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Title: Delineation of the Composition of an Indigenous Soil Population of *Rhizobium meliloti* in Root Nodules of Uninoculated, Field Grown *Medicago sativa* L.

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Abstract approved: Peter J. Bottomley

Agronomic and microbiological studies were conducted on "Anchor" alfalfa (*Medicago sativa* L.) established from uninoculated seed on three Aridisols in central Oregon. Significant responses to 230 Kg N per ha (NH_4NO_3), which was applied in mid-April and after each first and second harvests, were observed in yield parameters of the third harvest in five of seven post-establishment cuttings. Of the isolates of *Rhizobium meliloti* obtained from nodules of ten-week old plants at one of the sites 20% were ineffective, 16% were of intermediate effectiveness and 64% were highly effective. In the second post-establishment season no ineffective nodule isolates were recovered, but nodule numbers declined from 66 to 11 per plant between the first and third harvests. All isolates recovered from plants at the third harvest were of intermediate effectiveness. Microbiological studies were focused on the development of methods to discriminate between and

identify R. meliloti isolates occupying nodules of alfalfa at one of these field sites.

A collection of 300 field isolates of R. meliloti were subdivided into seven groups based on intrinsic antibiotic resistance characteristics. Two hundred and four and 55 isolates were placed into two groups, C and F, respectively. Group C isolates dominated the root nodule population in all but one of nine quadrants analyzed. Antiserum raised to a group C isolate, #31, cross agglutinated with 46 of 55 group C isolates. There were no cross reactions between isolates from any of the other six groups. Thirty three of 35 cross agglutinating field isolates from group C had the same sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) protein profile pattern as isolate #31 whereas non-agglutinating isolates had different protein profiles from the latter.

Further analysis of 32 isolates obtained from ten-week old plants in one replicate plot by SDS-PAGE showed that 12 and 6 isolates were represented by two protein profiles, gel types A and B, respectively. The remaining 14 isolates were represented by unique gel types. Antiserum raised to #31, a gel type A isolate, cross agglutinated with the 12 gel type A isolates and only with two of the other 20 isolates, each of which was represented by a distinct gel type. Gel-immune-diffusion analysis showed that 9 of the 12 gel type A isolates were antigenically identical to #31. Analysis of 79 nodule isolates obtained from a systematic sampling

of plants from the same replicate plot during the second post-establishment season showed that 53% of these isolates were identical to #31 and were distributed in nodules of plants growing throughout the plot. Isolates identical to #31 by serological and SDS-PAGE methodologies could still be subdivided further into two symbiotic effectiveness classes. The data suggest the need for complementary methods to identify unequivocally field isolates of R. meliloti, that some members of the diverse indigenous population are more dominant nodule occupants than others, and that turnover and change of nodule occupants may be a factor in limiting symbiotic N_2 fixation, and in the yield potential of alfalfa in the latter part of the growing season in this region of Oregon.

Delineation of the Composition of an Indigenous Soil Population of *Rhizobium meliloti* in Root Nodules of Uninoculated Field Grown *Medicago sativa* L.

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CHAPTER I

Introduction

Medicago sativa L., commonly called alfalfa, is a perennial forage legume, which, in a symbiotic association with the bacterium *Rhizobium meliloti*, can fix N_2 at an annual rate of up to 300 kg N per ha. (Bell and Nutman, 1971; Phillips, 1980). It is one of the most effective of N_2-fixing legume-*Rhizobium* symbioses known. Agricultural exploitation of alfalfa's capacity to produce excellent forage and accrue N is ancient (Bolton *et al.* 1972), although symbiotic N_2-fixation itself was not definitively demonstrated until the late 19th century by Hellriegel (1887). Increased interest in alfalfa is evident still today in increased acreage planted to alfalfa, as well as in the increase in demand for alfalfa as a high protein feed for the dairy and cattle industries. Thus interest in establishing *M. sativa* L.-*R. meliloti* symbiosis in the field is great especially in those regions where alfalfa production is on the increase such as central and eastern Oregon. Over the past twenty years there has been much interest in the idea of enhancing biological N_2-fixation as is evident in the number of reviews and books on the subject (Quispel, A., 1974; Newton and Hyman, 1975; Hardy *et al.* 1977; Hardy and Gibson, 1977; Hardy and Silver, 1977; Hollaender *et al.* 1977; Newton *et al.* 1977;

Döbereiner et al. 1978; Dommergues et al. 1978; Gordon et al. 1979; Subba Rao, 1979; Newton and Orme-Johnson, 1980). Since establishing a productive stand of alfalfa entails the development of an effective symbiotic N_2-fixing relationship with R. meliloti which is either introduced as inocula or which comprises an indigenous nodulating soil population it is imperative that one or the other can lead to optimal yields.

Alfalfa is a perennial legume, and thus, its behavior and management during the establishment season are quite distinct from subsequent post-establishment seasons. Establishment seasons in central Oregon produce a single harvest of about 2000 kg ha^{-1} of herbage dry weight whereas post establishment seasons can produce three harvests of 12,000 to 14,000 kg ha^{-1}. Weeds will reduce alfalfa yields during the establishment season (Peters and Peters, 1972). Inoculation responses which are seen at the establishment season may not be observed in post-establishment seasons either because inoculant strains fail to survive in an otherwise R. meliloti-free soil, or the indigenous soil population nodulates with increased frequency at the expense of the inoculant strains. In either case the indigenous soil population of R. meliloti is a significant factor in alfalfa's establishment and subsequent yield potential.

The importance of indigenous populations of R. meliloti in alfalfa inoculation trials and to post-establishment year of production was shown from studies conducted during the

International Biological Program. Inoculation responses were seen in the year of establishment, but in subsequent years poorly nodulated plants in uninoculated plots became well nodulated (Bell and Nutman, 1971; Brockwell, 1971). Jansen van Rensburg and Strijdom (1982b) chose strains of R. meliloti considered to be highly competitive nodulators for field inoculation studies in a soil containing an indigenous population of R. meliloti. The strains did not establish themselves in nodules, or persist as nodule occupants in subsequent years to the extent that was predicted from preliminary sterile soil experiments. Hardarson et al. (1982) observed a decline in the percentage of nodules occupied by an inoculant strain of R. meliloti even during the establishment year. An increase in the percentage of nodules occupied by members of the indigenous population accompanied the decline of the inoculant strain. However, the effectiveness status of isolates from a resident population of R. meliloti may be suboptimal as has been reported (Schiffman, 1958; Hely and Brockwell, 1961; Brockwell, 1962; Hely and Brockwell, 1966; Bottomley and Jenkins, 1983). Their presence nevertheless may make the frequency of nodulation by highly effective inoculant strains sufficiently low to have no enhancing effect on production.

The literature on the distribution and behavior of R. meliloti in soils is scant and somewhat contradictory. Reports have characterized R. meliloti as being sensitive to acidity and not able to colonize extensively acidic soils (Bryan, 1923; Peterson

and Gooding, 1941; Obaton, 1971; Rice et al. 1977; Lowendorf et al. 1981). Barber (1980) found that in Oregon soils a decrease in numbers of R. meliloti gram^{-1} of soil correlated significantly with decreasing soil pH. Though Vincent (1954) indicated that R. meliloti was not very saprophytically competent since it became seriously depleted from soils not cultivated to Medicago spp., Hely and Brockwell (1962) showed that R. meliloti may be widely distributed in soils with no previous history of alfalfa. In fact, Bergersen (1970) reported that R. meliloti survived for eleven years in soils in the total absence of plants from the genus Medicago. A few studies have shown that R. meliloti can withstand extremes of heat and dessication (Brockwell and Whalley, 1962; Brockwell and Phillips, 1965, 1970; Brockwell and Whalley, 1970). The effectiveness status of isolates from soils which have either never been planted or not cultivated to medics for several years can be significantly lower than commercial inoculant strains when challenged against commercial alfalfa cultivars (Brockwell and Hely, 1961, 1966). A survey of established alfalfa fields in which the symbiotic effectiveness status of nodule occupants was measured showed that the majority of the R. meliloti isolates obtained were suboptimally effective relative to standard commercial inoculant strains (Bottomley and Jenkins, 1983). If reliable means of identifying distinct strains of R. meliloti had been developed as they had in the studies of R. japonicum and R. trifolii in which serological techniques in particular have been successfully

applied, perhaps there would have been more ecological studies of R. meliloti.

Several important ecological studies of R. japonicum and R. trifolii were undertaken in order to investigate the pragmatic problem of establishing inoculant strains as nodule occupants and determining their ability to persist in soil over multiple growing seasons (Johnson et al. 1965; Brockwell and Dudman, 1968; Holland, 1970; Gibson et al. 1976; Roughly et al. 1976; Brockwell et al. 1982). Taking R. trifolii as an example, in most cases a strain of R. trifolii nodulated well, i.e. occupied >50 percent of the nodules tested, at establishment; but in subsequent seasons the inoculant strains occupied a smaller number of the nodules tested. The indigenous soil population of R. trifolii which nodulated the host would occupy the vast majority of nodules. Since field trials showed that some inoculant strains nodulated better against an indigenous population of Rhizobia than others, investigations into factors which may relate to competitive and persistance abilities were undertaken.

These investigations generally compared two isolates against each other on a host seedling under axenic conditions and attempted to correlate relative frequency of nodulation with such factors as root surface colonization and speed of nodulation (Pinto et al. 1974; Labandera and Vincent, 1975; Franco and Vincent, 1976; Jansen van Rensburg and Strijdom, 1982a). There have been several reports of differential competition between effective and ineffective

isolates (Nicol and Thornton, 1941; Robinson, 1969; Russell and Jones, 1975a and 1975b; Franco and Vincent, 1976; Amarger, 1981a and 1981b). A general point of the latter studies was to determine if there was a significant correlation between competitive abilities and effectiveness status of the isolates tested. In general, it was found that competitiveness was independent of effectiveness. In related studies data were obtained from which it was postulated that lines of alfalfa selected for superior N_2-fixing ability can themselves select for the more effective isolates from a mixed population of R. meliloti (Hardarson et al. 1981; Hardarson et al. 1982). Many other studies which show the influence of the host plant on the competitive characteristic of individual isolates have been documented in the literature (Nicol and Thornton, 1941; Johnson and Means, 1960; Holland, 1966; Caldwell and Vest, 1968; Robinson, 1969; Masterson and Sherwood, 1974; Russell and Jones, 1975a; Diatloff and Brockwell, 1976; Roughley et al. 1976; Jones and Hardarson, 1979). Abiological factors such as temperature (Weber and Miller, 1972; Hardarson and Jones, 1979; Roughley et al. 1980), soil types (Vincent and Waters, 1954; Damirgi et al. 1967), soil pH (Barber, 1980; Jansen van Rensburg and Strijdom, 1982b), antagonism between competing strains (Schwinghamer, 1971), and levels of inoculation (Vincent and Waters, 1953; Hely, 1965; Ireland and Vincent, 1968) have been studied. Though the factors involved in an isolate's competitive abilities in terms of relative frequency of nodulation are still

unelucidated, studies aimed at measuring an isolate's competitive abilities are reported without any attempt to address the true nature of the problem (Amarger, 1981b). Such studies seem futile in light of experiments by Jansen van Rensburg and Strijdom (1982b), in which the competitive abilities of commercial inoculant strains, predetermined under sterile soil conditions, were tested in the field against an indigenous, nodulating population the results of which did not correspond with established ranking in competitive abilities.

Few studies have been attempted to characterize an indigenous, nodulating, soil population of a Rhizobium sp. Interest was not aroused until it was documented that in the midwest inoculation responses were not observed in soybean cultivation (Johnson et al. 1965; Ham et al. 1971a). It was discovered that inoculated strains of R. japonicum could not compete with the resident soil populations of R. japonicum. Further analysis of indigenous soil populations of R. japonicum indicated that isolates of serogroup 123 invariably occupied the majority of the soybean nodules (Damirgi et al. 1967; Ham et al. 1971b). Despite this fact Reyes and Schmidt (1979, 1981), using strain specific immunofluorescence, could find no evidence for either numerical proliferation of R. japonicum in the rhizosphere of soybeans relative to the bulk soil or that serogroup 123 was more numerous than any other serogroup in the rhizosphere. Though the ability to proliferate in the rhizosphere may not be a factor in the competitive capacity of the

slow-growing R. japonicum, there is evidence that selective proliferation is a factor in R. meliloti associations (Jansen van Rensburg and Strijdom 1982a). It has been shown that some individual strains of R. leguminosarum (Amarger, 1981b; May and Bohlool, 1983) and R. phaseoli (Robert and Schmidt, 1983) appear to be competitively superior to other individual strains when paired one-on-one and also demonstrate a competitive superiority over indigenous soil populations of nodulating Rhizobium. This may not be the case for R. meliloti although it has been documented in only two papers that uncharacterized members of the indigenous nodulating soil population of R. meliloti dominate as nodule occupants (Hardarson et al. 1982; Jansen van Rensburg and Strijdom, 1982b).

Though the ideal inoculant strain of R. meliloti would be both highly effective at symbiotic N_2-fixation, and able to nodulate with a dominating frequency over an indigenous, nodulating, soil population, it is evident that on the whole only an inoculant strain's ability to fix N_2 symbiotically may be counted on. However, laboratory studies have shown that strains of R. trifolii, for example, can lose completely or express inferior symbiotic effectiveness after repeated subculturing on agar plates, repeated passage through nodules of Trifolium repens (Djordjevic et al. 1982), or exposure to supra-optimum growth temperature (Zurkowski, 1982). Decline in effectiveness was associated with either impaired replication or modification of plasmids carrying

determinants of symbiotic N_2-fixation. In a recent study, Brewin et al. (1983) demonstrated that there is no genetic link between the high effectiveness status of an isolate and its competitive abilities. They showed that plasmids containing determinants of N_2-fixation (sym plasmids) are not associated with competitive characteristics, but that determinants of competitive superiority are associated with the genetic material of the organism which was the recipient of the sym plasmid. Since the efficacy of rhizobial inoculants regarding both stability of symbiotic effectiveness and competitive abilities is questionable there are several reasons for studying an indigenous soil population of R. meliloti. Determining the symbiotic N_2-fixing capacity of an indigenous soil population would indicate the necessity of applying rhizobial inoculants. Delineating and characterizing the components of an indigenous soil population of R. meliloti would indicate the breadth of diversity of the population and reveal if any organisms dominate and persist as nodule occupants. Such information may indicate any limitations an indigenous soil population of R. meliloti would have on alfalfa production; and identifying dominant and persistant organisms may lead to improvements of inoculant strains, and inoculation strategies.

In general there are two fundamental methods of characterizing an indigenous soil population of R. meliloti, agronomic and microbiological. The agronomic approach entails a comparison of yield parameters between field grown alfalfa relying on the

nodulating N_2-fixing capacity of the resident population of R. meliloti and alfalfa supplemented with optimum quantities of fertilizer nitrogen. The microbiological characterization would entail an estimation of the effectiveness status of isolates obtained from a subsample of the nodules counted and collected (Bell and Nutman, 1971). It would also entail a delineation of the distinct members of the indigenous soil population of R. meliloti which would be based on means of strain identification. The agronomic methods would generate data on the herbage dry weight yields, amount of N removed with the dry matter, and hay quality, i.e. percent tissue N. These data would reflect any limitations that the indigenous soil population of R. meliloti would have on production in relation to management practices such as multiple harvests in the post-establishment growing seasons.

In addition to the agronomic parameters various methodologies have been developed in an attempt to discriminate between, or to verify the identity of the nodule isolates of Rhizobium spp. Identification procedures have centered around either specific serological or antibiotic resistance characteristics as features for discriminating or confirming the identity of selected strains of Rhizobium spp. (Schwinghamer and Dudman, 1980). Three additional methods of Rhizobium strain identification have been described recently in the literature. Intrinsic antibiotic resistance patterns (Josey et al. 1979; Beynon and Josey, 1980; Kremer and Petersen, 1982), and separation of cellular proteins by

sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Leps et al. 1980; Noel and Brill, 1980; Roberts et al. 1980) were shown to be capable of discriminating between isolates of indigenous soil populations of various Rhizobium spp. DNA colony hybridization has also been reported to be a discriminating method of Rhizobium strain identification (Hodgson and Roberts, 1983), though there are no reports of its use for investigating indigenous soil Rhizobium populations. Of course, an important additional criterion for successful methodologies of strain identification is the capacity of moderately skilled personnel to handle a large number of isolates.

For many years serological methods have been used to identify and discriminate between strains of Rhizobium spp. (Dudman, 1977; Schwinghamer and Dudman, 1980; Vincent, 1941, 1982), and have been used successfully in ecological studies of R. japonicum (Date and Decker, 1965; Schmidt, 1974; Reyes and Schmidt, 1979, 1981), and R. trifolii (Vincent, 1954). Although serological methods have been used successfully to show that different isolates of R. meliloti are antigenically heterogeneous (Stevens, 1923, 1925; Vincent, 1941), even within the same field and on the same plant (Hughes and Vincent, 1942; Purchase et al. 1951b), very little use has been made of this method in recent years for identification purposes. The reason for this hiatus of interest is that the serological nature of R. meliloti is inherently problematical. Reports have appeared which show extensive cross reaction between distinct

isolates (Humphrey and Vincent, 1975; Wilson, 1975; Sinha and Peterson, 1980) and anomolous behavior of both antigens (Gibbins, 1967) and antisera (Kishinevsky and Gurfel, 1980) of R. meliloti.

Although antibiotic resistance markers have been introduced into strains of R. meliloti (Schwinghamer, 1964, 1967; Obaton, 1971; Schwinghamer and Dudman, 1973; Danso and Alexander, 1974; Hardarson et al. 1981) the technique has only rarely been reported for the purpose of identifying specific strains in field situations (Hardarson et al. 1982; Jansen van Rensburg and Strijdom, 1982b). Antibiotic markers, or any other genetic marker, are useful essentially only for following the fate of inoculant strains, and are not applicable to the problem of discriminating between components of an indigenous soil population. In recent studies variations in intrinsic antibiotic resistance patterns have been used to discriminate between individual isolates of R. phaseoli (Josey et al. 1979; Beynon and Josey, 1980; Kremer and Peterson, 1982). In these reports the collection of isolates in question were, as a whole, rather sensitive to low concentrations of antibiotics, and contrasted with similar observations made with collections of slow-growing Rhizobia spp. which showed higher intrinsic levels of resistance (Pankhurst, 1977; Cole and Elkan, 1979; Kremer and Peterson, 1982). Observations made with a few laboratory strains of R. meliloti showed that they were, in general, inherently more resistant to antibiotics than isolates of other fast-growing species of Rhizobium which were tested

(Schwinghamer and Dudman, 1973). The usefulness of intrinsic antibiotic resistance patterns is in its capacity to discriminate between components of a resident soil population of a Rhizobium sp. It also characterizes the antibiotic sensitivities and resistances of a Rhizobium population as a whole. If many isolates from a population are intrinsically resistant to spectinomycin, for example, then spectinomycin may not be an appropriate marker for an inoculant strain under such conditions.

One-dimensional (SDS)-polyacrylamide gel electrophoresis as a means of Rhizobium strain identification was used specifically to discriminate between isolates of an indigenous soil population of R. japonicum and laboratory strains of R. japonicum representing certain serogroups (Noel and Brill, 1980). Isolate identification by this "protein profile" method is a fairly unequivocal means of discerning between components of an indigenous population of Rhizobium spp. provided that the protein profile patterns of the strains of interest are reproducible over time.

Gibson et al. (1971) showed that R. japonicum serogroup 123 was not a uniform population, as it was considered at the time, but rather a heterogenous population. Noel and Brill (1980) demonstrated the same using SDS-PAGE. These reports indicate that more than one method of strain identification ought to be employed if an isolate's identity is to be as unequivocal as possible. Since R. meliloti has a history of being problematical when using serological methods of identification, a preliminary delineation of

a population by such means as SDS-PAGE would give a researcher the opportunity to choose isolates which have unique "protein profiles" for antisera production. Such an opportunity could lead perhaps to antisera with more apparent antigenic specificity and less cross reaction and equivocal data.

Application of complementary methods of strain identification made possible the delineation of a resident soil population of nodulating R. trifolii (Dughri and Bottomley, 1983). As stated previously the results of such a delineation would indicate if any isolates of the same identity dominate as nodule occupants, and if any persist as such over successive growing seasons. Since information on the diversity of R. meliloti isolates occupying nodules of field grown alfalfa is sparse (Hughes and Vincent, 1942; Purchase et al. 1951a; Purchase et al. 1951b), identifying dominant and persistent members of a resident soil population of R. meliloti would be a first step towards a systematic analysis of what physiological and ecological factors may be involved in competitive dominance and persistence.

To this end my research has investigated the connection between the extent of nodulation and effectiveness status of the indigenous soil population of R. meliloti surveyed and production of field grown alfalfa. Thus, a first objective of this study was to estimate the symbiotic N_2-fixing performance of three indigenous soil populations of R. meliloti for at least two post-establishment seasons. The agronomic parameters of herbage dry weight yields,

amounts of N removed, and total % tissue N were collected for each harvest of alfalfa. Comparisons of the yield data for each harvest between alfalfa relying on symbiotic N_2-fixation as a major source of N and alfalfa adequately supplemented with fertilizer N were made to estimate the symbiotic performance of the indigenous soil R. meliloti. Measurements of the number of nodules per plant and the effectiveness status of isolates, which were obtained from subsamples of the nodules counted, for the establishment season and a post-establishment season were made. These measurements along with the yield data characterize the symbiotic N_2-fixing capacity of indigenous soil population of R. meliloti under field conditions. Applying complementary methods of strain identification to the collection of R. meliloti obtained as described above two other objectives of this study were met. First, a delineation of the components of a nodulating indigenous soil population of R. meliloti was made and concurrent with the delineation of the population the extent of diversity and field distribution of the members of the population investigated was measured. The second objective was to recognize any R. meliloti isolates which dominated as nodule occupants in the field during the establishment season, and to determine if such dominating isolates persist as such over post-establishment seasons.

CHAPTER II

Seasonal response of uninoculated alfalfa to nitrogen fertilizer: possible relationship to soil nitrogen, nodule turnover, and symbiotic effectiveness of *Rhizobium meliloti*[1]

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ABSTRACT

'Anchor' alfalfa was established from uninoculated seed on three moderately deep (0.6 m) Aridisols in central Oregon. Fertilizer nitrogen (NH_4NO_3) was applied at rates of up to 230 kg of N/ha in mid-April and immediately after each of the first and second harvests of each post-establishment growing season. Responses to fertilizer N were seen as increases in either dry weight, reduced N removed, or percentage N in herbage of the third harvest in five of the seven post-establishment cuttings. No significant responses to fertilizer N were seen in the first harvests and were seen only in two of the seven second harvests. The mineral N levels in the soil profiles under the non-fertilized plants at two of the sites declined during the growing season. The magnitude of this decline (35 and 40 kg/ha of N) approximated the difference between the total reduced N removed in the herbage of N-fertilized and non-fertilized plants. Symbiotic effectiveness tests of isolates of Rhizobium meliloti taken from nodules of 10 week-old plants during the establishment year showed that 20% of the isolates were ineffective, 16% were of intermediate effectiveness, and 64% were highly effective. In the second post establishment growing season although no ineffective nodule isolates were recovered, nodule numbers declined from 66 to 11 per plant between the first and third harvests and no isolates recovered from nodules at the third harvest were highly effective. The influence of nodule turnover, nodule occupants of

sub-optimum effectiveness, and the magnitude of the contribution of soil N to alfalfa production during post-establishment seasons requires more study.

INTRODUCTION

In Oregon approximately 400,000 acres are currently in alfalfa production. Much of this land lies east of the Cascade mountain range where variations in soil type and depth, elevation, irrigation practices, and cultivars in production make it extremely difficult to predict yield potentials and to diagnose problems. Although there is information in the literature on the effects of important environmental variables such as temperature (Cralle and Heichel, 1982) harvesting (Vance et al., 1979; Cralle and Heichel, 1981; Heichel, et al., 1981) and combined nitrogen (Fishbeck and Phillips, 1981) upon nodulation, nodule senescence, and nitrogen fixing activity of alfalfa, most of this information has been obtained from either greenhouse studies or during the establishment year of field-grown plants. Very little information exists on these phenomena in post-establishment seasons when the economic gains from this perennial forage legume are realistically expected.

The impact of Rhizobium meliloti on post-establishment seasons deserves more attention. Although Vance et al. (1979), saw no evidence for nodule turnover in response to harvest of alfalfa in a greenhouse experiment, field observations have provided evidence for nodule turnover. Bell and Nutman (1971) showed that increased production of alfalfa in post-establishment seasons correlated with an increased frequency of highly effective N_2 fixing strains of R. meliloti in nodules. Turnover of nodule occupants in the post-establishment season was observed when inoculant strains of R.

meliloti were found to be replaced by members of the indigenous soil population (Jansen van Rensburg and Strijdom, 1982b). This phenomenon has been documented extensively in the literature of Trifolium - R. trifolii associations (Read, 1953; Dudman and Brockwell, 1968; Roughley et al., 1976; Brockwell et al., 1982).

The importance of using N-fertilized plants as controls to assess the performance of symbiotic N_2 fixation in greenhouse and field experiments has been emphasized (Vincent, 1970; Bell and Nutman, 1971; Brockwell, 1980). In the case of alfalfa there are numerous reports showing either a positive or a lack of yield response to fertilizer nitrogen (Gerwig and Ahlgren, 1958; Giddens, 1959; Ward and Blaser, 1961; Doll, 1962; Markus and Battle, 1965; Brockwell, 1971; Lee and Smith, 1972; Hoglund et al., 1974). An explanation for such discrepancies was provided by the greenhouse studies of Fishbeck and Phillips (1981), where they showed that the yield of effectively nodulated alfalfa was less than N fertilized plants in three consecutive harvests after planting but the magnitude of this difference became less over time and disappeared by the fourth harvest.

Preliminary field trials carried out in central Oregon provided no evidence that the yield of alfalfa would respond to inoculation or fertilizer N in the establishment year even though the symbiotic effectiveness of the majority of nodule isolates of R. meliloti taken from several alfalfa production fields in this region was sub-optimum when compared to commercial inoculant

strains (Bottomley and Jenkins, 1983). The objectives of this investigation were to compare yield parameters of alfalfa established from uninoculated seed in soils possessing indigenous populations of R. meliloti with N-fertilized alfalfa in post-establishment growing seasons and to compare the symbiotic effectiveness of isolates taken from nodules in the establishment and post-establishment seasons.

MATERIALS AND METHODS

In June, 1979 two field experiments were established at the central Oregon Agricultural Experiment Station in Madras, Jefferson County, and Powell Butte, Crook County, respectively. The soil at the Madras site was a Madras sandy loam (fine-loamy, mixed, mesic Xerollic Durargid). The soil is typical of this region being moderately deep (0.5-0.6 m), well-drained, and suitable for alfalfa under irrigation. The soil at Powell Butte (site 1) was an Ayres sandy loam (loamy, mixed, mesic, shallow Xerollic Durargid). This soil is 0.5-0.6 m deep and well-drained. The field experiment established in June, 1980 at Powell Butte (site 2) was on a Deschutes sandy loam (coarse-loamy, mixed, mesic, Xerollic Camborthid). This soil comprises approximately 250,000 acres in central Oregon, is moderately deep (0.5-0.6 m) and well drained. Much of the alfalfa produced in this region is grown on soils of these types and under irrigation management. Approximately 30 x 0.1 kg samples of soil were taken with a sterile tube auger to a depth of 0.3 m at each site. The samples were mixed, air dried, sieved (<2 mm) and their chemical properties determined by standard procedures of the soil testing laboratory, Dept. of Soil Science, Oregon State Univ. (Berg and Gardner, 1978). Important characteristics of the three soils prior to seeding were: Madras, pH, 7.2; cation exchange capacity (CEC), 19.1 cmol (NH_4^+) kg^{-1}; base saturation, 100%; organic matter, 1.3%; organic N, 0.08%; extractable P, 96 mg kg^{-1}; nitrate-N, 4.4 mg kg^{-1}; ammonium-N, 5.5

mg kg^{-1}. Ayres: pH, 5.7; CEC, 15.6 cmol (NH_4^+) kg^{-1}; base saturation, 84%; organic matter, 2.8%; organic N, 0.14%; extractable P, 76 mg kg^{-1}; nitrate-N, 39 mg kg^{-1}; ammonium-N, 6 mg kg^{-1}. Deschutes: pH 5.9; CEC, 13.5 cmol (NH_4^+) kg^{-1}; base saturation, 81.8%; organic matter, 2.3%; organic N, 0.1%; extractable P, 38 mg kg^{-1}; nitrate-N, 6.4 mg kg^{-1}; ammonium-N, 4.4 mg kg^{-1}.

Most probable number (MPN) determinations. Subsamples (0.01 kg) of each of the composite soil samples from each site were diluted consecutively in 0.15 M NaCl over the range 10^{-1} to 10^{-6}. Seeds of 'Anchor' alfalfa were surface sterilized, germinated and transferred to test tubes (30 x 3 cm) containing nitrogen-free mineral salts agar composed of per liter: $CaCl_2$, 0.6 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; NaCl, 0.1 g; ferric citrate, 0.1 g; K_2HPO_4, 0.5 g; KH_2PO_4, 0.25 g; 10 mL of a trace element mixture (Evans, 1974) and 15 g of Bacto agar (Difco Laboratories, Detroit, Mich.). Ferric citrate was dissolved in a small quantity of HCl before adding to the growth medium; the phosphate salts were autoclaved separately in 100 mL of distilled H_2O and added to the remainder (900 mL) of the growth medium after cooling to provide a final pH of 6.8-7.0. Duplicate seedlings were inoculated with 0.2 mL portions of each of the soil dilutions and were grown in a glass house with a temperature range of 30 (day) to 18°C (night). Natural daylight was supplemented with 16 h of illumination from fluorescent F48/T12/D/RS lamps suspended 0.5 m above the growth

tubes to provide an irradiance of 80 W m^{-2} at plant height. After four weeks of growth plants were scored for nodulation and the MPN of R. meliloti was determined by standard procedures (Vincent, 1970). Values of 10^5, 10^6, and 10^6 R. meliloti/kg dry soil were obtained for the Madras, Ayres, and Deschutes soils respectively.

Establishment of the field trials. The soils at each site were ploughed, disced and 90 kg ha^{-1} of S in the form of granular gypsum (14% wt/wt sulfur) were broadcast onto the soil and worked into the surface layers. In June, 1979 surface sterilized uninoculated seed of 'Anchor' alfalfa were sown at a rate of 31.4 kg ha^{-1} into six plot areas (2.4 x 1.5 m) and worked into the upper 1 to 2 cm of soil with ethanol dipped flame sterilized rakes. Irrigation was applied immediately at a rate of 0.8 cm h^{-1} for a 12 h period and repeated at 8 to 10 d intervals throughout the months of June, July, August, and early September. The second site at Powell Butte was established in a similar manner in June, 1980. Sulfur was applied at the same rate as described above and uninoculated, surface sterilized seeds were broadcast into two sets of triplicate plot areas (4.9 x 3.1 m) with irrigation as described above. Soil tests were conducted at all sites during November of 1979 through 1982. Sulfur (90 kg) in the form of gypsum was applied annually to each site in March. In March, 1981, 186 kg ha^{-1} of K in the form of KCl, 39 kg ha^{-1} of P in the form of triple superphosphate (45% wt/wt P_2O_5) were applied to both the sites at Powell Butte.

Fertilizer N applications. Initially 115 kg ha^{-1} of N in the form of NH_4NO_3 were applied to three of the six plots at both Madras and Powell Butte (site 1) in June, 1979. In 1980, 115 kg ha^{-1} of N were applied in March followed by 115 kg and 230 kg ha^{-1} of N after the first and second harvests respectively at both sites. In 1981 and 1982 230 kg ha^{-1} of N was applied in mid-April and after the first and second harvests. At Powell Butte (site 2), 230 kg ha^{-1} of N were applied at establishment and immediately after each harvest. At all three sites the first harvest of each season was made when the plants were at the full bud stage of growth or at the appearance of first flowers and subsequently at the full bud stage of growth for the remainder of each growing season. Alfalfa was swathed with a sickle bar mower (0.9 m wide) down the center of each plot, and approximately 4 cm above the soil surface. The alfalfa was weighed immediately and large sub-samples (1-1.5 kg) were taken for dry weight determinations and tissue analysis. Nitrogen and phosphorus were determined on Kjeldahl digests and Ca, Mg, and K by atomic absorption spectrophotometry after perchloric acid digestion. Sulfur was determined by the method of Tabatabai and Bremner (1970). N-fertilized plots were weeded periodically by hand during the establishment year, and although weeds were not a serious problem thereafter they were accounted for in the herbage dry weight determinations.

Isolation of R. meliloti from field plants. In August, 1980 at Powell Butte (site 2) five plants (10 weeks of age) were chosen

randomly and removed from the ground using metal cores made from muffler pipe, 0.013 m^2 x 0.28 m in length. Individual plants, and their associated soil cores were placed in ziploc bags, stored on ice, and transported to the laboratory. All the nodules were collected from each plant within three to four days of sampling. Forty nodules (approximately one third of the total per five plants) were chosen randomly for purification of the occupants. Nodules were surface sterilized, the contents streaked for purity, and maintained on slants of yeast extract mannitol (YEM) agar (Vincent, 1970). Isolates were stored on slants under sterile aqueous glycerol (20% v/v) at -20°C until required.

In June, 1982, immediately prior to the first harvest, six plant-soil cores were removed systematically from two rows, 1 m apart, in the plot area with a distance of 1 m between cores. All the nodules were collected from each core (which usually included two or three plants) and as many isolates as possible were purified. In September 1982, prior to the third harvest, another six plant soil cores were removed, nodules collected, and the isolates purified. From the isolates obtained from four of the cores in June and six of the cores in September ten isolates were subsampled and analysed for symbiotic effectiveness.

Symbiotic effectiveness tests. Seedlings of 'Anchor' alfalfa germinated from surface sterilized seed, were transferred to slants of mineral salts agar contained in large test tubes (30 x 3 cm). Seedlings were grown under the glasshouse conditions described

above for seven days prior to inoculation so that unhealthy individuals could be culled before establishing the inoculation treatments. Six seedlings were inoculated with 5×10^8 viable cells of each field isolate. Uninoculated plants were included to serve either as minus N controls or for subsequent fertilization with nitrate. Three milliliters of sterile KNO_3 (18 mM) were added to each N control seedling 10 d after inoculation. The nitrate supplements were repeated 24 and 31 d after inoculation. Preliminary experiments had established that this concentration of KNO_3 and this application regime provided optimum growth under these conditions. Each inoculated and uninoculated control seedling received 3 mL of sterile distilled water at the same time intervals. Shoots were harvested 35 d after inoculation, dried at 60°C for 5 d and weighed. Analysis of variance was performed on the data and a Dunnet's two-tailed range test was used to make comparisons of treatment.

RESULTS

Data summarized in Table 1 represent the herbage dry weight, total reduced N content, and percentage of reduced N in herbage tissue for the three experimental sites between the 1980 and 1982 post establishment growing seasons. The herbage dry weight of the first harvest in all seven cases was not affected by the application of up to 230 kg/ha of fertilizer N in the spring after the breaking of winter dormancy. The yield of this first harvest comprised between 37 and 47% of the total seasonal yields. Only in two of the seven second harvests did fertilizer N increase yield parameters whereas in five of the seven third harvests taken between 25 Aug. and 10 Sep. fertilizer N resulted in increases in one or more of the growth parameters measured. In five of the seven cases these differences were not evident in the total seasonal yields since the third harvests were usually the smallest of the season and ranged between 15.5 and 26.5% of the total seasonal yield. In three of these third harvests the responses of herbage dry weight to fertilizer N were highly significant ranging from 35.7 to 52.7% increases above the nonfertilized plots whereas in two of the four other harvests the differences in herbage dry weight, albeit not statistically significant, were approximately 20% increases.

Although visual observations of the plants at the field sites showed no obvious evidence of nutrient deficiencies, tissue analysis was performed on plant samples taken from each harvest of

the 1982 growing season at the Madras site and at Powell Butte site 2. The data summarized in Table 2 show no evidence for nutrient deficiencies nor of obvious stimulatory uptake of these nutrients due to interactions with the fertilizer N.

Analysis of the levels of soil mineral nitrogen throughout the growing season of 1982 was performed. This was undertaken because of the possibility that these relatively shallow soils of low organic N content may not be capable of maintaining an even supply of mineral N throughout the growing season. This has been shown to contribute significantly to the N nutrition of alfalfa (Heichel et al., 1981). Data in Table 3 show there was a measurable decline in the quantity of available mineral N as the growing season progressed, despite the regular irrigation of the stands. These differences extrapolated to approximately 35 kg ha^{-1} and 40 kg ha^{-1} of N in the soil profiles at Powell Butte (site 2) and Madras respectively. These values are similar to the seasonal differences in total reduced N content of N-fertilized and non N-fertilized alfalfa in 5 of the 7 post establishment seasons (40 to 70 kg ha^{-1} of N) (Table 1). It is possible therefore that the response of the third harvest of alfalfa to N fertilizer may be a result of the inability of symbiotic N_2 fixation to compensate for the decline in availability of soil nitrogen as the growing season progressed.

Evidence in support of this was obtained from an analysis of the nodulation of plants and the symbiotic effectiveness status of nodule isolates in establishment and post-establishment seasons at

Powell Butte site 2 (Table 4). Prior to the first harvest in June, 1982, the average number of nodules per plant was 63 and three times greater than the number on the 10 week old plants sampled in the establishment year (1980). However, the sampling of plants prior to the third harvest in September showed a six-fold decline in nodule numbers relative to the numbers found before the first harvest in June. Furthermore, information obtained on the symbiotic effectiveness of individual nodule occupants relative to N fertilized control plants (Table 5) showed that although a small percentage (20%) of nodules were occupied by ineffective strains in 1980, these had been eliminated by 1982. In September, 1982 all the isolates were of sub-optimum effectiveness in contrast to only 42% being of that status in June. These data provide further evidence that the N_2 fixing capacity of the plants had declined as the growing season progressed and there was increasing need for combined nitrogen to supplement the N budget of the plants if maximum growth was to be obtained.

Table 1. Yield parameters of field grown alfalfa either supplemented or not supplemented with NH_4NO_3 at Madras (Ma), and the two Powell Butte field sites (PB-1, PB-2).

			Harvest 1			Harvest 2			Harvest 3			Total	
Site	Treatment	Year	Yield†	N‡	%N§	Yield	N	%N	Yield	N	%N	Yield	N
PB-1	-N¶	1980	6.89	0.21	3.09	5.51	0.16	2.94	2.69	0.09	3.23	15.10	0.46
	+N#		6.16	0.20	3.23	5.66	0.19	3.31	4.11**	0.14**	3.42	15.92	0.53
	-N	1981	5.01	0.15	2.94	4.46	0.12	2.62	2.49	0.07	2.76	11.96	0.33
	+N		5.75	0.19	3.36	4.92	0.16	3.33*	3.72**	0.14**	3.68**	14.39	0.49*
Ma	-N	1980	5.40	N.D.	N.D.	5.36	0.15	2.74	3.19	0.10	2.98	13.95	I.D.
	+N		4.68	N.D.	N.D.	3.45	0.10	2.80	4.33*	0.14*	3.21	12.46	I.D.
	-N	1981	5.62	0.17	3.00	4.69	0.13	2.80	3.71	0.11	2.90	14.02	0.41
	+N		5.15	0.18	3.27	5.23	0.16	3.03	3.53	0.11	3.19	13.91	0.45
	-N	1982	6.56	0.18	2.75	5.42	0.15	2.79	3.52	0.11	3.09	15.51	0.44
	+N		6.77	0.19	2.81	6.29	0.18	2.85	4.21	0.14	3.34*	17.28	0.51
PB-2	-N	1981	7.04	0.24	3.40	4.24	0.14	3.26	3.80	0.11	3.00	15.08	0.49
	+N		7.21	0.24	3.39	4.69	0.17	3.60	4.62	0.15*	3.14	16.53	0.56
	-N	1982	5.71	0.18	3.16	5.08	0.14	2.68	1.98	0.08	4.14	12.78	0.40
	+N		6.06	0.20	3.36	5.91	0.17*	2.88	2.10	0.10	4.59	14.07*	0.47*

†Mean herbage dry weight (Mg ha^{-1}). ‡Mean reduced N in herbage tissue (Mg ha^{-1}). §Mean of percent N in herbage tissue. ¶-N Uninoculated alfalfa not supplied with fertilizer N. #+N Uninoculated alfalfa supplied with fertilizer N. *Significantly different at p = 0.05. **Significantly different at p = 0.01.

Table 2. Nutrient analysis of herbage from post-establishment harvests of alfalfa either supplemented or not with NH_4NO_3 at the PB-2 and Madras (Ma) sites during the 1982 growing season.

Site	Treatment	Harvest	S	P	K	Ca	Mg	N/S
			—— Percent herbage dry wt ——					
PB-2	-N†	1	0.24	0.28	2.32	1.22	0.26	12.8
	+N‡		0.26	0.29	2.33	1.35	0.32	13.0
Ma	-N	1	0.23	0.28	4.00	1.46	0.39	12.2
	+N		0.27	0.29	2.81	1.18	0.40	10.2
PB-2	-N	2	0.27	0.27	2.70	1.28	0.33	9.6
	+N		0.28	0.27	2.60	1.30	0.37	10.0
Ma	-N	2	0.32	0.28	2.65	1.73	0.44	9.0
	+N		0.37	0.27	2.37	1.49	0.47	10.2
PB-2	-N	3	0.36	0.39	3.05	1.51	0.36	11.5
	+N		0.37	0.40	2.93	1.59	0.43	12.5
Ma	-N	3	0.38	0.32	3.07	1.57	0.43	8.0
	+N		0.35	0.31	3.01	1.63	0.51	9.4

†Uninoculated alfalfa, no fertilizer N.

‡Uninoculated alfalfa, supplemented with fertilizer N.

Table 3. Seasonal levels of soil mineral N at the Madras (Ma) and Powell Butte (PB-2) sites during the 1982 growing season.†

Site	Sampling Time	NO_3^-	NH_4^+	$NO_3^- + NH_4^+$	Organic N
		mgN/kg soil			(Percent)
Ma	Nov. 1981	3.2	3.1	6.3	0.09
	Jun. 1982	1.3 ± 0.2	9.4 ± 0.5	10.6 ± 0.6	0.08
	Jul. 1982	1.4 ± 0.3	4.7 ± 0.5	5.7 ± 0.3	0.07
	Sep. 1982	2.7 ± 0.3	3.0 ± 0.5	5.7 ± 0.5	0.08
PB-2	Nov. 1981	7.8 ± 1.1	3.6 ± 0.3	11.4 ± 0.9	0.11
	Mar. 1982	2.2 ± 0.2	13.5 ± 2.2	15.7 ± 2.4	0.12
	Jul. 1982	4.3 ± 1.0	6.4 ± 0.5	10.7 ± 1.1	0.11
	Sep. 1982	5.8 ± 0.6	4.0 ± 0.7	9.8 ± 1.1	0.11

†Soil samples were taken from each of the three nonfertilized replicate plots at each site. S.E. of mean is given for the three replicate samples.

Table 4. Nodulation status of alfalfa plants immediately before the harvest in the establishment season (1980), and before first and third harvests of the 1982 post-establishment season at the PB-2 site.

Sampling Date	Mean No. of Nodules Plant	No. Plants Examined	Total No. of Nodules
Sep. 1980	23 ± 1.8	15	341
Jun. 1982	63 ± 7.5	18	1140
Sep. 1982	11 ± 1.3	26	285

Table 5. Symbiotic effectiveness of isolates of R. meliloti obtained from nodules excised from plants immediately prior to harvest in the establishment year (1980) and prior to the first and third harvests of the post-establishment season (1982) at the PB-2 site.

Sampling date	Effectiveness categories			Total no. of isolates
	E_1[†]	E_2[‡]	E_3[§]	
	—— Percent of total isolates ——			
Sep. 1980	64	16	20	92
Jun. 1982	58	42	0	40
Sep. 1982	0	100	0	59

[†]Represents those isolates producing plant growth (≥27 mg) that was not significantly less than the mean yield of nitrate grown plants at $p = 0.05$. The mean dry weight of nitrate grown plants was 39 ± 6 mg.

[‡]Represents those isolates producing symbiotically derived plant growth (18-27 mg) that was significantly less than the mean yield of nitrate grown plants, and significantly greater than uninoculated plant growth at $p = 0.05$.

[§]Represents those isolates producing symbiotically derived plant growth (8-18 mg) that was not significantly different from the mean yield of uninoculated plants (8 mg ± 2 mg) at $p = 0.05$.

DISCUSSION

These data provide both confirmatory and novel observations with regard to N_2 fixation and alfalfa production. That the total seasonal yield in five of seven cases was not increased by the application of large portions of fertilizer N confirms observations of others (Lee and Smith, 1972) that application of fertilizer N to alfalfa is an uneconomical proposition. However in the context of the importance of N-fertilized controls in the study of the efficiency of N_2 fixation in legumes our observations are of interest. Fishbeck and Phillips (1981) observed that NH_4NO_3 enhanced the first herbage yield of seedling alfalfa and thereafter this difference became less until no significant yield differences were seen by the fourth harvest. In contrast, our observations at these field sites showed a trend for the yields during the post-establishment seasons to decline as the season progressed and to be increasingly responsive to nitrogen fertilizer. Lee and Smith (1972) observed significant increases in crude protein in the third harvest of N fertilized alfalfa but no increase in yield. In our third harvests we observed significant increases in herbage dry weight in three of seven cases, in amount of reduced N removed in four of seven cases and in % N of herbage tissue in two of seven harvests.

It has been documented that alfalfa can obtain a significant portion of its N requirements from the mineralizable soil nitrogen during the establishment year (Heichel et al., 1981). We observed

a small but distinct decline in the pool of mineral N ($NH_4^+ + NO_3^-$) throughout the growing season which could be interpreted most simply to mean that the release of mineral N from organic N did not keep in balance with the rate of its utilization by the alfalfa. The seasonal decline in the quantity of mineral N amounted to 35 and 40 kg N/ha in 0.6 m of soil depth at the two sites and were of a similar magnitude to the differences between the total reduced N content of the fertilized and non-fertilized herbage. The N supplying ability of these low organic N containing Aridisols is unknown and in need of more detailed study. However, the levels of soil mineral N measured were substantially less than those reported in temperate Mollisols of the mid-west where other N related studies on alfalfa have been performed and where mineral N can make a substantial contribution to the N nutrition of alfalfa (Lee and Smith, 1972; Heichel et al., 1981). We can infer that factors limiting the N_2 fixing capacity under these soil conditions might impact more seriously upon field production than in soils with adequate N-supplying capacity.

The studies on nodulation provided information on field grown alfalfa not heretofore reported in the literature. Although previous greenhouse studies showed that nodule numbers did not decline, or declined to a small degree, after harvest of alfalfa (Vance et al., 1979; Cralle and Heichel, 1981), a dramatic 6-fold decrease in numbers of nodules was observed during the 1982 post establishment growing season. We deliberately allowed our plots be

exposed to management practice which included a 14-16 d interval between the final irrigation prior to cutting and the first irrigation after the curing and baling of the hay in the surrounding production fields. This may have been a significant factor contributing toward the decline in nodule numbers. Certainly it emphasizes that caution should be taken in extrapolating data obtained from well-irrigated greenhouse grown plants to field situations.

Data obtained on the symbiotic effectiveness of the field isolates was interesting. The appearance of completely ineffective isolates of *R. meliloti* in the nodules in the establishment year and their complete disappearance by the second post-establishment season supports the observations of Bell and Nutman (1971) that nodule turnover occurs on alfalfa. Furthermore, the decline of superior N_2 fixing isolates and either their replacement by, or the better persistence of, sub-optimally effective isolates could also be a factor contributing to the decline in the N_2 fixing capacity of the plants. Although the literature of *Trifolium/R. trifolii* associations is well documented to show turnover of nodule populations through subsequent growing seasons (Dudman and Brockwell, 1968; Brockwell *et al.*, 1982) scant attention has been paid to other perennial forage legumes. Certainly situations where this turnover could be contributing to the decline of N_2 fixation, concomitant with a decline in field production and longevity of the stand are worthy of more intensive field-oriented studies.

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CHAPTER III

Composition and field distribution of the population of *Rhizobium meliloti* in root nodules of uninoculated field grown alfalfa.

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ABSTRACT

Only four antibiotics (kanomycin, spectinomycin, ampicillin and novobiocin) of ten tested were capable of discriminating between root nodule isolates of Rhizobium meliloti obtained from uninoculated field-grown alfalfa (Medicago sativa L.). The 300 isolates in the collection were subdivided into seven groups based on their intrinsic antibiotic resistance characteristics with 204 and 55 isolates placed into two groups, C and F, respectively. Isolates from group C dominated the root nodule population on plants in eight of the nine quadrants analyzed. Furthermore they were one of the two dominant groups found in nodules formed on plants grown in a plant infection-soil dilution experiment and challenged with a composite soil sample from the field site. Antiserum raised to a group C isolate (#31) cross-agglutinated with 46 of 55 group C isolates to a titer identical with that of the parent antigen. There were no cross-reactions between isolates from any of the other six groups. Only 9 of 34 isolates from group F cross agglutinated with antiserum raised to a group F isolate (#17). Thirty-three of 35 cross agglutinating field isolates from group C had the same SDS-PAGE protein profile pattern as isolate #31 whereas non-agglutinating isolates from the same group had distinctly different protein profile patterns. The data suggest that intrinsic antibiotic resistance characteristics can be a useful complementary tool to be used in conjunction with other methods for the identification and discrimination of isolates of R.

meliloti. It should not be used on its own as a strain identification method.

INTRODUCTION

Various methodologies have been developed in an attempt to discriminate between, or to verify the identity of isolates of *Rhizobium* spp. Identification procedures have centered around either specific serological or antibiotic resistance characteristics of salient strains (Schwinghamer and Dudman, 1980). In this connection *Rhizobium meliloti* has been a problematical organism. Although serological methods were used successfully to show that strains of *R. meliloti* are antigenically heterogeneous (Stevens, 1923, 1925; Vincent, 1941) even within a field and on the same plant (Hughes and Vincent, 1942; Purchase *et al.*, 1951), very little use has been made subsequently of this technique for identification purposes. Reports have appeared which show extensive cross reaction between strains (Humphrey and Vincent, 1975; Wilson *et al.*, 1975; Sinha and Peterson, 1980) and which show anomolous behavior of both antigens (Gibbins, 1967) and antisera (Kishinevsky and Gurfel, 1980) of *R. meliloti*.

Although antibiotic resistance markers have been introduced successfully into strains of *R. meliloti* (Schwinghamer, 1964, 1967; Obaton, 1971; Schwinghamer and Dudman, 1973, Danso and Alexander, 1974; Hardarson *et al.*, 1981) the technique has only rarely been used to identify specific strains in field situations (Hardarson *et al.*, 1982; Jansen van Rensberg and Strijdom, 1982b). Recently, variations in intrinsic antibiotic resistance patterns have been used to discriminate between strains of *Rhizobium phaseoli* (Josey

et al., 1979; Beynon and Josey, 1980; Kremer and Peterson, 1982). In these reports the collections of isolates in question were, as a whole, rather sensitive to very low concentrations of antibiotics, and contrasted with similar observations made with collections of slow-growing Rhizobium spp. where somewhat higher intrinsic levels of resistance were observed (Pankhurst, 1977; Cole and Elkan, 1979; Kremer and Peterson, 1982). Interestingly, observations made with a few laboratory strains of R. meliloti showed that they were in general inherently more resistant to antibiotics than isolates of other fast growing species of Rhizobium which were tested (Schwinghamer and Dudman, 1973).

The objectives of this study were to ascertain the antibiotic resistance characteristics of field isolates of R. meliloti taken from nodules formed on uninoculated alfalfa (Medicago sativa L. cv. 'Anchor') sown into a field site. Secondly, determine if antibiotic resistance characteristics would correlate with serological and protein profile pattern methods of identification. Finally, determine the extent of diversity of the strains in this nodule population and to ascertain if any strains were dominating the nodules.

MATERIALS AND METHODS

Soil and site description. The field site was located on the central Oregon Agricultural Experiment Station, Powell Butte, Oregon. The soil was a moderately deep (0.6 m), well drained, sandy loam of the Deschutes series; a member of the coarse loamy, mixed, mesic Xerollic Camborthids. The soil was formed in mixed alluvium with pumice and volcanic ash over basalt bedrock and had been in an alfalfa-cereal rotation for many years. Approximately thirty-0.1 kg samples of soil were taken randomly throughout the plot area to a depth of 0.3 m. The samples were mixed thoroughly, air dried and sieved (<2 mm). Chemical properties were determined by the standard techniques of the soil testing laboratory of the Dept. of Soil Science, Oregon State University (Berg and Gardner (1978). Some of the specific characteristics are as follows: pH 5.9 ± 0.1; organic matter 2.3%; cation exchange capacity 13.5 cmol (NH_4^+) kg $soil^{-1}$; % base saturation, 82; 38 mg extractable P, kg $soil^{-1}$; 3.8 cmol Ca kg $soil^{-1}$; 1.6 cmol Mg kg $soil^{-1}$; 0.3 cmol K kg $soil^{-1}$. The most probable number (MPN) of *R. meliloti* was determined by standard procedures (Vincent, 1970) to be 1.0 ± 0.3 x 10^6 kg^{-1} dry soil.

Establishment of stand. The soil was ploughed, disced and 90 kg S ha^{-1} in the form of granular gypsum (14% wt/wt sulfur) was broadcast and worked into the seed bed. Surface sterilized uninoculated seed of alfalfa cv. 'Anchor' was sown at the rate of 31.4 kg ha^{-1} into 18 plot areas (4.9 m x 3.05 m) and worked into

the upper 1-2 cm with rakes. Irrigation was applied immediately to the plot areas at a rate of 0.8 cm h^{-1} per 12 h period with irrigations of similar duration repeated at 8-10 day intervals throughout June, July, August, and early September.

Isolation of R. meliloti from nodules. Five plants (10 weeks old) were selected randomly from each of nine replicate plots also chosen at random. Each individual plant, plus the accompanying soil core, was placed into a ziploc bag, and transferred on ice to the laboratory. All nodules were collected from each plant within three to four days of sampling. From each plot 40 nodules (approximately one third to one half of the total per five plants) were chosen at random for purification of the occupants. Isolates were also obtained from the nodules formed on plants grown in the greenhouse on mineral salts agar in the plant infection-soil dilution experiment used to determine the most probable number of R. meliloti in a composite soil sample. All nodules (approximately 40) were collected from plants where nodulation had occurred and included those challenged with 0.2 ml portions of 10^{-1} and 10^{-2} dilutions of the soil sample. Nodules were surface sterilized, the isolates streaked for purity and maintained on slants of yeast extract mannitol (YEM) agar (Vincent, 1970). Isolates were stored on slants under glycerol (20% v/v) at -20°C until required.

Methods of identification. Two media were used to grow the isolates. YEM, adjusted to pH 7.0, was used to grow isolates for determining antibiotic resistance patterns and for determining

protein profile patterns by SDS-PAGE. A defined medium (GM) was used to grow isolates for serological analysis and was composed of the following: mannitol, 10 g; L-(+)-glutamic acid, monosodium salt, monohydrate, 1 g; K_2HPO_4, 0.5 g; $MgSO_4$ $7H_2O$, 0.2 g; NaCl, 0.1 g; $CaCl_2$, 0.08 g; calcium pantothenate, 10 mg; thiamine hydrochloride, 10 mg; biotin, 0.3 mg; and was adjusted to pH 7.0 prior to autoclaving.

Determination of antibiotic resistance/sensitivity patterns. Ten antibiotics were used in preliminary experiments to provide compounds with a variety of target sites and included: kanamycin sulfate, neomycin sulfate, streptomycin sulfate, ampicillin, polymyxin B sulfate, rifampicin, novobiocin sodium salt, nalidixic acid (all from Sigma Chemical Co., St. Louis, Mo.), spectinomycin hydrochloride (Upjohn Ltd., Kalamazoo, Mich.), and carbenicillin disodium salt (Roerig, Pfizer, New York, N.Y.). Stock solutions of the antibiotics were made up in distilled water immediately prior to use and filter sterilized through millipore filters (0.4 µm pore size). Stock solutions of rifampicin were prepared by dissolving in a minimum quantity of absolute ethanol and then diluting to volume with water. Appropriate quantities of the antibiotic stock solutions were added to portions of melted YEM agar held at 48°C, mixed thoroughly, and the latter solutions were then poured into large petri plates (15 cm. diam.). Isolates were grown in YEM broth for 48 h and the optical density at 560 nm was determined. A portion of each culture was diluted ten fold in 0.15 M NaCl to a

cell density ranging between 1.6 and 3.1 x 10^8 viable cells ml^{-1}, unless stated otherwise, and portions (0.2 ml) dispensed into sterile Durham tubes (5 x 0.6 cm). From preliminary experiments it was determined that each needle of the multiple point inoculator transferred between 0.53 and 1.02 x 10^4 cells from such suspensions to the agar plates. Duplicate plates of each concentration of antibiotic were incubated for 48 h at 30°C and the isolates scored for growth by visual inspection. A maximum of 40 isolates were screened in each experiment.

Serological analysis

Preparation of antisera. Isolates 31 and 17, which were represented by two different antibiotic resistance patterns (C and F) respectively were grown at 30°C in medium GM for 48 h on an orbital shaker. Cells were harvested by centrifugation, washed three times in 0.15 M phosphate buffered saline, pH 7.0 and resuspended to 2 x 10^9 cells/ml in 0.15 M sterile NaCl. Formalin was added to the suspensions to a 0.3% (v/v) concentration. The suspensions were heated at 100°C for 30 min to denature flagellar (H) antigens. The heat-treated suspensions were emulsified in equal volumes of Freund's Complete Adjuvant (Difco Laboratories, Detroit, Mich.) and then 1 ml (1 x 10^9 cells) of each antigen was injected intramuscularly in the rear thigh of three white New Zealand male rabbits 10 weeks of age. Four weeks later rabbits were given a second injection of cells prepared as above, except the antigen suspensions were emulsified in Freund's Incomplete

Adjuvant (Difco). Ten days following the second injection 5 ml of blood was collected aseptically from the marginal ear vein, and the sera were assayed by tube agglutination for specific antibody titer. In both cases adequate titers were obtained (1:320 for isolate 17; 1:1280 for isolate 31). There were no cross agglutination reactions of either antigen when challenged reciprocally with the heterologous antisera. Subsequently 60-90 ml of blood was collected aseptically from each rabbit by cardiac puncture 14 and 16 days following the secondary immunization. Blood was allowed to clot overnight at 23°C and sera obtained by centrifugation at 582 x g. The sera were heat-inactivated at 56°C for 30 min and stored at -20°C in 5 ml portions until required.

Whole-cell tube agglutination. The preparation of antigens was essentially as described above except that cells were washed in 0.15 M phosphate buffered saline containing 0.15 M NaCl, and finally resuspended in 83 mM NaCl containing 0.5% v/v formalin. These suspensions were heated at 95°C for 40 min. Portions (0.4 ml) of the antigen suspension were incubated with an equal volume of consecutive two-fold dilutions of antiserum 31 ranging from 1:10 to 1:5120 dilutions of the original antiserum. In the case of antiserum 17 the dilution series ranged from 1:10 to 1:640 dilution of original antiserum. Controls included non-immunized rabbit serum and antigen suspension without antiserum. Tubes were incubated partially submerged at 52°C for four hours. Titers were recorded as the greatest serum dilution in which agglutination was clearly evident.

Preparation of cell extracts for analysis by SDS-PAGE. Each isolate was grown at 30°C in 50 ml of YEM broth on an orbital shaker at 200 rpm to the early stationary phase of growth (2.0 x 10^9 cells ml^{-1}). Cultures were harvested by centrifugation at 25,000 x g for 10 min. The cell pellets were resuspended and washed three times in ice cold 10 mM Tris-HCl buffer, pH 7.6 to remove mucilage. After the first wash it became easier to pellet the cells by centrifugation and 12,000 x g for 10 min would suffice. The final cell pellet was resuspended in 0.5 ml of the same Tris buffer, transferred to a polypropylene microfuge tube (1.5 ml capacity) and the cells disrupted by sonication whilst immersed in crushed ice. The sonication protocol involved using a Bronson sonifer, Model 200 fitted with a double step microtip and consisted of three separate 30s bursts at 40W output with two min. of cooling between bursts. After disruption 0.5 ml of "sample" buffer (Laemmli, 1970) was added to the sonicate, mixed gently and incubated at 37° for 2 h. Extracts were stored at -20°C until required for electrophoresis.

Gel preparation. Slab gels (0.8 mm thick) were cast between two 10 x 8 cm microscope slides (A. H. Thomas Co., Philadelphia, PA). The formulation of Laemmli (1970) were used to cast a resolving gel (8 x 6 cm) of 11% (w/v) acrylamide. After the gel was polymerized it was clamped into a home-made electrophoretic apparatus similar to that described by Matsudaira and Burgess (1978). A stacking gel (8 x 1.5 cm) of 5% (w/v) acrylamide was cast onto the top of the resolving gel according to the formulation of Laemmli (1970).

Electrophoresis. Cell extracts were thawed, mixed, and centrifuged for three minutes in a Beckman microfuge B. Samples (3-7 µl) of supernatant (25-50 µg protein) were loaded into appropriate wells of the stacking gel. Gels were electrophoresed at 4°C for 2.5 to 3 h with an initial voltage of 45 V and a current of 12.5 mA. Gels were then stained for 2 h at room temperature in 0.1% (w/v) Coomassie brilliant blue R-250, 7.5% (v/v) glacial acetic acid, and 4% v/v ethanol. They were destained for 10 min in methanol:acetic acid:water (50:10:40 v/v) and then for 50 min in methanol:acetic acid:water (7.5:10:82.5 v/v). Gels were recorded photographically using Kodak Technical Pan film with an orange filter over the lens of the camera and then dried for storage.

RESULTS

From a preliminary screening of 10 antibiotics with a variety of target sites it became apparent that the majority showed little ability to discriminate the isolates in our collection. The isolates as a whole were exceedingly sensitive to some antibiotics, for example to streptomycin at concentrations ≤ 0.5 mg l^{-1}, and to rifampicin and polymyxin B at concentrations $\leq$ 2 mg l^{-1}. Alternatively the isolates showed resistance to rather high levels of certain antibiotics, for example to carbenicillin (100 mg l^{-1}) and to nalidixic acid (40 mg l^{-1}). Of the antibiotics tested we found that four (kanamycin, spectinomycin, ampicillin, and novobiocin) provided the most unequivocal ability to separate the isolates into groups. Data in Table 6 show the seven antibiotic resistance patterns into which the 300 isolates were placed. In most cases discrimination was based conservatively upon either complete sensitivity or resistance to one or more specific antibiotics over the range of concentrations tested. Although five of the antibiotic resistance patterns (A, B, D, E, and G) were of this type they represented only 40 of the 300 isolates and no more than 13 isolates were represented by any one of these five patterns. In contrast, the majority of the isolates were represented by the antibiotic resistance patterns C (205 isolates) and F (55 isolates). The feature which discriminated between the latter two antibiotic resistance patterns was the degree of sensitivity/resistance to the antibiotic ampicillin. This is more

clearly seen in the data represented in Table 7 where isolate PB17, representing group F, was significantly more resistant than isolate PB18, representing group C, to 40 mg/liter of ampicillin. It should be noted however that stoichiometric growth of the two isolates was not observed during this plating experiment indicating that all of the cells in the inocula were not intrinsically resistant to the antibiotic at these concentrations.

Data presented in Table 8 show the distribution of nodule isolates with the seven antibiotic resistance patterns throughout nine replicate plots plus those obtained from the plant infection-soil dilution experiment. In eight of the nine plots isolates represented by pattern C dominated the nodule population. In two plots (2 and 3) isolates represented by pattern F were in large numbers whereas in the remainder they were either minor components or were absent. Isolates of patterns A, B, D, E, and G were either minor occupants or absent from the nodules in all plots. Isolates obtained from the nodules formed in the plant infection-soil dilution experiment were of interest. Isolates from group C were again found to be a dominant component of these nodules and yet isolates in Group B, characterized by being very sensitive to spectinomycin, were also major nodule occupants. Only one organism possessing this latter phenotype was recovered from nodules on field grown plants.

Antisera were raised to a field isolate from both group C (#31) and group F (#17). Data in Table 9 show the whole-cell tube

agglutination behavior of these sera against all of the 88 isolates collected from three of the plots. Antiserum to isolate #31 cross-agglutinated with 35 of 43 isolates from group C and did not agglutinate with isolates from any other group. In addition 11 of the 12 isolates placed in group C from the plant infection-soil dilution experiment agglutinated with antiserum 31 while no isolate from the other three groups (A, B, and F) reacted (Table 10). In each of the four populations of C-type isolates analyzed a few organisms did not react with the antiserum showing that some isolates, although possessing the same antibiotic resistance pattern, were not antigenically related. In the case of antiserum to #17, a minority of the isolates (9 of 34) from group F cross-agglutinated, with six of these isolates originating from the same plot as #17 (Table 9). However, in no case did an isolate with a different antibiotic resistance pattern agglutinate.

The isolates which cross-agglutinated with antiserum 31 were analyzed further by determining their protein profile patterns by SDS-PAGE. Data summarized in Table 11 show that the same protein profile pattern represented the isolates cross-agglutinating with antiserum 31 within each plot and those isolates from group C which did not agglutinate possessed different protein profile patterns to the cross-reacting strains. Data in Fig. 1 show the protein profile patterns of three cross agglutinating group C isolates (one from each plot), and suggests that the isolates from the different, and spatially separated, plots were indeed identical. All six

isolates in plot 3, which agglutinated with antiserum 17, were also found to possess an identical profile; the protein profile patterns of three of such isolates (Q_4, Q_5, Q_6) are also shown in Fig. 1. The latter profile pattern is distinct from the profile of isolates from group C.

Table 6. Antibiotic resistance patterns of the isolates of R. meliloti.

Antibiotic Resistance Pattern	No. of Isolates[†]	Antibiotic Conc. (mg/liter)											
		Kan			Spec			Amp		Nov.			
		20	30	40	20	30	40	20	40	10	20	30	
A	8	-[‡]	-	-	-	-	-	-	-	+[§]	-	-	
B	13	+	+	+	-	-	-	-	-	+	+	+/-	
C	205	+	+	+	+	+	+	+/-[¶]	-	+	+	+	
D	5	+	+	+	+	+	+	+/-	-	-	-	-	
E	2	-	-	-	+	+	+	+/-	-	+	+	+	
F	55	+	+	+	+	+	+	+	+	+	+	+	
G	12	+	+	+	+	+	+	+	+	-	-	-	

[†]A total of 300 isolates were analyzed.

[‡]Designates no visible growth after 48 h of incubation.

[§]Designates visible growth after 48 h of incubation.

[¶]Designates either inconsistent growth on replicated plates or, inconsistent growth in repeated experiments.

Table 7. Differential sensitivity of representative isolates of *R. meliloti* from groups C and F to ampicillin.

Antibiotic Resistance Pattern	Isolate	Log_{10} Inoculum size	Ampicillin conc. (mg/liter) 20	40
			No. of colonies†	
C	PB18	5.76	Lawn	0
		4.76	37±6	0
		3.76	5±2	0
F	PB17	5.68	Lawn	245±51
		4.68	Lawn	27±7
		3.68	182±15	3±2

† Number of colonies represents the mean of 5 replicates ± the standard deviation of the mean.

Table 8. Distribution of isolates of *R. meliloti* with the different antibiotic resistance patterns within the 9 replicate plots and from the soil dilution experiment.

Plot	No. of isolates	Antibiotic Resistance Patterns†						
		A	B	C	D	E	F	G
		No. of isolates						
1	31	0	0	21	0	1	8	1
2	33	0	0	16	2	0	13	2
3	24	1	1	6	0	0	13	3
4	32	0	0	23	0	0	7	2
5	27	0	0	27	0	0	0	0
6	32	2	0	25	2	0	3	0
7	28	1	0	24	0	0	1	2
8	26	0	0	23	0	0	3	0
9	36	0	0	28	1	1	4	2
Soil Dilution	31	4	12	12	0	0	3	0
Total	300	8	13	205	5	2	55	12

† See Table 1 for description of antibiotic resistance patterns.

Table 9. Behavior of isolates from three replicate plots in whole-cell tube agglutination with antiserum 17 and 31.

Plot		Antibiotic Resistance Patterns[†]						
		A	B	C	D	E	F	G
		No. of Isolates						
1	No. of isolates	0	0	21	0	1	8	1
	No. aggln. with[¶] antiserum 31	0	0	17	0	0	0	0
	No. aggln. with[††] antiserum 17	0	0	0	0	0	2	0
2	No. of isolates	0	0	16[‡]	2	0	13	2
	No. aggln. with antiserum 31	0	0	14	0	0	0	0
	No. aggln. with antiserum 17	0	0	0	0	0	1	0
3	No. of isolates	1	1	6	0	0	13[§]	3
	No. aggln. with antiserum 31	0	0	4	0	0	0	0
	No. aggln. with antiserum 17	0	0	0	0	0	6	1

[†] See Table 1 for description of antibiotic resistance patterns.

[‡] Group from which isolate #31 was selected.

[§] Group from which isolate #17 was selected.

[¶] Isolates agglutinating against antiserum 31 to a titer identical to that of the parent antigen ± a two fold dilution of serum.

[††] Isolates agglutinating against antiserum 17 to a titer identical to that of the parent antigen ± a two fold dilution of serum.

Table 10. Behavior of the isolates obtained from the plant infection-soil dilution experiment against antisera 17 and 31.

	Antibiotic Resistance Patterns			
	A	B	C	F
	No. of Isolates			
No. of isolates	4	12	12	3
No. aggln. with antiserum 31	0	0	11	0
No. aggln. with antiserum 17	0	0	0	0

Table 11. Identification and discrimination of the field isolates possessing antibiotic resistance patterns C and F by protein profile patterns.

Plot	Antibiotic Resistance Pattern	No. of Isolates	Agglutination with as31	Zero Agglutination with as31
			No. of isolates	
1	C	21	17[†][17][‡]	4[§][4][¶]
2	C	16	14[12]	2[2]
3	C	6	4[4]	2[2]
3	F	13	6[#][6]	7[††][6]

† Number of isolates which agglutinated with antiserum 31 to the same titer as the homologous antigen.

‡ Number of isolates having a protein profile pattern identical to isolate 31.

§ Number of isolates which did not agglutinate with antiserum 31.

¶ Number of protein profile patterns different from isolate 31 which represented the non-agglutinating isolates.

Number of isolates which agglutinated with antiserum 17 to the same titer as the homologous antigen. Number in parentheses indicates all isolates had the same protein profile pattern as isolate 17.

†† Number of isolates which did not agglutinate with antiserum 17. Number in parentheses indicates the number of protein profile patterns different from isolate 17 which represented the non-agglutinating isolates.

Fig. 1. Three protein profile patterns (Q_1 Q_2 Q_3) representing an isolate from each of 3 replicate plots which cross reacted serologically with antiserum 31 and three protein profile patterns (Q_4, Q_5, Q_6) representing isolates which cross reacted serologically with antiserum 17. The gel was composed of 11% (w/v) polyacrylamide.

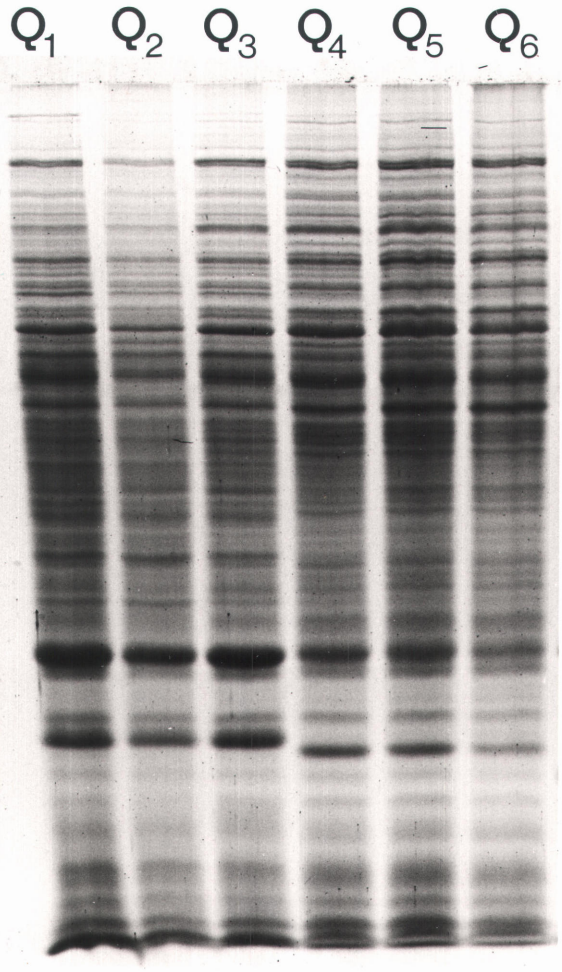

Fig. 1.

DISCUSSION

The need for methods of identification of large numbers of isolates of Rhizobium species which are rapid, inexpensive, and capable of being carried out by moderately skilled technicians has led to the introduction of several methods over the years (Schwinghamer and Dudman, 1980). Although the use of elevated antibiotic resistance markers remains popular, concerns over pleiotropic effects, such as a decline in symbiotic effectiveness and competitive ability (Schwinghamer, 1967; Pankhurst, 1977; Bromfield and Jones, 1979) led to the introduction of discrimination by intrinsic antibiotic resistance characteristics (Josey et al., 1979). This method makes no attempt to modify the strains of interest and has been considered to have the additional advantage of providing information on the composition of indigenous populations of rhizobia.

In this study we have shown that the majority of nodule occupants from within this indigenous soil population of R. meliloti possessed rather high intrinsic resistance to a broad spectrum of antibiotics. Josey et al., (1979) and Beynon and Josey (1980) showed that in general the majority of isolates of R. phaseoli and R. leguminosarum which they studied were rather sensitive to a spectrum of antibiotics at concentrations ≤ 10 mg l^{-1}. In contrast, there are several reports in the literature where isolates of slow growing rhizobia were found to be distinctly more resistant to antibiotics than the fast growers

(Pattison and Skinner, 1974; Pankhurst, 1977; Cole and Elkan, 1979; Kremer and Peterson, 1982). Our observations agree with results obtained from a more limited study (Schwinghamer, 1967) where two isolates of R. meliloti were found to have intrinsic antibiotic resistance more in common with a slow-growing species, R. japonicum, than with other species of fast growing rhizobia.

Of interest was the high degree of sensitivity displayed by our population to streptomycin. Since the success of monitoring inoculant strains resides in being able to distinguish unambigously the inoculant from the indigenous population, our data suggest that streptomycin markers could be successful at this site. Indeed, several workers have made extensive use of streptomycin markers in R. meliloti (Obaton, 1971; Danso and Alexander, 1974; Amarger, 1981a, 1981b; Hardarson et al., 1981; Lowendorf et al., 1981; Amarger and Lobreau, 1982; Hardarson et al., 1982; Jansen van Rensburg and Strijdom, 1982a, 1982b). Of interest was the response seen to spectinomycin; an equally popular antibiotic used in studies of R. meliloti (Schwinghamer and Dudman, 1973). Two hundred and sixty eight of the field isolates analyzed possessed intrinsic resistance to concentrations of 40 mg l^{-1} of spectinomycin and yet, isolates from one of the dominant groups (B) obtained from the plant infection-soil dilution experiment were highly sensitive to spectinomycin. Such data casts some doubt on the ability of the extensively used plant infection-soil dilution technique (Brockwell, 1963; Vincent, 1970) to portray the true

magnitude and/or character of the members of an an indigenous soil population of R. meliloti which will nodulate under field conditions. The discriminatory power of the antibiotic ampicillin, and the lack of such ability in carbenicillin in this particular population is of interest. Although resistance of rhizobia to penicillin has been well documented (Schwinghamer, 1967; Pattison and Skinner, 1974; Mahler and Bezdicek, 1978; Hagedorn, 1979), the large number of variants in the penicillin family (Perlman, 1967) should not be discounted as possible alternatives to test for the discrimination of isolates.

The results obtained from serological and protein profile typing illustrate both the limitations and potential of intrinsic antibiotic resistance typing. The fact that each of the two antisera only reacted with isolates of a specific antibiotic resistance pattern suggests that the latter technique can be useful for a preliminary screening of field populations to identify potential dominant and nondominant groups of nodule occupants. However, since several isolates from such groups did not cross-agglutinate, it must be inferred that isolates with the same antibiotic resistance patterns were not necessarily identical. Certainly a larger number of antibiotics could have perhaps increased the resolving power of the technique provided that they gave stable and discriminating responses. The protein profile patterns confirmed the serological evidence that isolates with the same antibiotic resistance character were indeed a heterogeneous

mixture of organisms and that some were identical to each other. Thus, we are in agreement with others (Stein et al., 1982) that intrinsic antibiotic resistance cannot prove unequivocally the identity of strains. SDS-PAGE in conjunction with serological techniques, provided the more substantial evidence for isolate identity.

It remains to be seen if these dominant organisms within serogroup 31 possess common, identifiable characteristics other than antigenic and antibiotic resistance phenotypes which account for their dominance of the nodules. Of equal interest are those isolates which were either minor components of the nodules on field plants or which were only a major component of nodules on plants grown in the soil dilution system. Only by identifying isolates which are either poor or superior soil colonizers and highly or poorly competitive nodulators will we be able to determine the physiological and, subsequently, the genetic characteristics linked to these ecologically and agriculturally important attributes.

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CHAPTER IV

Identification of a strain of *Rhizobium meliloti* dominating the root nodules of uninoculated field-grown alfalfa.[1]

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ABSTRACT

Analysis of thirty-two isolates of *Rhizobium meliloti* obtained from root nodules of uninoculated field grown 'Anchor' alfalfa (*Medicago sativa* L.) by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) showed that 12 and 6 isolates were represented by two protein profile patterns (gel types A and B) respectively. The remaining 14 isolates were each represented by unique gel types. Antiserum raised to an isolate with a gel type A (#31) cross-agglutinated with the 12 isolates possessing gel type A and only with 2 of the remaining 20 isolates represented by distinctly different gel types. Gel-immune-diffusion analyses showed that 9 of the 12 isolates with gel type A were antigenically identical to the parent isolate, #31. Analysis of 79 nodule isolates obtained two years later from the same plot area showed that forty-two (53%) of these isolates were identical to #31 and were found in nodules on plants growing throughout the whole plot area. Two isolates not represented by a gel type A, cross-agglutinated with the antiserum, whilst three isolates of the 45 possessing gel type A were antigenically non-identical to isolate #31. Isolates identical to #31 could be subdivided further into two effectiveness classes. Preliminary screening of field isolates of *R. meliloti* by SDS-PAGE can lead to identification of isolates of *R. meliloti* which are antigenically distinct, and which possess competitive and persistent phenotypes.

INTRODUCTION

There are few reports in the literature on the ecology and characteristics of indigenous populations of Rhizobium meliloti. This species can occur in soils where agriculturally important Medicago species have never been grown but are often sub-optimally effective at fixing N_2 on commercially important cultivars (Schiffmann, 1958; Brockwell and Hely, 1961, 1966; Hely and Brockwell, 1962; Bottomley and Jenkins, 1983). The importance of indigenous populations of R. meliloti in alfalfa inoculation trials and to post-establishment year production of alfalfa was shown from studies conducted during the International Biological Program. Inoculation responses were seen in the year of establishment, but in subsequent years poorly nodulated plants in uninoculated plots became well nodulated (Bell and Nutman, 1971; Brockwell, 1971). Jansen van Rensberg and Strijdom (1982b) chose strains of R. meliloti considered to be highly competitive nodulators for field inoculation studies in a soil containing an indigenous population of R. meliloti. The strains did not establish themselves in nodules, or persist as nodule occupants in subsequent years to the extent that was predicted from preliminary sterile soil experiments. Hardarson et al. (1982), observed a decline in the percentage of nodules occupied by an inoculant strain of R. meliloti even during the establishment year. An increase in the percentage of nodules occupied by members of the indigenous population accompanied the decline of the inoculant strain.

The objectives of our study were to characterize an indigenous population of *R. meliloti* found in root nodules on field grown uninoculated plants of 'Anchor' alfalfa (*M. sativa* L.) and to determine if any members of that population were dominant nodule occupants at establishment and capable of persisting as dominant nodule occupants in subsequent growing seasons.

MATERIALS AND METHODS

Soil/Field site description: The field site was located at the central Oregon Agricultural Experiment Station, Powell Butte, Oregon. The soil was a moderately deep (0.6 m), well drained sandy loam of the Deschutes series, a member of the coarse loamy, mixed, mesic Xerollic Camborthids. The soil was formed in mixed alluvium with pumice and volcanic ash over basalt and had been in a cereal-alfalfa rotation for many years. Approximately thirty-0.1 kg samples of soil were taken randomly throughout the plot area with a tube auger probe to a depth of 0.3 m, mixed thoroughly and passed through a 2 mm sieve. Chemical and physical properties of the soil were determined using standard methods of the soil physics and soil testing laboratories of the Dept. of Soil Science, Oregon State University (Berg and Gardner, 1978) and are described in Table 12.

Establishment of trial: The soil was ploughed, disced and 90 kg ha^{-1} of sulfur in the form of granular gypsum (14% wt/wt sulfur) were broadcast onto the soil and worked into the surface layer. Other mineral nutrients were determined to be adequate based on soil tests and fertilizer guide recommendations. In June, 1980, surface sterilized, uninoculated seed of 'Anchor' alfalfa (obtained from Dr. J. Moutray, North American Plant Breeders, Ames, Iowa) were sown at a rate of 31.4 kg ha^{-1} into a 4.9 x 3.1 m area and worked into the upper 1-2 cm of soil using alcohol dipped flame sterilized rakes. Irrigation was applied immediately to the plot area at a rate of 0.8 cm h^{-1} for a 12 h period, and repeated at 8

to 10 d intervals throughout the months of June, July, August and early September. In the fall of 1980 a chemical analysis of the soil resulted in 186 kg ha^{-1} of K in the form of KCl, 39 kg ha^{-1} of phosphorus in the form of triple phosphate (45% wt/wt P_2O_5) and 90 kg ha^{-1} of sulfur in the form of granular gypsum (14% wt/wt sulfur) being applied in March, 1981. Soil tests performed in the fall of 1981 showed that mineral nutrient levels were adequate and only the sulfur application was repeated in March, 1982.

Most probable number (MPN) determinations. A subsample (0.01 kg) of the composite soil sample was diluted consecutively in sterile 0.15 M NaCl over the range 10^{-1} to 10^{-6}. Seeds of 'Anchor' alfalfa were surface sterilized, germinated and transferred to test tubes (30 x 3 cm) containing nitrogen-free mineral salts agar composed of: $CaCl_2$, 0.6 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; NaCl, 0.1 g; ferric citrate, 0.1 g; K_2HPO_4, 0.5 g; KH_2PO_4, 0.25 g; 10 mL of a trace element mixture (Evans, 1974) and 15 g of Bacto Agar (Difco Laboratories, Detroit, Mich.) per liter. The ferric citrate was dissolved in a small quantity of HCl before adding to the growth medium, the phosphate salts were autoclaved separately and added to the remainder of the neutralized, sterile medium after cooling. Duplicate seedlings were inoculated with 0.2 mL portions of the soil dilutions and were grown in a glasshouse with a temperature range of 30 (day) to 18°C (night). Natural daylight was supplemented with 16 h of illumination from fluorescent F48/T12/D/RS lamps suspended 0.5 m above the growth tubes to

provide an irradiance of 80 W m^{-2} at plant height. After four weeks of growth, plants were scored for nodulation and the most probable number of R. meliloti per gram of dry soil calculated (Brockwell, 1965).

Isolation of R. meliloti from field plants. Five plants (10 weeks of age) were chosen randomly and removed from the ground using metal cores made from muffler pipe, 0.013 m^2 x 0.28 m in length. Individual plants, and their associated soil cores were placed in ziploc bags, stored on ice, and transported to the laboratory. All the nodules were collected from each plant within three to four days of sampling. Forty nodules (approximately one third of the total per five plants) were chosen randomly for purification of the occupants. Nodules were surface sterilized, the contents streaked for purity, and maintained on slants of yeast extract mannitol (YEM) agar (Vincent, 1970). Isolates were stored on slants under sterile aqueous glycerol (20% v/v) at -20°C until required.

In June, 1982, twelve plant soil cores were removed systematically from two rows, 1 m apart, in the plot area with a distance of 1 m between cores. All the nodules were collected from each core (which usually included two or three plants) and as many isolates as possible were purified. The populations obtained from eight of the cores were chosen at random and ten isolates subsampled from each of these populations for analysis. In addition, ten individual colonies arising from each of five nodules were picked to assess the possibility of mixed infections of individual nodules.

Symbiotic effectiveness tests. Seedlings of alfalfa cv. 'Anchor', germinated from surface sterilized seed, were transferred to slants of mineral salts agar contained in large test tubes (30 x 3 cm). Seedlings were grown in the glasshouse for seven days prior to inoculation so that unhealthy individuals could be culled prior to establishing the inoculation treatments. Six seedlings were inoculated with 5×10^8 viable cells of each field isolate. Uninoculated plants were included to serve either as minus N controls or for subsequent fertilization with nitrate. Three milliters of sterile KNO_3 (18 mM) were added to each nitrogen control seedling 10 d after inoculation. The nitrate supplements were repeated 24 and 31 d after inoculation. Each inoculated and uninoculated control seedling received 3 mL of sterile distilled water at the same time periods. Shoots were harvested 35 d after inoculation, dried at 60°C for 5 d and weighed. Analysis of variance was performed on the data and a Dunnet's two-tailed range test was used to make comparisons of treatments.

Methods of identification of isolates. Two growth media were used: YEM, adjusted to pH 7.0, was used to grow isolates for determining their protein profile patterns by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). A defined medium (GM) was used to grow isolates for serological analysis and was composed of the following: mannitol, 10 g; L-(+) glutamic acid, monosodium salt, monohydrate, 1 g; K_2HPO_4, 0.5 g; $MgSO_4$ $7H_2O$, 0.2 g; NaCl, 0.1 g; $CaCl_2$, 0.08 g; calcium pantothenate 0.01 g;

thiamine hydrochloride, 0.01 g; and biotin, 3×10^{-4} g per liter; the medium was adjusted to pH 7.0 prior to autoclaving.

Preparation of cell extracts for analysis by SDS-PAGE. Each isolate was grown at 30°C in 50 mL of YEM broth on an orbital shaker at 200 rpm to the early stationary phase of growth (2.0×10^9 cells mL^{-1}). Cultures were harvested by centrifugation at 25,000 x g for 10 min and the cell pellets were resuspended and washed three times in ice cold 10 mM Tris-HCl buffer, pH 7.6 to remove mucilage. After the first wash it became easier to pellet the cells by centrifugation and 12,000 x g for 10 min was sufficient. The final cell pellet was resuspended in 0.5 mL of the same Tris buffer, transferred to a polypropylene microcentrifuge tube of 1.5 mL capacity (VWR Scientific) and the cells disrupted by sonication. The sonication protocol involved using a Bronson sonifer (Model 200) fitted with a double step microtip and consisted of three separate 30s bursts at 40W output with one min. of cooling between bursts. After disruption 0.5 mL of "sample" buffer (Laemmli, 1970) was added to the sonicate, mixed gently, and incubated at 37°C for 2 h. Extracts were stored at -60°C until required for electrophoresis.

Gel preparation. Gel slabs, 0.8 mm thick, were cast between two large microscope slides, 10 x 8 cm (A. H. Thomas Co., Philadelphia, PA). Using the gel formulations of Laemmli (1970), a resolving gel (6 x 8 cm) of 11% (wt/v) acrylamide was cast into the gel plate sandwich. After polymerization the gel was clamped into

a home-made electrophoretic apparatus similar to that described by Matsudaira and Burgess (1978), and a stacking gel, 8 x 1.5 cm, was cast onto the top of the resolving gel.

Electrophoresis. Cell extracts were thawed, mixed and centrifuged for 3 min in a Beckman microfuge B. Three to seven microliters of supernatant (25-50 µg protein) were loaded into appropriate wells of the stacking gel. The gels were electrophoresed at 4°C for 2.5 to 3 h with an initial voltage of 45 V and a current of 0.013A. Gels were then stained for two hours at room temperature in 0.1% (wt/v) Coomassie brilliant blue R-250, 7.5% (v/v) glacial acetic acid, and 4% v/v ethanol. They were destained for 10 min in methanol:acetic acid:water (50:10:40 v/v) and then for 50 min in methanol:acetic acid:water (7.5:10:82.5 v/v). Gels were recorded on photographs using Kodak Technical Pan film and then dried for storage.

Serological analysis

Preparation of antisera. Isolate #31 was chosen to represent those isolates with the protein profile pattern, gel type A. It was grown at 30°C in medium GM for 48 h on an orbital shaker. Cells were harvested by centrifugation, washed three times in 0.15 M phosphate buffered saline, pH 7.0 (NaCl, 8.5 g; Na_2HPO_4, 21.3 g; KH_2PO_4, 20.4 g L^{-1}) and resuspended to 2 x 10^9 cells/mL in 0.15 M sterile NaCl. Formalin (0.3% v/v final concentration) was added to the suspension which was heated at 95°C for 30 min to denature flagellar (H) antigens. The heat-treated suspension was emulsified

in an equal volume of Freund Complete Adjuvant (Difco Laboratories, Detroit, Mich.) and then 1 mL (1 x 10^9 cells) of each antigen was injected intramuscularly in the rear thigh of three white New Zealand male rabbits 10 weeks of age. Four weeks later rabbits were given a second injection (1 mL) of freshly grown cells prepared as described above, except that the antigen suspension was emulsified in Freund Incomplete Adjuvant (Difco). Ten days following the second injection 5 mL of blood was collected aseptically from the marginal ear vein, and the sera were assayed by whole-cell tube agglutination for specific antibody titer. Adequate end-point titers were obtained in all rabbits (1/1280 dilutions of antiserum). Subsequently 60-90 mL of blood was collected aseptically from each rabbit by cardiac puncture 14 and 16 d following the secondary immunization. Blood was allowed to clot overnight at 23°C and sera obtained by centrifugation at 582 x g. The sera were heat-inactivated at 56°C for 30 min and stored at -20°C in 5 mL portions until required.

Whole-cell tube agglutination. The preparation of antigens was essentially as described above except that cells were washed in 0.15 M phosphate buffered saline and finally resuspended in 0.085 M NaCl containing 0.5% v/v formalin. These suspensions were heated at 95°C for 40 min. Portions (0.4 mL) of the antigen suspension were incubated with an equal volume of consecutive two-fold dilutions of antiserum 31 ranging from 1/10 to 1/5120 dilutions of the original antiserum. Controls included non-immunized rabbit

serum and antigen suspensions without antiserum. Tubes were incubated partially submerged at 52°C for 4 h. Titers were recorded as the greatest serum dilution in which agglutination was clearly evident.

Gel-immune-diffusion. The system was basically an Ouchterlony double diffusion plate method. Gels were composed of 0.9% (wt/v) agarose (type IV Sigma Chem. Co., St. Louis, Mo.), and 0.025% wt/v sodium azide in physiologically buffered saline pH 7.2 which was poured to a depth of 4 mm in 50 x 9 mm petri plates (no. 1006, Falcon Plastics, Oxnard, Calif.). Wells, (4 mm diam.) were cut using a stainless steel cork borer in a pentagonal arrangement around the center well (4 mm diam.) with a distance of 9 mm between them and the center well. Antigens were grown and harvested as described above and resuspended in PBS containing 0.5% v/v formalin to a constant concentration of 30-40 mg of dry weight/mL. The packed cell volume of the final cell pellet of each isolate was used to determine the volume of diluent required to obtain a standard concentration of each antigen. Cells were disrupted by sonication as described above. Forty two microliter portions of undiluted antiserum were placed into the center wells and the same volume of antigen suspension in the outer wells. The plates were incubated at 23°C in a humidified chamber and development of precipitin lines monitored. The lines were clearly visible after 2 to 3 d of incubation. Data were recorded photographically using indirect light and dark field illumination upon Kodak Technical Pan Film.

RESULTS

Analysis by SDS-PAGE revealed that 16 distinct protein profile patterns represented the 32 isolates from the nodules sampled in 1980. Eight of these profiles are presented in Fig. 2 and are arranged in such a way that similarities and dissimilarities are readily apparent. Only two of the 16 protein profile patterns (A and B) were common to more than one isolate; the remaining 14 were unique (Table 13). Twelve isolates (37.5% of the total) were found to have the same protein profile pattern, hereafter referred to as gel type A. The protein profile patterns of eight of these isolates are represented in Fig. 3. The identity of these protein profile patterns was confirmed by electrophoresing the extracts on polyacrylamide gels of different single concentrations of acrylamide and on gels of different linear gradient concentrations.

Serological evidence was sought to confirm the identity of the isolates possessing gel type A by raising antiserum to one of these isolates, #31. The results of whole-cell tube agglutination assays of the 32 isolates when challenged against antiserum 31 are presented in Table 13. All of the isolates with gel type A cross agglutinated with antiserum 31 to the same titer as the parent antigen. Only two of the remaining 20 isolates cross agglutinated with that antiserum. Gel-immune-diffusion serological assays were performed to determine further the antigenic characteristics of those isolates which cross agglutinated with antiserum 31. Nine of the 12 isolates which possessed a gel type A were antigenically

identical to isolate 31. However, several different immunoprecipitin reactions were also observed in gel-immune-diffusion assays (Fig. 4). Isolate 23, represented by gel type E, and isolate 30, represented by gel type G (data not shown), were antigenically non-identical to isolate 31. In addition, isolates 1 and 35, despite possessing a gel type A also showed antigenic reactions of non-identity.

Analyses of nodule occupants taken from the eight plant soil cores in 1982 are summarized in Table 14. Forty-two (53.2%) of the 79 isolates analyzed were identical to isolate 31. Although the percentage of such isolates within each core varied widely (11-90%), in seven of the eight cores, no less than 40% of the isolates were identical to 31. In three cores (6, 7, and 8) a total of five isolates were found that did not complement fully the characteristics of isolate 31. For example, in plant soil core No. 6 two isolates which agglutinated with antiserum 31 did not show a protein profile pattern of gel type A. In each of the plant soil cores 6, 7, and 8, one isolate alone showed non-identical antigenic behavior to antiserum 31 in gel-immune-diffusion despite possessing a gel type A protein profile pattern and despite reacting in whole-cell tube agglutination to a titer equal to that of the parent isolate. These isolates could be considered analogous to the five isolates from the collection made in the establishment year that also did not fully complement the characteristics of isolate 31. Some evidence was obtained to suggest that nodules occupied by

serogroup 31 type isolates did not contain other distinctly different isolates. Analysis of multiple isolates obtained from individual nodules known to possess an organism identical to #31 showed that each isolate possessed a gel type A and agglutinated with antiserum 31 to a titer equal to that of the parent isolate (data not shown).

Information obtained on the symbiotic effectiveness of the isolates identical to #31 shows that these organisms could be subdivided into two effectiveness classes (Table 15). Although all the isolates from each of six of the eight plant soil cores were of the same effectiveness, two cores (3 and 4) possessed isolates of superior effectiveness (E_1) and four cores (5,6,7, and 8) possessed inferior (E_2) organisms. In the other two cores (2 and 3) isolates of both effectiveness classes were found suggesting there was further subdivision of isolates identical to #31 even within the soil surrounding one or two plants.

Figure 2. Representative protein profile patterns of eight isolates of R. meliloti displayed on an 11% (wt/v) polyacrylamide gel. ps; protein standards are displayed from top to bottom of the gel in order of decreasing molecular wt. in kilodaltons. Bovine albumin, 66 kd; egg albumin, 45 kd; trypsinogen, 24 kd; β lactoglobulin, 18.4 kd; lysozyme, 14.3 kd.

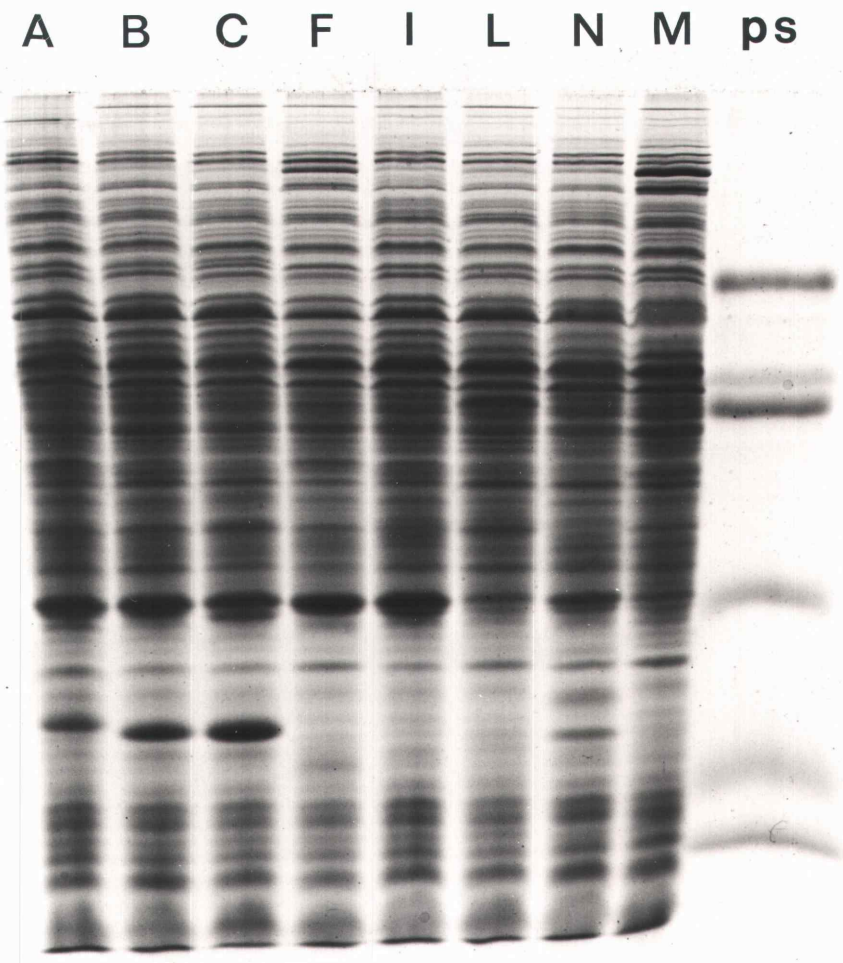

Fig. 2.

Figure 3. Eight different isolates showing the same protein profile pattern (gel type A) on an 11% (wt/v) polyacrylamide gel.

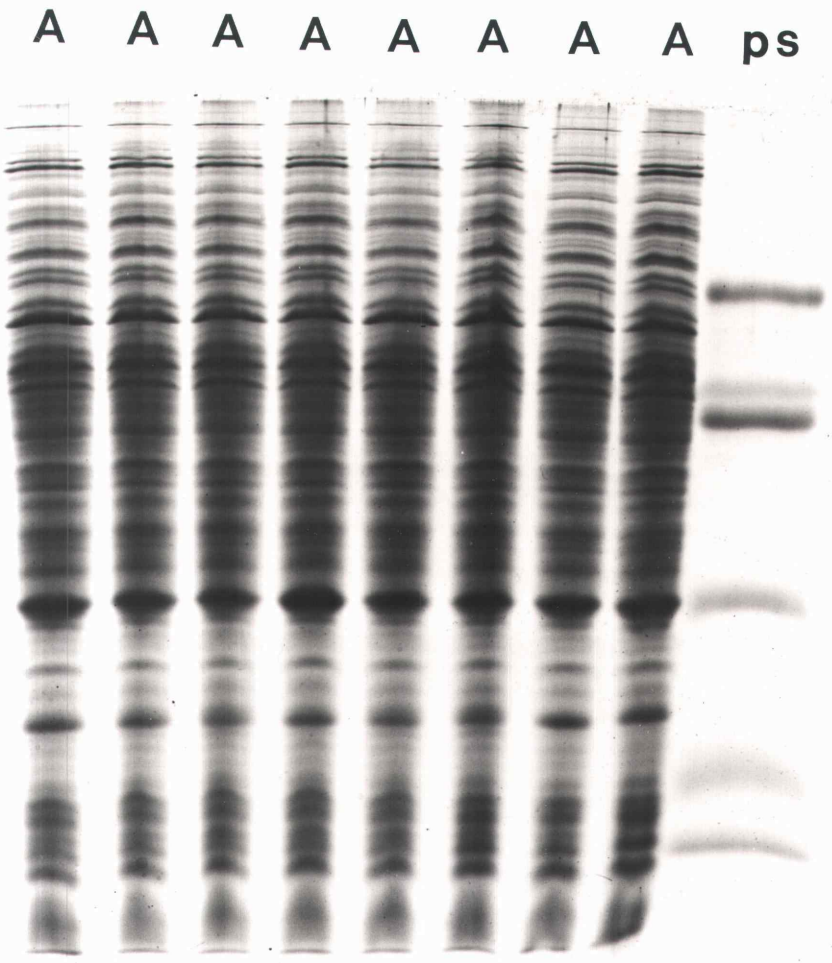

Fig. 3.

Figure 4. Representative serological reactions in gel-immune-diffusion of isolates cross-agglutinating with antiserum 31. Antiserum is in the center well; antigens are in the outer wells. Isolates 1, 31, and 35, represent organisms with a gel type A protein profile pattern; isolate 23, represents an organism with a gel type E protein profile pattern.

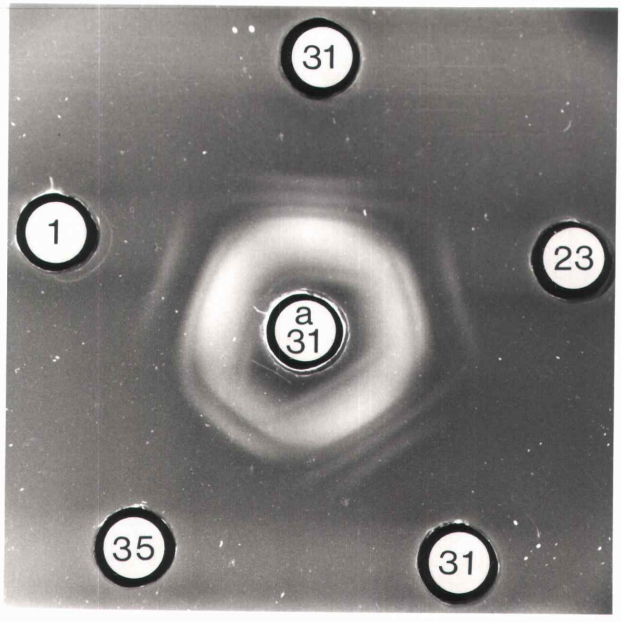

Fig. 4.

Table 12. Characteristics of the soil at the experimental site.[†]

Soil Texture			Organic	Base		Cation Exchange	Exchangeable Bases			P (Olsen	MPN of *Rhizobium meliloti*
Sand	Silt	Clay	Matter	Saturation	pH	Capacity (CEC)	Ca	Mg	K	Procedure)	
%						$cmol\ kg^{-1}$				$mg\ kg^{-1}$	Log_{10} $No.\ kg^{-1}$
73	13	14	2.3	81.8	5.9	13.5	3.8	1.6	0.3	38	6.0

[†]Deschutes sandy loam : coarse loamy, mixed mesic Xerollic Camborthid.

Table 13. Gel types and serological characteristics of the isolates of R. meliloti collected in the establishment year (1980).

	Total No. of Isolates	Gel Types															
		A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P
No. of isolates in each gel type	32	12	6	1	1	1	1	1	1	1	1	1	1	1	1	1	1
No. of Isolates antigenically identical to #31 in whole cell agglutination†	14	12	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0
No. of Isolates antigenically identical to #31 in gel-immune-diffusion‡	9	9	—	N.D.¶	—	N.I.§	N.D.	N.I.	———					N.D.	———		

† Isolates showing whole-cell agglutination with antiserum 31 to a titer equal to that of the homologous antigen (1/640 to 1/1280 dilution of antiserum). Isolates showing a zero response showed no agglutination with 1/10 dilution of antiserum.

‡ Isolates showing immunoprecipitin reactions of complete identity.

§ N.I. = isolates showing immunoprecipitin reactions of non-identity.

¶ N.D. = not determined.

Table 14. Identification of isolates of R. meliloti collected in 1982.

Plant Soil Core (PSC) No.	Total No. of Isolates	Number of Isolates Identical to #31 Based Upon: Whole-Cell Agglutination	Gel Type A	Gel-Immune-Diffusion	Complete #31 Character
		Number of isolates			
1	10	7	7	7	7
2	10	9	9	9	9
3	10	5	5	5	5
4	10	4	4	4	4
5	10	8	8	8	8
6	10	7	5	4	4
7	9	2	2	1	1
8	10	5	5	4	4
Total Isolates	79	47	45	42	42

Table 15. Symbiotic effectiveness of the isolates of R. meliloti identical to #31 collected in 1982.

Plant Soil Core No.	No. of 31-type Isolates	Effectiveness Categories E_1†	E_2‡
		No. of Isolates	
1	7	1	6
2	9	3	6
3	5	5	-
4	4	4	-
5	8	-	8
6	4	-	4
7	1	-	1
8	4	-	4

† Isolates produced shoot dry weights equal to those of nitrate fertilized plants at the 5% level of probability; 65% ≯ of the latter value.

‡ Isolates produced shoot dry weights significantly less than nitrate fertilized plants at the 5% level of probability; between 45 and 65% of the latter value.

DISCUSSION

These data provide both novel and supportive information to that already known about R. meliloti. To the best of our knowledge this is the first report showing the wide diversity of different strains of R. meliloti found on alfalfa plants growing in a field situation. These data support recent observations that indigenous soil populations of Rhizobium phaseoli (Beynon and Josey, 1980) and Rhizobium japonicum (Noel and Brill, 1980) can be composed of a wide diversity of different strains. Isolates identical by the three complementary methods of analysis, were found to be the dominant nodule occupants of an otherwise diverse nodule population. Only one report has appeared previously in the literature which showed (a) antigenic heterogeneity within the population of R. meliloti in nodules of Medicago minima growing in one location and (b) that representatives of one serogroup dominated the nodules on plants of M. sativa, M. hispida and Melilotus alba growing at another location (Hughes and Vincent, 1942).

The use of the SDS-PAGE methodology to develop protein profile patterns of the field isolates allowed us to make the preliminary observations on the diversity of R. meliloti and the dominance of the isolates identical to #31. Our ability to raise an antiserum which showed agglutinating activity against all 57 isolates with a gel type A and only against four of the remaining 58 isolates with different gel types, is worthy of comment. Despite the earlier

success in obtaining antisera to isolates of R. meliloti which showed agglutinating specificity (Stevens, 1923, 1925; Vincent, 1941; Purchase et al. 1951a and b) very little use has been made subsequently of serological techniques with this species of Rhizobium. In part this can be attributed to reports on the extensive cross reactivity of different strains of R. meliloti to specific antisera (Humphrey and Vincent, 1975; Wilson et al. 1975; Sinha and Peterson, 1980; Bauer, 1981) and to anomalous behavior of antisera raised to R. meliloti antigens (Gibbins, 1967; Kishinevsky and Gurfel, 1980). Although our results are restricted to observations made with one strain and at one specific location we propose that surveying a population or collection of R. meliloti by SDS-PAGE will enable researchers to choose distinctly different strains for raising antisera. Indeed, with this approach we have been successful in obtaining other strain specific antisera to isolates of R. meliloti (Fuquay, Bottomley, and Jenkins, unpublished observations), and R. trifolii (Dughri and Bottomley, 1983).

The protocol used for sampling nodules in 1980 resulted in a composite sample of isolates from five randomly selected plants and prevented us from determining whether isolates identical to #31 were uniformly or randomly distributed within the plot area. From the samples obtained in 1982 we were able to show that isolates identical to #31 were distributed in nodules on plants throughout the plot area and, with one exception, that such isolates occupied

at least 40% of the nodules obtained from each plant soil core. Since 53% of the total isolates analyzed in 1982 were identical to #31 and 28% of the total in 1980, there was evidence for an increase in the contribution of such isolates to the nodule population as the plants aged. This can be related to the general lack of persistence of inoculant strains of R. trifolii and R. meliloti in nodules of clovers and medics in subsequent growing seasons (Read, 1953; Dudman and Brockwell, 1968; Brockwell et al. 1972; Roughley et al. 1976; Brockwell et al. 1977; Jansen van Rensburg and Strijdom, 1982b). In contrast, inoculant strains of R. trifolii have been observed to persist successfully in nodules throughout subsequent growing seasons in soils harboring indigenous rhizobial populations (Gibson et al. 1976; Brockwell et al. 1982; Materon and Hagedorn, 1983). We believe that identifying indigenous organisms such as #31 is the first essential step to be made if systematic progress is to occur in this area.

In conclusion, the variation in the symbiotic effectiveness of isolates identical to #31 supports the observations that antigenically identical strains of R. trifolii do not necessarily have the same symbiotic effectiveness characteristics (Vincent, 1944 and 1945; Purchase and Vincent, 1949). In more recent literature there is evidence from laboratory studies to show that strains of R. trifolii can lose completely or express inferior symbiotic effectiveness after (i) repeated subculturing on agar plates, (ii) repeated passage through nodules of T. repens

(Djordjevic et al. 1982) or (iii) exposure to supra-optimum growth temperatures (Zurkowski, 1982). Evidence was found to link the decline in effectiveness to impaired replication and/or modification of plasmids. Certainly, the impact of the soil/plant environment on the stability of a superior N_2 fixing trait is of agricultural significance and in need of further study.

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