Populations of *Rhizobium meliloti* in Areas with Rangeland Alfalfa

W.L. LOWTHER, M.D. RUMBAUGH, AND D.A. JOHNSON

Abstract

The perpetuation of legumes in rangeland pasture plantings is dependent upon natural seeding and nodulation of the seedlings by naturalized soil rhizobia. This study determined the distribution and effectiveness of *Rhizobium meliloti* Dang. isolates in mature stands of rangeland alfalfa (*Medicago sativa* L.). Five sites in northern Utah and southern Idaho were studied. At each site, soil samples were taken from beneath the crown area of 10 plants and also at 50 and 100-cm intervals downslope and at right angles to the slope. Populations of *R. meliloti* ranged from 1.7×10^2 to more than 1.7×10^5 per g of soil in all soil samples taken from within the crown area of established plants. The distribution of *R. meliloti* in soil adjacent to established plants differed with site and sampling position. *R. meliloti* were present in all the interplant spaces sampled on 1 site while the percentage of interplant samples with *R. meliloti* varied from 20% to almost 100% on the other 4 sites. Movement of *R. meliloti* downslope was detected only on 1 site. It was concluded that failure of *R. meliloti* to spread into the interplant spaces in rangeland alfalfa stands may limit natural reseeding.

Key Words: bacteria, *Medicago sativa*, nitrogen fixation, rhizobia, rhizosphere, soil microbiology, symbiosis

Alfalfa (*Medicago sativa* L. and *M. falcata* L.) is widely recommended for range improvement (Kneebone 1959, Townsend et al. 1975). Alfalfa has been shown to live up to 23 years in semiarid environments (Kilcher and Heinrichs 1965, Rumbaugh and Pedersen 1979) and is capable of self-perpetuation through reseeding at sites with as little as 28 cm of precipitation (Rumbaugh 1982). However, stand decline was identified as a major problem in a survey conducted in Montana (Gomm 1974).

Little is known of the influence of the *Rhizobium*/host symbiosis on the productivity and longevity of rangeland alfalfa, even though this symbiosis is known to play an important role in other environments. In acid soil, survival of rhizobia is a major limitation to alfalfa nodulation (Rice et al. 1977), while the presence of ineffective rhizobia has been attributed as a cause of poor alfalfa growth in Washington (Weber and Leggett 1966). In Oregon, Bottomley and Jenkins (1983) found high proportions of ineffective rhizobia in nodules on alfalfa plants over a wide range of soil pH (5.9 to 8.8).

Adequate populations of effective *R. meliloti* are required in the host rhizosphere to ensure continued nodulation and symbiotic N-fixation in established alfalfa plants on rangelands. In addition, adequate populations of rhizobia are required in the interplant spaces for nodulation of seedlings established by natural seeding. Natural seeding is important in perpetuating the stand and in increasing stand density where initial alfalfa establishment was low.

In the present study, 5 rangeland alfalfa stands between 7 and 31 years old in northern Utah and southern Idaho were sampled to determine the spread and symbiotic effectiveness of *R. meliloti*.

Authors are agronomist, Invermay Agricultural Research Centre, Research Division, Ministry of Agriculture and Fisheries, Private Bag, Mosgiel, New Zealand; and research geneticist and plant physiologist, respectively, USDA Agricultural Research Service, Forage and Range Research, Utah State University, Logan 84322-6300.

Cooperative investigations of the USDA Agricultural Research Service and the Utah Agricultural Experiment Station, Logan 84322-6300. Approved as paper No. 3287.

Manuscript accepted 18 December 1986.

Materials and Methods

Field Sampling

A description of the 5 alfalfa sites sampled is given in Table 1. Annual precipitation at the sites was estimated to range from 280 mm at the Curlew Valley site to 460 mm at the Logan Canyon site. At the Snowville, Curlew, and Logan sites, seed had been drilled into soil while at Orem and Pocatello seed had been broadcast onto the soil surface. No conclusive information is available on seed inoculation at any of the sites. However, the absence of *R. meliloti* from adjoining areas (Lowther et al. 1987) indicates that inoculation would have been necessary for nodulation.

Sampling was conducted during July and August 1985. At each site 10 plants were selected at random and soil samples were taken within the crown of the plant. In addition, samples were taken at 50 and 100 cm from the outer edge of the crown at right angles to the slope and also downslope from each plant. Each sample consisted of 5 cores of soil, each 1.5 cm in diameter and 10 cm deep. Sampling corers were sterilized between samples by flaming. Samples were placed in plastic bags and stored at 4° C until processed, normally within 7 days.

Rhizobia Distribution

The 5 cores of each soil sample were thoroughly mixed in the plastic bag and a subsample of 10 g taken for rhizobia counts. Enumeration of *R. meliloti* was done by the plant infection method using the N-free seedling agar of Jensen (Date and Vincent 1962). The soil subsample was added to 90 ml of the 1/4 strength solution of McKnight (McKnight 1949) containing glass beads, and the solution was shaken for 15 minutes. A 10-fold dilution series to 10^{-5} was prepared and a 1-ml aliquot at each dilution was used to inoculate each of 2 tubes (20 × 150 mm) containing a sterile seedling of Spredor 2 alfalfa growing on sloped seedling agar (Jensen 1942). The dilution series enabled detection of a minimum population of 6 rhizobia per gram of soil. Detection of numbers lower than this by increasing the amount of soil added to the test tubes is not reliable (Thompson and Vincent 1967). After 6 to 8 weeks, the most probable numbers (MPN) of *R. meliloti* in the soil samples were calculated from the number of plants forming nodules.

Symbiotic Effectiveness

Rhizobia were isolated from nodules formed in the distribution tests at the lowest dilution of soil samples taken under alfalfa crowns and at 50 and 100-cm distances at right angles to the crowns. The 50 and 100-cm samples were combined. Sampling at the lowest dilution allows for the possibility of host-plant selection (Masterson and Sherwood 1974). After excision, a random sample of 20 nodules was taken for rhizobia isolation. Nodules were surface sterilized by immersion momentarily in 96% ethanol, in 3% calcium hypochlorite for 5 min, and then rinsed in sterile water (Somasegaran and Hoben 1985). Nodules were crushed in 1/4 strength nutrient solution, streaked on congo red yeast mannitol (YM) agar (Vincent 1970), and incubated at 20° C. After a second streaking, single colony isolates were streaked on YM agar slopes and stored at 4° C after incubation. Inoculant suspensions were prepared by streaking isolates on 9 ml of sloped agar in 20 × 150-mm culture tubes, incubating for 7 days at 20° C, and then

Table 1. Characteristics of the 5 sites sampled.

Name	Year seeded	Elevation (m)	Soil pH	General vegetation type before seeding[1]	Land use
Snowville	1954	1,420	7.4	Sagebrush steppe	Livestock grazing
Orem Bench	1959	1,750	7.6	Mountain mahogany-oak scrub	Soil conservation
Logan Canyon	1973	1,540	7.1	Douglas fir forest	Revegetation of highway right-of-way
Pocatello Valley	1974	1,800	6.1	Sagebrush steppe	Livestock grazing
Curlew Valley	1978	1,580	7.9	Juniper-pinyon woodland	Wildlife forage

[1]From Küchler (1964).

suspending in 12 ml of 1/4 strength nutrient solution. Each Spredor 2 alfalfa seedling growing on sloped seedling agar in 20 × 150-mm test tubes was inoculated with 1 ml of inoculant suspension. Each isolate was replicated 10 times. Isolates were compared with commercial inoculant strains 102F51 and 102F77 obtained from the Nitragin Co.[1] (Milwaukee, Wis.), an uninoculated control, and an uninoculated combined-N control which received 3 ml of 18 mM KNO_3 solution 14 d and 28 d after sowing (Bottomley and Jenkins 1983). At 28 d after sowing, each inoculated seedling received 3 ml of sterile deionized water. Plants were grown in a randomized complete block design in a shaded greenhouse with day/night temperatures maintained at approximately 30/15° C. At 52 or 54 d after sowing, plants were harvested and weighed. The symbiotic effectiveness of the isolates was expressed as a percentage of the mean yield of the 2 commercial inoculant strains.

The homogeneity of the sites and of the sampling positions with respect to the frequency of samples containing or not containing rhizobia was tested by Chi-square analysis of appropriate contingency tables. Differences among the sites and positions in symbiotic effectiveness of the isolates were evaluated by analysis of variance procedures. Effectiveness of each isolate also was compared to that of the commercial inoculant strains by means of Student's *t* test.

Results

The pattern of occurrence and size of populations of *R. meliloti* are presented in Figures 1 and 2. A Chi-square test of the presence or absence of *R. meliloti* indicated heterogeneity among the 5 sites and among the 5 sampling positions ($P<0.001$; Table 2). *R. meliloti* were found within the crown area of all established alfalfa plants, and populations ranged from 1.7×10^2 to more than 1.7×10^5 rhizobia per gram of soil. On the Logan Canyon site, *R. meliloti* were present in all samples taken at right angles and downslope from established alfalfa plants; the minimum population was 5.8×10^3 rhizobia per gram. On the Snowville site, *R. meliloti* were detected in the majority of samples (80 to 100%) although there were as few as 6 *R. meliloti* per gram in some instances. On the remaining 3 sites, the percentage of samples with detectable populations of *R. meliloti* varied widely. As few as 20 to 30% of the samples from some positions on each site contained the bacterium. Orem was the only site where the occurrence of *R. meliloti* varied with sampling position. Rhizobia were present in 90% of the samples taken at 50 cm downslope from the plant compared with only 30 to 40% of samples taken at right angles or 100 cm downslope.

Site, position, and a site by position interaction were significantly ($P<0.01$) related to the symbiotic effectiveness of the isolates. The mean symbiotic effectiveness of the isolates was lower on the Orem site (80%) than on the other 4 sites (106% to 121%; Table 2) due to a higher percentage of isolates classified as inferior N-fixers. On the Orem and Snowville sites, the mean effectiveness of isolates from areas adjacent to alfalfa plants was higher than from soil under the crown. This pattern reversed on the other 3 sites.

[1]Mention of a trademark, proprietary product, or vendor does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture, and does not imply its approval to the exclusion of other products or vendors that may also be suitable.

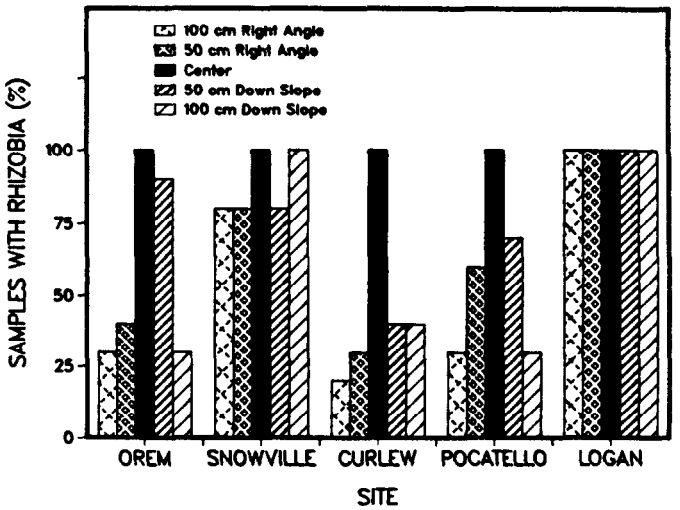

Fig. 1. *The effect of site and sample position around alfalfa plants on the percentage of samples with detectable (>6 per gram of soil)* Rhizobium meliloti.

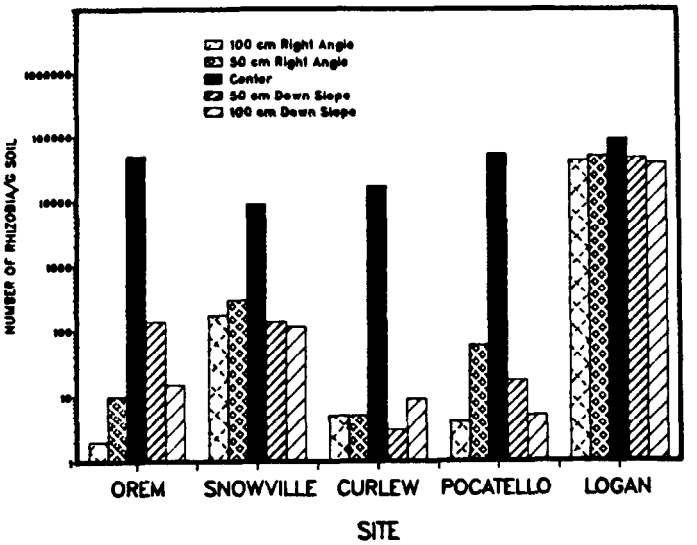

Fig. 2. *The effect of site and sample position around alfalfa plants on the number of* Rhizobium meliloti *per g of soil.*

Table 2. Symbiotic effectiveness of isolates of *R. meliloti* from soil under the crown or adjacent to established alfalfa plants on 5 rangeland sites.

Site	Sample position	No. of isolates	Percent of isolates classed as[1]			Percent effectiveness[2]	
			Superior	Effective	Inferior	Mean	Range
Orem	Crown	20	0	65	35	74	-1-178
	Adjacent	20	0	80	20	86	21-146
	Mean	40	0	73	28	80	-1-178
Snowville	Crown	20	5	90	5	118	74-172
	Adjacent	20	10	90	0	125	64-168
	Mean	40	8	90	2	121	64-172
Pocatello	Crown	20	10	90	0	120	74-173
	Adjacent	20	0	95	5	92	48-128
	Mean	40	5	93	2	106	48-173
Curlew	Crown	20	20	75	5	124	46-176
	Adjacent	19	11	89	0	111	67-156
	Mean	39	15	82	3	118	46-176
Logan	Crown	20	10	90	0	131	68-171
	Adjacent	20	15	70	15	106	50-174
	Mean	40	13	80	7	119	50-174

[1]Superior isolates were defined as those producing plant yields significantly ($P<0.05$) higher than the yield of control plants inoculated with commercial strains, effective when not significantly different from the control, and inferior when significantly lower than the control.

[2]Percent effectiveness defined as $\frac{\text{yield of isolate} - (-\text{N control})}{\text{yield of controls} - (-\text{N control})} \times 100$

Discussion

Lack of survival of *R. meliloti* in the rhizospere of established plants appears unlikely to be a limitation to the long-term persistence of rangeland alfalfa. We found rhizobia in the soil under the crown of plants established for up to 31 years. The number of *R. meliloti* in soil has been shown to vary during the season (Mahler and Wollum 1982), and our samples taken during early summer probably contained fewer rhizobia than would have been found during spring when there is more soil moisture. The failure of *R. meliloti* to spread into the interplant spaces in some of the rangeland alfalfa stands indicates the possibility of nodulation failure, which may limit natural reseeding.

Factors such as wind, water, animal and insect movement may affect rhizobial spread (Parker et al. 1977). The limited distribution of *R. meliloti* in the undisturbed rangelands of the Intermountain West of the U.S. led Lowther et al. (1987) to suggest that movement of rhizobia in windborne dust was not a rapid means of spread. This was attributed to the failure of rhizobia to survive on the soil surface due to detrimental effects of high temperature and desiccation (Danso and Alexander 1974, Van Rensburg and Strijdom 1980). The occurrence of *R. meliloti* in at least some of the interplant spaces on all of the sites may be due to the survival and persistence of rhizobia introduced at sowing or to the spread of rhizobia from established alfalfa plants. Only on the Orem site was there evidence of the downslope movement of *R. meliloti* in water. However, movement was apparent only 50 cm downslope from the alfalfa plants. Because rhizobia can be transported in percolating water (Madsen and Alexander 1982) or move in soil moisture (Hamdi 1971), the limited spread of *R. meliloti* downslope in the present study is surprising. Although the sites are in semiarid environments, rhizobia movements could be expected during the period of favorable soil moisture in winter and spring.

The occurrence of high populations of *R. meliloti* in scattered areas suggests that movement or initial establishment was limited rather than persistence. A similar conclusion was reached after a survey of *R. meliloti* in undisturbed rangelands (Lowther et al. 1987). It is not known why *R. meliloti* was found in interplant spaces only at the Logan Canyon and Snowville sites, particularly since there was limited spread at the other 3 sites with a similar cultural history. The soil pH of all sites was above the 5.5 to 6.0 range shown to restrict multiplication of *R. meliloti* in soil (Rice et al. 1977).

The difference in symbiotic effectiveness of isolates from different alfalfa stands is in agreement with the results of Bottomley and Jenkins (1983). The lower mean effectiveness and higher percentage of inferior isolates from the Orem site indicate that some of the isolates may be from populations naturalized in the Intermountains rangelands (Lowther et al. 1987). The high mean effectiveness and the extremely low occurrence of inferior isolates from the other 4 sites suggest that the rhizobia at these sites probably originated from initial seed inoculation. The range of symbiotic effectiveness of the isolates may be a result of differential symbiosis with the test host cultivar (Burton and Wilson 1939) inoculated with the different strains of *R. meliloti* found in mixed strain commercial inoculants. The persistence of inoculant strains of rhizobia in these rangeland situations, in the absence of ineffective naturalized soil *R. meliloti*, indicates the importance of seed inoculation with highly effective strains.

In view of the potential importance of natural reseeding to improve stand density and long-term persistence of alfalfa on rangeland, research is required to delineate and overcome the limited spread of *R. meliloti*. Selection of strains of *R. meliloti* better able to colonize soil (Chatel et al. 1968, Robson and Loneragan 1970) should be investigated.

References

Bottomley, P.J., and M.B. Jenkins. 1983. Some characteristics of *Rhizobium meliloti* isolates from alfalfa fields in Oregon. Soil Sci. Soc. Amer. J. 47:1153-1157.

Burton, J.C. and P.W. Wilson. 1939. Host plant specificity among the *Medicago* in association with root-nodule bacteria. Soil Sci. 47:293-303.

Chatel, D.L., R.M. Greenwood, and C.A. Parker. 1968. Saprophytic competence as an important character in the selection of *Rhizobium* for inoculation. Proc. 9th Int. Congr. Soil Sci., Adelaide, 1968, Vol. 2. p. 65-73.

Danso, S.K.A. and M. Alexander. 1974. Survival of 2 strains of *Rhizobium* in soil. Soil Sci. Soc. Amer. Proc. 38:86-89.

Date, R.A. and J.M. Vincent. 1962. Determination of the number of root-nodule bacteria in the presence of other organisms. Aust. J. Exp. Agr. Anim. Husb. 2:5-7.

Gomm, F.B. 1974. Forage species for the northern Intermountain Region. A summary of seeding trials. U.S. Dep. Agr. Tech. Bull. No. 1479.

Hamdi, Y.A. 1971. Soil-water tension and the movement of rhizobia. Soil Biol. Biochem. 3:121-126.

Jensen, H.L. 1942. Nitrogen fixation in leguminous plants. I. General characteristics of root-nodule bacteria isolated from species of *Medicago* and *Trifolium* in Australia. Proc. Linn. Soc. N.S.W. 66:98-108.

Kilcher, M.R., and D.H. Heinrichs. 1965. Persistence of alfalfa in mixture with grasses in a semi-arid region. Can. J. Plant Sci. 46:163-167.

Kneebone, W.R. 1959. An evaluation of legumes for western Oklahoma rangelands. Oklahoma Agr. Exp. Sta. Bull. B-539.

Küchler, A.W. 1964. Potential natural vegetation of the conterminous United States. Amer. Geographical Soc. Special Pub. No. 36. New York.

Lowther, W.L., D.A. Johnson, and M.D. Rumbaugh. 1987. Distribution and symbiotic effectiveness of *Rhizobium meliloti* in rangeland soils of the Intermountain West. J. Range Manage. 40:264-267.

Madsen, E.L., and M. Alexander. 1982. Transport of *Rhizobium* and *Pseudomonas* through soil. Soil Sci. Soc. Amer. J. 46:557-560.

Mahler, R.L. and A.G. Wollum. 1982. Seasonal variation of *Rhizobium meliloti* in alfalfa hay and cultivated fields in North Carolina. Agron. J. 74:428-431.

Masterson, C.L. and M.T. Sherwood. 1974. Selection of *Rhizobium trifolii* strains by white and subterranean clovers. Irish J. Agr. Res. 13:91-99.

McKnight, T. 1949. Efficiency of isolates of *Rhizobium* in the cowpea group, with proposed additions to this group. Queensland J. Agr. Sci. 6:61-76.

Parker, C.A., M.J. Trinick, and D.L. Chatel. 1977. Rhizobia as soil and rhizosphere inhabitants. p. 311-352. *In:* R.W.F. Hardy and A.H. Gibson (eds.). A treatise on dinitrogen fixation. IV. Agronomy and ecology. John Wiley & Sons, New York.

Rice, W.A., D.C. Penney, and M. Nyborg. 1977. Effects of soil acidity on rhizobia numbers, nodulation and nitrogen fixation by alfalfa and red clover. Can. J. Soil Sci. 57:197-203.

Robson, A.D. and J.F. Loneragan. 1970. Nodulation and growth of *Medicago truncatula* on acid soils. II. Colonization of acid soils by *Rhizobium meliloti*. Aust. J. Agr. Res. 435-445.

Rumbaugh, M.D. 1982. Reseeding by 8 alfalfa populations in a semiarid pasture. J. Range Manage. 35:84-86.

Rumbaugh, M.D. and M.W. Pedersen. 1979. Survival of alfalfa in five semiarid range seedings. J. Range Manage. 32:48-51.

Somasegaran, P. and H.J. Hoben. 1985. Methods in Lebume-*Rhizobium* Technology. NIFTAL, Univ. of Hawaii.

Thompson, J.A. and J.M. Vincent. 1967. Methods of detection and estimation of rhizobia in soil. Plant Soil 26:72-84.

Townsend, C.E., G.O. Hinze, W.D. Ackerman, and E.E. Remmenga. 1975. Evaluation of forage legumes for rangelands of the central Great Plains. Colorado Agr. Exp. Sta. Gen. Series 942.

Van Rensburg, H.J. and B.W. Strijdom. 1980. Survival of fast- and slow-growing *Rhizobium* spp. under conditions of relatively mild desiccation. Soil Biol. Biochem. 12:355-356.

Vincent, J.M.1970. A manual for the practical study of root-nodule bacteria. IBP Handbk. No. 15. Blackwell Scientific Publ., Oxford, England.

Weber, D.F. and G.E. Leggett. 1966. Relation of rhizobia to alfalfa sickness in eastern Washington. USDA-ARS Res. Rep. 41-117.