0 0 0 1 0 0
16	1 0 1 0 1 1 1 0 0 1 0 0 0 1 0 1	61	1 0 0 0 1 1 1 0 0 1 0 0 0 1 0 1
17	1 0 1 0 1 1 1 0 1 1 0 1 0 0 0 0	62	1 0 1 0 1 1 1 0 1 1 0 0 0 1 0 0
18	0 0 1 1 0 1 1 0 1 1 0 0 0 0 0 0	63	1 0 1 0 1 0 1 0 1 0 1 0 0 1 0 0
19	0 0 0 0 0 1 0 0 0 1 0 1 0 0 0 0	64	1 0 0 0 1 0 0 0 1 0 0 0 1 0 0 0
20	1 0 0 0 1 1 0 0 1 1 0 1 0 1 0 0	65	1 0 1 0 0 0 1 0 1 0 1 1 0 1 0 0
21	1 0 1 1 1 1 1 0 1 1 0 1 0 1 0 0	66	0 0 0 0 0 1 0 0 1 1 0 0 0 0 0 0
22	1 0 1 0 1 1 1 0 1 1 1 1 0 1 0 0	67	0 0 0 0 1 0 0 0 0 0 0 0 0 0 0 0
23	0 0 0 0 0 0 0 0 0 0 0 1 0 0 0 0	68	1 0 1 0 1 0 1 0 1 0 0 0 0 1 0 0
24	0 0 0 0 0 1 0 0 0 1 0 0 0 0 0 0	69	1 0 0 0 1 1 0 0 0 1 0 1 0 1 0 1
25	0 0 0 0 1 1 0 0 1 1 0 1 0 0 0 1	70	1 0 0 0 1 1 0 0 0 0 0 0 0 1 0 0
26	0 0 1 0 0 0 1 0 0 0 0 1 0 0 0 0	71	0 0 0 0 1 0 0 0 0 0 0 1 0 0 0 0
27	1 0 1 0 1 1 1 0 1 1 0 1 0 1 0 0	72	0 0 0 0 0 0 0 0 0 1 0 0 0 0 0 0
28	1 0 1 0 1 1 1 0 1 0 0 0 0 1 0 0	73	1 0 0 0 1 1 0 0 1 0 0 1 0 0 0 0
29	0 0 0 0 0 1 0 0 0 1 0 1 0 0 0 1	74	1 0 0 0 1 0 0 0 0 0 0 0 1 0 0 0
30	1 1 1 1 0 0 1 0 0 1 0 1 0 1 0 0	75	1 0 1 0 1 0 1 0 1 1 0 0 0 1 0 1
31	0 0 1 0 0 1 1 0 1 1 0 1 0 0 0 0	76	0 0 0 0 0 0 0 0 1 0 1 0 0 0 0 0
32	0 0 1 0 0 0 1 0 1 0 0 0 0 0 0 0	77	0 0 0 0 0 0 0 0 1 0 0 0 0 0 0 0
33	1 0 1 0 1 1 1 0 1 0 0 1 0 1 0 0	78	0 0 1 0 0 1 1 0 1 1 0 0 0 0 0 1
34	0 0 1 0 1 0 1 0 1 0 0 1 0 0 0 0	79	0 0 1 0 0 0 1 0 1 0 1 1 0 0 0 0
35	0 0 1 0 0 0 1 0 0 0 0 0 0 0 0 0	80	1 0 0 1 1 0 0 0 0 1 0 0 0 1 0 0
36	1 0 0 0 0 0 0 0 0 0 0 0 1 1 0 0	81	0 0 1 0 0 1 1 0 1 0 0 1 0 0 0 0
37	0 1 1 1 0 1 1 0 1 1 0 0 0 0 0 0	82	1 0 1 0 1 0 1 0 1 1 1 1 0 1 0 0
38	0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0	83	1 0 1 0 1 0 1 0 1 0 0 0 0 1 0 1
39	1 1 1 0 1 0 1 0 0 0 0 0 0 1 0 0	84	1 0 1 0 1 1 1 0 0 0 0 0 0 1 0 1
40	0 0 1 0 1 1 1 0 1 1 0 1 0 0 0 0	85	1 0 1 0 1 0 1 0 1 1 0 1 0 1 0 1
41	1 0 0 0 1 0 0 0 1 0 0 1 0 1 0 0	86	1 0 0 1 1 0 0 0 1 1 0 0 1 0 1 0 0
42	1 0 1 0 0 1 1 0 1 1 0 0 0 1 0 1	87	0 0 0 0 0 1 0 0 0 1 0 0 0 0 0 1 0
43	0 0 0 0 1 1 0 0 0 1 0 1 0 0 0 1	88	1 0 1 0 1 0 0 0 1 0 0 0 0 1 0 1
44	1 0 1 0 0 0 1 0 1 0 0 0 0 0 0 0	89	0 0 1 0 0 0 1 0 1 0 0 0 0 0 0 1
45	0 0 1 0 0 0 1 0 1 1 0 0 0 0 0 1		

[a] 1, Sensitive, lysis; 0, no reaction. Each phage sensitivity pattern represents *R. meliloti* reactivity with phages, respectively, 1, T1, 7a, 27, 43, N2, N3, N4, N9, A3, A8, SP, M3, M4, M5, and 70. Phage type 26 represents *R. meliloti* 2011str[r].

from that at site B. At site A, phage types 1 and 3 predominated and accounted for 53% of all indigenous nodule isolates. Phage types 4, 6, 25, 27, 29, 33, and 40 also featured consistently at this location, but each accounted for only 3 to 5% of the naturalized isolates of *R. meliloti*; the remaining 46 indigenous phage types were detected infrequently. At site B, 56 of the 65 indigenous phage types occurred at low frequency, whereas types 6, 31, 32, 38, 49, 54, 57, 62, and 68 were more abundant, each representing between 3 and 15% of the indigenous isolates from this location. With the exception of phage type 6, the most frequently encounterd indigenous types differed between locations. A minority of the indigenous isolates of *R. meliloti* from both sites were resistant (phage type 38) to the typing phages: <1 and 15% from sites A and B, respectively. Variation in the frequency of the most commonly encountered indigenous phage types between replicates at each site appeared to be small, indicating that the populations of *R. meliloti* sampled were uniformly distributed throughout the experimental areas in any one year.

Weighted least-squares analyses were used to summarize data for sites A and B. For the purposes of the analyses, all indigenous phage types encountered at low total frequency were amalgamated to form a composite class; to assess the effect of inoculation on the relative abundance of indigenous phage types, analyses were carried out with and without the inoculant strain (phage type 26). Because the distribution of phage types differed between sites, the data for each location were analyzed separately.

The summarized data and statistical analysis (excluding phage type 26) for site A (Tables 3 and 4) show that the frequency of indigenous phage types differed ($P < 0.01$) between years but not between *M. sativa* cultivars or inoculation treatments. Statistical analysis of the data including phage type 26 gave similar results, but due to recovery of type 26 only from inoculated cultivars, there was an additional, but expected difference ($P < 0.001$) between inoculation treatments. The difference between years was probably due to the higher frequency of phage types 1 and 3 and the lower frequency of types 25, 33, and 40 and the composite group in 1983 than 1984. Because the composite group contained many diverse phage types, an analysis of the data (Table 3) was carried out omitting this class; the patterns of statistical significance for all main effects and interactions were the same as those observed when the composite class was included in the analysis. Analysis of the frequencies of phage type 26 in nodules of inoculated cultivars Apollo and Saranac in 1984 indicated differential recovery ($P < 0.01$) of the inoculant strain from these cultivars.

Summarized data (excluding phage type 26) for the incidence of phage types at site B are presented in Table 5. Since the isolates comprising phage type 38 were potentially heterogeneous, they were included in the composite class for the purposes of the statistical analysis (Table 6). The relative occurrence of indigenous phage types differed ($P < 0.05$) between years, probably due to fluctuations in the frequency of types 49, 57, and 62. The higher frequency of phage type 32 and the lower frequency of types 31, 49, and 68 in nodules of cultivar Apollo than Saranac appear to account for the difference ($P < 0.01$) between cultivars. Overall differences between inoculation treatments ($P < 0.05$) are probably attributable to the differential occurrence of phage types 31 and 49. The cultivar × inoculation interaction ($P < 0.05$) suggests differential representation by phage types 32, 49, 57, and 68 and the composite group in nodules of *M. sativa* cultivars in response to inoculation (Fig. 2). Analysis of the data for site B including phage type 26 provided results

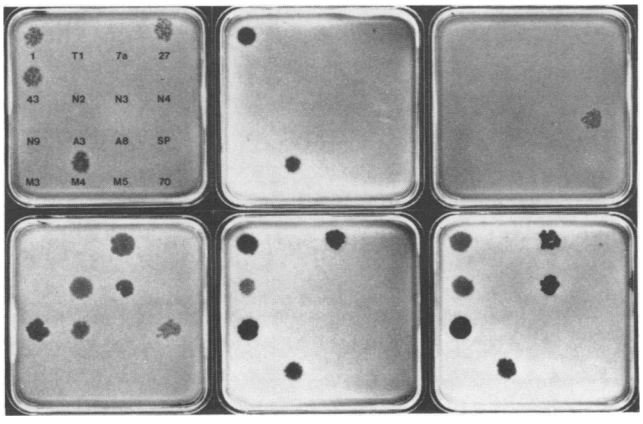

FIG. 1. Phage sensitivity patterns of six phage types of indigenous *R. meliloti*. (Left to right) Phage types 11, 13, and 23 (upper row) and 31, 60, and 68 (lower row). The relative position of each typing phage is designated on the upper left petri dish (phage type 11).

TABLE 2. Distribution of phage types of indigenous *R. meliloti* in nodules of *M. sativa* cv. Apollo and *M. sativa* cv. Saranac grown at two sites for 2 years, with and without inoculation

Phage type	No. of *R. meliloti* isolates in phage type from															
	Site A[a]								Site B							
	Year 1[a]				Year 2				Year 1				Year 2			
	AO[b]	SO[b]	AI[b]	SI[b]	AO	SO	AI	SI	AO	SO	AI	SI	AO	SO	AI	SI
1	42	38	36	32	25	29	26	32	1	1	2					
2	6		2	1		1	5	4						1		
3	33	30	21	24	31	22	21	19								
4	3	4	2	4	3	4	2	5								
5	2	1		1	1		3	1								
6	2	5	3	7	3	9	6	4	4	6	4	5	5	4	3	
7		1		1	1			1	1							
8		1			2			1								
9	2	3					1	5	1		1	3	1	1		2
10	1	1		1	3	4		1					1			
11	2			2		1										
12				1							1		1	1	1	
13	2	1	1		1		2	1						1	1	
14	1					14			1							
15	1								2				1	1	2	
16			1			1	1	2		1					1	
17	2															
18				2												
19	1	1							1			2				
20	1			1							3					
21		2					1	2	1					1		2
22	1	4		1	2	1										1
23	1	1	1		1						1		1	2	1	1
24		2								2					1	
25	2	3	1		10	5	6	1								
26			27	26			31	12			24	16			46	41
27	6	4	6	6	6	4	2	7	4				4			
28	1		2	1	2	1		1								
29	4	6	4	2	6		2	2					1			
30	1															
31	1			1	2	4	1		1	11	3	8	7	14		4
32		1							17	3	6	6	10	8	5	4
33		2	5		8	3	3	3								
34		1			1											
35		1			1									1		
36		1	2			1	1	1								
37		1														
38			2	2	1		1	1	18	28	10	12	21	14	10	9
39	1		1				1	1								
40	1		3	2	4	3	2	9					2			1
41		4			1				2		1					
42		1		1	2		1	1								
43				1					1							
44									2	2						
45									3	1	1		1		2	
46									1	1	2	1				
47									1		1					2
48											2					
49						2			12	6	8	22	3	3	4	14
50									1	3	3				1	
51							1			2				2		
52										3			1		2	2
53						2			1	4			2	1		
54									5	3		3	4	6	3	2
55									2		1	1		1		
56									1	1						
57									1	2	2		3	7	4	2
58										1						
59									1	1			1	2	1	
60									1	1		5	1	2	1	2
61									1				2			
62								2	14	12	8	8	17	14	12	10
63										1	3			6	1	

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TABLE 2—*Continued*

Phage type	No. of *R. meliloti* isolates in phage type from															
	Site A[a]								Site B							
	Year 1[a]				Year 2				Year 1				Year 2			
	AO[b]	SO[b]	AI[b]	SI[b]	AO	SO	AI	SI	AO	SO	AI	SI	AO	SO	AI	SI
64										3	1	1				
65											2					
66									3		1					
67											1		1	1	1	
68									11	20	15	20	19	16	6	16
69											1					
70									1							
71									3	1	2	1	1			1
72												4		4		
73											1					
74												1			1	2
75											8	1	4	2	2	
76											1		1			
77															1	
78															2	
79					1	1										
80					1											
81					1											
82						1										
83						1								1	4	2
84						3										
85						1							2	1	1	
86								1								
87						2										
88													2			
89														2		

[a] Sites A and B located on Central Experimental Farm, Ottawa, without and with previous history of *M. sativa* cultivation, respectively. Year 1, 1983; Year 2, 1984.

[b] Treatment combination abbreviations: A, cultivar Apollo; S, cultivar Saranac; O, not inoculated; I, seed inoculated with *R. meliloti* 2011str[r] (phage type 26). Each site, year, cultivar and inoculation treatment combination represents a total of 120 *R. meliloti* isolates, consisting of three replicates.

similar to those above (Table 6), except that the difference between inoculation treatments was highly significant ($P < 0.001$) due to recovery of phage type 26 only from inoculated plots. Analysis of the data (Table 5), omitting the composite class, revealed the same pattern of statistical significance for all main effects and interactions, with the exception of the cultivar × inoculation interaction which was not significant ($P = 0.16$). There were differences ($P < 0.01$) in the occurrence of phage type 26 between 1983 and 1984 for both cultivars. This indicates a significant increase in recovery of the inoculant strain in year 2 relative to year 1.

Indigenous isolates of *R. meliloti* from sites A and B invariably produced growth on YEM medium which was translucent, misty in appearance, and accompanied by copious gum formation, whereas the inoculant strain, *R. meliloti* 2011str[r], produced growth which was nongummy, opaque, and white in appearance. Therefore, the use of growth morphology and streptomycin resistance markers to distinguish inoculant from indigenous isolates provided a means of independently confirming the actual stability of phage sensitivity in *R. meliloti*. All nodule isolates from *M. sativa* grown with inoculation in 1983 and 1984, which were recognized as

TABLE 3. Summarized data for the relative abundance of phage types of indigenous *R. meliloti* at site A[a]

Year	Cultivar and inoculation treatment[b]	Proportion of nodule isolates representing phage type[c]:										Total isolates[c]
		1	3	4	6	25	27	29	33	40	Composite[d]	
1983	AO	0.350	0.275	0.025	0.017	0.017	0.050	0.033	0	0.008	0.225	120
1983	AI	0.387	0.226	0.022	0.032	0.011	0.065	0.043	0.054	0.032	0.129	93
1983	SO	0.317	0.250	0.033	0.042	0.025	0.033	0.050	0.017	0	0.233	120
1983	SI	0.340	0.255	0.043	0.074	0	0.064	0.021	0	0.021	0.181	94
1984	AO	0.208	0.258	0.025	0.025	0.083	0.050	0.050	0.067	0.033	0.200	120
1984	AI	0.292	0.236	0.022	0.067	0.067	0.022	0.022	0.034	0.022	0.213	89
1984	SO	0.242	0.183	0.033	0.075	0.042	0.033	0	0.025	0.025	0.342	120
1984	SI	0.296	0.175	0.046	0.037	0.009	0.065	0.019	0.028	0.083	0.241	108

[a] See Table 4 for weighted least-squares analysis.

[b] Designations as defined in Table 2, footnote *b*.

[c] Data exclude phage type 26.

[d] Composite consisting of 46 phage types of *R. meliloti*.

TABLE 4. Weighted least-squares analysis of data in Table 3[a]

Source of variation (df = 9)	Chi square	P
Year (Y)	23.40	0.005
Cultivar (C)	12.39	0.192
Inoculation (I)	12.59	0.282
Y × C	6.84	0.654
Y × I	4.15	0.902
C × I	7.65	0.570
Y × C × I	9.77	0.369

[a] See Table 3. Data exclude phage type 26.

TABLE 6. Weighted least-squares analysis of data in Table 5[a]

Source of variation (df = 8)	Chi square	P
Year (Y)	16.9	0.040
Cultivar (C)	23.98	0.002
Inoculation (I)	15.39	0.049
Y × C	4.42	0.817
Y × I	10.22	0.250
C × I	16.14	0.040
Y × C × I	10.84	0.211

[a] See Table 5. Data exclude phage type 26.

strain 2011str^r according to growth morphology, were also resistant to streptomycin (300 μg/ml) and revealed a phage sensitivity pattern characteristic of the inoculant strain (phage type 26). This suggests that all three identifying characteristics were stable, remaining so even after prolonged exposure to field environments and reisolation from nodules.

Analyses of variance of data for shoot dry-matter production by *M. sativa* cv. Apollo and *M. sativa* cv. Saranac (data not presented) showed no significant differences between inoculated and uninoculated cultivars at each site in either 1983 or 1984. Based on the low recovery of strain 2011str^r from inoculated cultivars (Table 2), the absence of significant differences in shoot yield between inoculation treatments was not unexpected and provides a general indication that the population of indigenous *R. meliloti* at each site was symbiotically effective.

DISCUSSION

Bacteriophages have been used previously for the classification of *Rhizobium* spp. (33, 34) and for the discriminative typing of strains (24) and mutants (20) of *R. meliloti*. Apart from one report on the relationship between native strains of *R. japonicum* and their bacteriophages (23), the potential of phage typing for delineating the composition of indigenous populations of *Rhizobium* spp. has been little realized. Our data demonstrate the resolving power of phage typing and its ability to analyze naturalized populations of *R. meliloti*: the 1,920 nodule isolates were subdivided into 88 unique phage types, of which 55 and 65 were encountered at the two field locations. This diversity is considerably greater than any reported previously for the serological analysis of nodule isolates of indigenous *Rhizobium* spp. from specific locations (7, 18, 21).

The frequency of occurrence of phage types of indigenous *R. meliloti* varied considerably within the nodule population at each location. This variation suggests that specific phage types at each location were either intrinsically more competitive for nodulation of the cultivars of *M. sativa* or, alternatively, were more abundant in the soil than others. These alternatives are currently being tested. Analogous differences in the predominance of members within populations of indigenous *R. japonicum* inhabiting nodules have been demonstrated with serology (5, 6, 15) and protein profile patterns (26).

The importance of environmental factors in influencing the composition of indigenous populations of *R. meliloti* was indicated by the marked differences in the distribution of phage types between locations in relatively close proximity but differing in agricultural history and soil characteristics. Previous studies have also shown variation in the serogroup distribution of *R. japonicum* between locations (7, 15, 21) and the variation attributed to soil factors or cropping history (7, 15).

It has been reported that cropping history can influence the population densities of indigenous *R. japonicum* in various soils (38). In this connection, it was unexpected that site A, which had no previous history of *M. sativa* cultivation, contained a relatively large population of *R. meliloti* before planting (7.8×10^3 per g of soil). These rhizobia may have originated from naturally occurring *Medicago* spp., in particular *M. lupulina*, which was abundant and well established in the vicinity of the field plots. A further effect of cropping history was indicated by the apparent increase in *R. meliloti* numbers in response to *M. sativa* cultivation for

TABLE 5. Summarized data for the relative abundance of phage types of indigenous *R. meliloti* at site B[a]

Year	Cultivar and inoculation treatment[b]	Proportion of nodule isolates representing phage type[c]									Total isolates[c]
		6	31	32	49	54	57	62	68	Composite[d]	
1983	AO	0.033	0.008	0.142	0.100	0.042	0.008	0.117	0.092	0.458	120
1983	AI	0.042	0.031	0.063	0.083	0	0.021	0.083	0.156	0.521	96
1983	SO	0.050	0.092	0.025	0.050	0.025	0.017	0.100	0.167	0.475	120
1983	SI	0.048	0.077	0.058	0.212	0.029	0	0.077	0.192	0.308	104
1984	AO	0.042	0.058	0.083	0.025	0.033	0.025	0.142	0.158	0.433	120
1984	AI	0.041	0	0.068	0.054	0.041	0.054	0.162	0.081	0.500	74
1984	SO	0.033	0.117	0.067	0.025	0.050	0.058	0.117	0.133	0.400	120
1984	SI	0	0.051	0.051	0.177	0.025	0.025	0.127	0.203	0.342	79

[a] See Table 6 for weighted least-squares analysis.
[b] Designations as defined in Table 2, footnote *b*.
[c] Data exclude phage type 26.
[d] Composite consisting of 57 phage types of *R. meliloti*.

two seasons at both field sites initially containing established *Rhizobium* populations. A similar effect was reported by Bell and Nutman (2), although this was at sites originally harboring few indigenous *R. meliloti*.

There is much evidence which attests to the variable performance of inoculants in soils containing populations of indigenous *Rhizobium* (12, 22, 31, 35). Even where inoculation has achieved initial success, this has generally been followed by a decline in nodule occupancy by inoculant strains and their replacement by indigenous rhizobia in the years after inoculation (9, 31, 35). Despite the relatively high rate of inoculation (5×10^4 *R. meliloti* per seed), the present data show low levels of inoculant recovery from nodules at sites with established populations of indigenous *R. meliloti* and also great variety of phage types. However, the inoculum showed no tendency towards decline in year 2, and at one location inoculant occupancy of nodules was greater ($P < 0.01$) than in year 1. A few other reports also indicate that the displacement of applied strains by indigenous rhizobia in the years succeeding inoculation need not invariably be the case (12, 25).

One objective of the present investigation was to determine whether the establishment of an introduced inoculant strain of *R. meliloti* would perturb the distribution of indigenous phage types inhabiting nodules. While the spectra of phage types from inoculated and uninoculated plots at each site were similar, the presence of the inoculant significantly ($P < 0.05$) influenced the frequency of certain indigenous types at site B. This contrasts in part with a previous report for *R. japonicum* (26) which indicated that the establishment of an inoculum in a small proportion of nodules did not affect either the variety or distribution of an indigenous population.

It has been suggested that heterogeneity per se of indigenous *R. japonicum* represents a potential barrier to the establishment of introduced strains (26). In the current situation, an inoculant strain of *R. meliloti* would have to compete successfully with an extremely wide array of indigenous types, varying in frequency, to become established in a majority of the nodules. Therefore, it is not surprising that an introduced strain, not selected for the particular environment and legume genotype combination, should fail to occupy most of the nodules.

The legume host has been reported to exert specific preferential or selective effects which determine the relative success in nodulation among competing *Rhizobium* strains in test-tube culture (27, 37). In a field situation, Caldwell and Vest (5) showed that several genotypes of *Glycine max* were differentially nodulated by serogroups of indigenous *R. japonicum*. In another study, different species of *Trifolium* were reported to selectively nodulate with symbiotically effective *R. trifolii* in a native population (29). Our data show a significant ($P < 0.01$) influence of *M. sativa* cultivar on the frequency of occurrence of certain phage types of naturalized *R. meliloti* in nodules at one site. Such host genotype effects emphasize that information on the distribution of rhizobia within indigenous populations derived from the use of a single legume genotype should be treated with caution; the use of several genotypes from the same cross-inoculation group should be considered for the provision of more representative data.

The interaction of cultivar and inoculation ($P < 0.05$) on the frequency of occurrence of phage types inhabiting nodules at site B (Fig. 2) indicates that cultivar preferences for naturalized *R. meliloti* were differentially affected by the introduction of an inoculant. To our knowledge, this is the first report of an apparent differential influence of an applied strain on nodulation preference by legume genotypes for members of an indigenous *Rhizobium* population.

The predominance of particular groups of *R. japonicum* from specific locations has been reported to vary between years or sampling times (6, 26). This is consistent with the present data for *R. meliloti*, which show differences in the frequency of certain phage types between sample years at both field sites. These population shifts probably reflect *M. sativa* nodulation preference for specific phage types, the influence of environmental variables, or both.

The results presented in this paper provide new insights into the diversity and dynamics of naturalized *R. meliloti* inhabiting nodules of *M. sativa* and suggest that development of methods for the selection of inoculant strains able to successfully establish and persist in competitive situations is only likely to be realized when more is understood about the nature of indigenous *Rhizobium* populations and the interactions among the biotic and abiotic factors that influence their composition.

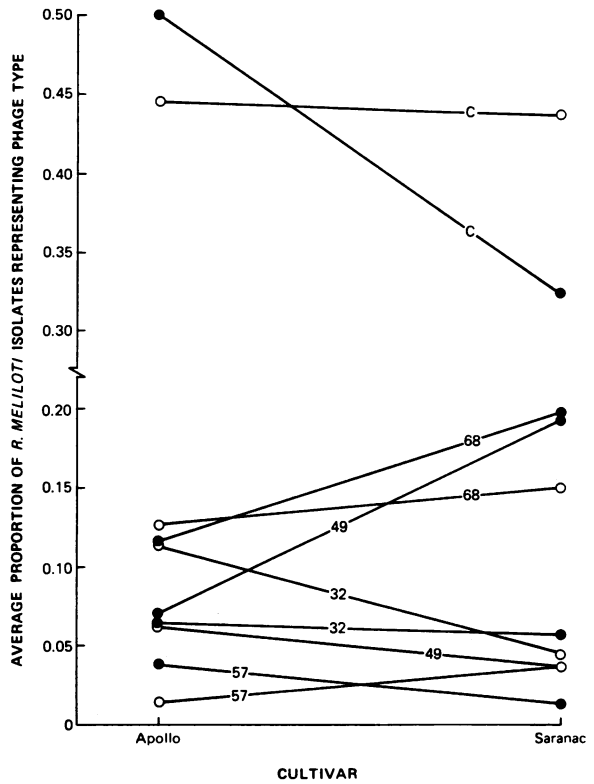

FIG. 2. Interaction of *M. sativa* cultivar and inoculation on the incidence of phage types of indigenous *R. meliloti* from site B. Open circles, uninoculated; closed circles, inoculated; numbers 32, 49, 57, and 68 refer to the phage type; C, composite class. Proportions represent averages of 2 years. Only those phage types showing differential occurrence on *M. sativa* cultivars in response to inoculation are presented (see Tables 5 and 6).

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