AN ABSTRACT OF THE THESIS OF

Blanca Valdivia de Capó for the degree of Master of Science in Microbiology presented on January 19. 1987. Title: Indigenous Rhizobium trifolii Isolated From Nodules of Red Clover Grown in Subclover Pasture Soils.

Abstract approved: Redacted for Privacy
Peter J. Bottomley

A series of identification methods were used to determine the composition of Rhizobium trifolii populations in nodules recovered from uninoculated red clover (Trifolium pratense L.) cv. 'florie' grown in two acidic Oregon soils. Both soils, Abiqua and Whobrey had been in subclover (T. subterraneum L.) pasture for thirty to forty years. The red clover isolates, 24 from Abiqua and 30 from Whobrey soils, were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to elucidate their protein profile patterns. Serological assays performed with antisera raised to two representative isolates from each soil type, revealed that 30 and 47% of the Whobrey isolates were clustered into two serogroups, WR 26 and WR 27, respectively. A third serogroup, AR 21, made up 50% of the Abiqua isolates.

Each set of isolates was also challenged to antisera raised to major serogroups previously recovered and identified from subclover grown in the respective soils. Gel-immune-diffusion studies indicated that none of the Whobrey isolates were antigenically identical to serogroups WS 1-01 or WS

2-01, previously discovered in this soil. A similar analysis of the Abiqua isolates revealed that only 2 isolates were antigenically identical to any of the 4 serogroups, AS 6, AS 16, AS 27 or AS 36, previously discovered in this soil. Three and two serotypes were characterized among the WR 26 and WR 27 serogroups, respectively. The isolates in the Abiqua serogroup, AR 21, were classified into 3 distinct serotypes.

The red clover isolates from Whobrey soil were also challenged with the antisera from the major Abiqua serogroups. Antigenic identity, confirmed by whole cell somatic tube agglutination and fluorescent antibody reactions, was observed between the 14 isolates in serogroup WR 27 and serogroup AS 16. An antigenic link was also established between serogroup WR 26 and the dominant red clover serogroup identified in Abiqua soil, AR 21.

Immunofluorescent direct counts confirmed the antigenic relationship between the Abiqua and the Whobrey populations. The population densities of some of the indigenous serogroups observed in Whobrey soil were significantly different ($P = 0.05$). Serogroup WR 27, occupant of almost half of the red clover nodules, was present in numbers significantly less ($P = 0.05$) than AS 6, which was recovered from only 3% of the nodules. The population sizes of three other serogroups found in this soil, WS 1-01, WS 2-01 and AS 36, were not different from that of WR 27, yet were totally absent from the red clover nodules.

Thirty eight and twenty five percent of the Whobrey and Abiqua red clover isolates, respectively, were highly effective (E_3) on red clover. When subclover was used as the host legume, 48% of the Whobrey isolates were incapable of

fixing nitrogen (E_1), although 24% remained highly effective. The Abiqua isolates however, were either ineffective or suboptimally effective (E_2) when evaluated on subclover. The symbiotic effectiveness traits of the isolates on the two hosts permitted even further discrimination within the Whobrey and Abiqua serotypes. Most of the isolates that displayed a single nitrogen fixing capability on red clover expressed at least 2 effectiveness categories when evaluated on subclover.

Indigenous Rhizobium trifolii Isolated From Nodules of Red Clover Grown in Subclover Pasture Soils

by

Blanca Valdivia de Capó

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of
Master of Science

Completed January 19, 1987

Commencement June 1987

APPROVED:

Redacted for Privacy

Associate Professor of Microbiology and Soil Science in charge of major

Redacted for Privacy

Chairman of the Department of Microbiology

Redacted for Privacy

Dean of Graduate School

Date thesis is presented January 19, 1987

Typed by Nina Vaught for Blanca Valdivia de Capó

ACKNOWLEDGEMENTS

I wish to express my sincere appreciation to my major professor, Dr. P. J. Bottomley, for his guidance and advice in conducting this study and preparing this thesis. Sincere thanks are extended to committee members, Drs. M Powelson, Sandine and Swanson for their assistance.

I also want to thank my parents for their constant encouragement. To Miguel, Yordi and Zinnia goes my love and gratitude for their emotional support during this period of graduate study.

Special thanks are extended to Muktar, Angela, Kam and Matt for their "scientific and technical help", but most of all for their friendship.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

INDIGENOUS Rhizobium trifolii ISOLATED FROM NODULES OF RED CLOVER GROWN IN SUBCLOVER PASTURE SOILS

CHAPTER I

INTRODUCTION

Biological N_2 Fixation

Leguminous crops have been cultured and valued both as food sources and soil enrichers for centuries. However, it was not until 1888, through the classical work of Hellriegel and Wilfarth, that biological nitrogen fixation by legumes was scientifically established. Over the past 20 years, the process by which certain bacteria convert atmospheric nitrogen gas into amonia has been extensively studied and reviewed (Quispel, 1974; Newton and Nyman, 1975; Hardy et al., 1977; Hardy and Gibson, 1977; Hardy and Silver, 1977; Hollaender et al., 1977; Newton et al., 1977; Dobereiner et al., 1978; Newton and Orme-Johnson, 1980; Gibson and Newton, 1981; Veeger and Newton, 1984; Evans et al., 1985).

Nitrogen is the plant mineral nutrient which most often limits crop production. Nitrogen fixing rhizobia, however, can counteract this limitation by establishing an effective symbiotic association with a legume host. Although the amount of nitrogen fixed by legumes is influenced by many biotic and abiotic factors, it has been calculated that between 23 and 100% of the total nitrogen

nitrogen assimilated during the growth of a leguminous plant is derived from N_2 fixation (Burns and Hardy, 1975; Rennie, 1985).

The most extensively studied symbiotic nitrogen-fixing association is the one between bacteria of the genus Rhizobium and legumes. Atmospheric nitrogen fixation occurs when these soil borne bacteria invade the roots of the legume and form nodules. Before the nodule is established, however, a series of complex events take place, about which very little is known. Nodulation genes in Rhizobium are induced by flavone-like molecules found in seed or root exudates (Peters et al., 1986). Although these early gene products have not been identified, their expression results in root hair curling, host recognition of the rhizobia, and division of cortical cells in preparation for bacterial invasion. The curled root hair undergoes invagination of its cell wall, and encloses the bacteria. Meristematic activity increases in advance of the infection thread and eventually a functional nodule is formed as the cortical cells divide and grow outward from the root. The rhizobia are released from the thread wall and undergo transformation into enlarged pleiomorphic bacteroids which fix atmospheric nitrogen through the action of the enzyme nitrogenase. The primary product of nitrogen fixation is ammonium which is exported from the bacteroid into the plant cell cytoplasm (O'Gara and Shanmugam, 1976), where it is incorporated into either amino acids or ureides (Rawsthorne et al., 1980). In return, the plant provides photosynthate, to be used by the rhizobia as its energy source.

Inoculant and Indigenous Strains

It has been a tradition for many years to routinely inoculate legume seed with the appropriate rhizobia. Inoculation is justified in areas where effective nitrogen fixing rhizobia are either totally absent from the soil or present in such low numbers that they might jeopardize host establishment. In particular it is imperative that inoculant strains, especially for permanent pasture legumes, be symbiotically effective, successful at nodulation and saprophytically competent in order to ensure its symbiotic performance and persistence into post establishment years.

Inoculant strains often perform poorly because they are incapable of competing with the indigenous rhizobia population. Johnson and Means (1964) found that Bradyrhizobium japonicum strain USDA 110 inoculated onto soybeans did not increase yield production when they were grown in soils with established B. japonicum populations. Other researchers (Johnson et al., 1965; Caldwell and Vest, 1968) found that the recovery of inoculant strains from nodules of soybeans grown in soils harboring indigenous B. japonicum communities ranged from 5 to 10%. Noel and Brill (1980) observed that B. japonicum inoculant strains were recovered from 15% of the nodules at the first sampling time, while none were found in later samplings.

Successful nodulation by inoculant strains of R. trifolii on various Trifolium species has been observed in both the establishment year (Gibson et. al., 1976; Brockwell et al., 1977; Hagedorn, 1979; Brockwell et al., 1982; Materon and Hagedorn, 1983) and in subsequent years (Brockwell et al., 1977; Brockwell et

al., 1982). This has not always been the case, however, as shown by the decline in nodule occupancy of the inoculant strain in the years after establishment (Read, 1953; Dudman and Brockwell, 1968; Brockwell et al., 1972; Roughley et al., 1976). In many instances, variation in performance by the inoculants was attributed to the unknown nature of the indigenous populations at the test sites. Therefore, the identification and characterization of indigenous Rhizobium populations can be considered of primary importance to understanding the limitations of legume productivity. The study of indigenous soil populations might allow us to determine the nitrogen-fixing capabilities of the Rhizobium strains, and identify those most dominant at nodulation and their saphrophytic competence over successive seasons.

Identification Methods

Since most agricultural soils already harbor an indigenous population of unknown origin and composition, it is necessary to verify the identity of the Rhizobium spp. recovered from root nodules. Identification methods, generally centered around serological or antibiotic properties, have been utilized to characterize the members of the Rhizobium communities (Schwinghamer and Dudman, 1980).

Serological techniques, which rely on the surface characteristics of the cell, are used to obtain insights into the nature of the bacteria and to define strains in terms of their antigenic composition. The three most widely utilized

serological methods are whole-cell tube agglutination (Vincent, 1942; Means et al., 1964; Chatel and Greenwood, 1973), gel-immune-diffusion (Dudman, 1964; Dudman and Brockwell, 1968) and fluorescent antibody techniques (Schmidt et. al., 1968). These techniques, like others, have their limitations. The researcher may be restricted by the number of antisera available, and furthermore, the cross-reaction among rhizobia (Graham, 1963) may make the identification process difficult and inconclusive. Serological techniques, however, have been successfully used in ecological studies of B. japonicum (Schmidt, 1974; Reyes and Schmidt, 1979, 1981) and of R. trifolii (Vincent, 1954). These methods have demonstrated that indigenous Rhizobium strains are antigenically heterogeneous (Hughes and Vincent, 1942; Purchase et al., 1951; Holland, 1966).

Fluorescent antibodies have been successfully used to identify strains in nodules (Trinick, 1969) and as a basis for quantitative determination of specific rhizobia in the soil (Bohlool and Schmidt, 1972; Schmidt, 1974). This technique provides a means of more accurately monitoring population dynamics under natural conditions. The extracting and flocculation procedure of Schmidt (1974) has been modified to extract rhizobia from different soil types (Kingsley and Bohlool, 1981; Demezas and Bottomley, 1986a). Quantitative immunofluorescence has been used by Schmidt and co-workers to study the response of indigenous serogroups of B. japonicum to plant rhizospheres (Reyes and Schmidt, 1979; Moawad et al., 1984; Robert and Schmidt, 1985).

High concentration antibiotic resistance markers have also been used to

identify strains of rhizobia present in nodules and in the soil (Schwinghamer and Dudman, 1973). The symbiotic and saphrophytic capabilities of these variants, however, may be modified by such markers (Pankhurst, 1977; Jones and Bromfield, 1978). In addition, they can only be used to follow the fate of inoculant strains.

A different approach to the study of indigenous populations of rhizobia involves the use of intrinsic antibiotic resistance characteristics (Josey et al., 1979). Beynon and Josey (1980) found that 56 different strains of R. phaseoli occupied the nodules of field grown beans. In general, fast growing Rhizobium spp. are rather sensitive to low concentrations of antibiotics, while slow-growing Bradyrhizobium species show higher intrinsic levels of resistance (Pankhurst, 1977; Cole and Elkan, 1979; Kremer and Peterson, 1982). The highly sensitive nature of the former group make this method susceptible to both technical error and antibiotic instability.

Another strain identification method compares the protein profile patterns of isolates produced by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). This technique has been used to characterize B. japonicum (Noel and Brill, 1980), R. trifolii (Dughri and Bottomley, 1983; Demezas and Bottomley, 1984) and R. meliloti (Jenkins and Bottomley, 1984). At this time, however, it would be reasonable to conclude that no single method is suitably definitive on its own and that a set of complementary methods is required.

Antigenic Relationships Among Strains

Once the members of the Rhizobium population are adequately identified they may be further characterized by comparing the antigenic properties of the strains. Rhizobium strains from different parts of the world can be antigenically similar, and in some cases, identical (Vincent, 1954). For example, B. japonicum strains isolated from soybeans grown in South Carolina, Iowa, Mississippi and Maryland were found to share common antigens (Johnson and Means, 1963). Wright et al. (1930) identified 6 serogroups among 156 B. japonicum isolates recovered from soybeans grown in such geographically isolated areas as Japan, Manchuria, Virginia, Mississippi and Louisiana. Several common serogroups were also detected in B. japonicum populations across 7 locations in 5 states in the southeastern USA (Caldwell and Hartwig, 1970).

Kingsley and Bohlool (1983) grouped 29 isolates of Rhizobium spp. recovered from chickpea (Cicer arietinum) and obtained from several culture collections throughout the world, into 4 distinct somatic serogroups. A similar study involving 55 strains of R. phaseoli of diverse geographical origin detected 5 serogroups and 11 somatic antigenic determinants (Robert and Schmidt, 1985). Mahler and Bezdicek (1980) determined that R. leguminosarum isolates geographically distributed along 9 Palouse catenas in Eastern Washington, were grouped into only 3 major serogroups. Furthermore, Dowdle and Bohlool (1985) have reported antigenic relationships between B. japonicum strains isolated from soybean fields located in the People's Republic of China and several USDA

strains.

In contrast, there are many reports in the literature that show considerable heterogeneity among Rhizobium strains in nodules on plants in close proximity in the same field and even from different nodules present on the same plant. This has been documented for R. trifolii (Hughes and Vincent, 1942; Purchase and Vincent, 1949; Purchase et al., 1951; Holland, 1966) and for B. Japonicum (Johnson and Means, 1963; Damirgi and Anderson, 1967; Caldwell and Hartwig, 1970; Caldwell and Weber, 1970).

Factors Influencing Strain Competition for Nodulation

The competitive ability of a rhizobial strain is determined by its capacity to infect and nodulate a legume host in the presence of other nodulating strains. The competitive properties of commercial inoculant strains, as predetermined under nonsoil or sterile soil conditions, have not necessarily correlated with field experiments where indigenous rhizobia strains are present (Jansen van Rensburg and Strijdom, 1982). Many studies have shown that either the host plant or the relative abundance of the strains in the inoculum are the major factors affecting strain competitiveness (Vincent, 1954, 1962, 1965; Nutman, 1965; Date and Brockwell, 1978).

Robinson's host selection hypothesis (1969) is based on the nodulation outcome of red clover (T. pratense) and subclover (T. subterraneum) planted in the same soil. He found that the host plant can "select" from a natural R. trifolii

population predominantly those strains with which it will establish an effective symbiotic association. The hypothesis was based on the following: the isolates from red clover nodules were effective on red clover but ineffective on subclover and vice versa. Masterson and Sherwood (1974) observed a similar trend with indigenous R. trifolii strains nodulating white clover and subclover. Nevertheless, the opposite has also been observed, that is, ineffective strains exhibiting greater nodulating success while effective strains vary in their nodulating competitiveness (Nicol and Thornton, 1941; Vincent and Waters, 1953). In this regard, Jones and Morley (1981) found that white clover (T. repens), cv. Milkanova, was not preferentially nodulated by effective R. trifolii strains. Furthermore, other studies carried out by Pinto et al. (1974) with two Medicago and three Trifolium spp. revealed that the R. meliloti and the R. trifolii strains that nodulated the respective hosts were not only of diverse symbiotic effectiveness, but that neither the speed of nodule formation nor the number of nodules produced could account for the competitive superiority of the strains tested.

Objectives

An approach to understanding if rhizobia are major limitations to legume productivity is the identification and categorization of the indigenous Rhizobium communities. Knowledge of the interrelationships among Rhizobium spp. in their natural habitat will help select those strains that are both competitive and highly effective. Such strains may be utilized as inocula in areas with a similar set of environmental conditions and that lack an effective rhizobia population. Thus, this research was conducted to: 1) identify and quantify the Rhizobium trifolii populations recovered from red clover grown in Abiqua and Whobrey subclover pasture soils; 2) determine if red clover nodules were occupied by strains antigenically distinct from those found in subclover nodules, and; 3) compare the symbiotic effectiveness of the isolates recovered from red clover nodules on subclover and red clover.

CHAPTER II

INDIGENOUS *Rhizobium trifolii* ISOLATED FROM NODULES OF RED CLOVER GROWN IN SUBCLOVER PASTURE SOILS

INTRODUCTION

Much information has been gathered during the past thirty years on the factors influencing the phenomenon of competition between strains of Rhizobium for nodulation of leguminous plants. Studies carried out with Trifolium (clover) species suggest that either the host plant itself or the relative abundance of strains in the rhizosphere zone can be the most influential factor (Vincent, 1965; Date and Brockwell, 1978; Bushby, 1982; Amarger, 1984).

Red clover (Trifolium pratense L.) has played an important, albeit often overlooked role, in the development of concepts regarding competitive nodulation. Much of the work which suggested that growth in the rhizosphere, and the relative abundance of strains therein dictated nodulation success, was carried out with red clover as the host plant (Nicol and Thornton, 1941; Purchase and Nutman, 1957; Purchase, 1958; Mulder and Van Veen, 1960; Rovira, 1961). On the other hand, studies implicating a dominant role for the host plant in selecting from mixtures of R. trifolii in non-soil (Vincent and Waters, 1953; Russell and Jones, 1975) and in soil environments (Robinson, 1969), involved red clover as the host.

Previous work from our laboratory had identified several antigenically distinct groups of R. trifolii occupying root nodules of subclover (T. subterraneum L.) sown noninoculated into two acidic soils (Dughri and Bottomley, 1983; Demezas and Bottomley, 1984). The relative abundance of these serogroups in nodules was influenced by the cultivar of subclover utilized (Dughri and

Bottomley, 1984; Demezas and Bottomley 1986a). Antigenically unrelated R. trifolii were also detected, and the proportion of nodules they occupied varied between experiments from a minor to major portion.

The objectives of this study were; 1) to identify and to quantify the soil populations of other antigenically distinct members of the indigenous R. trifolii population in the Abiqua and Whobrey pasture soils; 2) to determine if red clover selected strains antigenically distinct from those previously identified in subclover nodules, and; 3) to compare the symbiotic effectiveness of the isolates recovered from red clover nodules on subclover and red clover.

MATERIALS AND METHODS

Soils

Two soils, representative of the Abiqua and Whobrey series, were used for this study. Both are silty clay loams, acidic, low in extractable phosphate, of high cation exchange capacity, and have been described in detail elsewhere (Dughri and Bottomley, 1983; Demezas and Bottomley, 1984). Using sterile utensils, 15 Kg samples of each soil were collected to a depth of 0.3 m from four locations in the fields. They were mixed thoroughly, sieved (2 mm) and stored at 4^{o} C.

Isolation of R. trifolii

Seven hundred grams of each soil type were placed into each of four plastic pots. Each pot was amended with monobasic potassium phosphate (25 mg P Kg^{-1}) and sodium molybdate (1 mg Mo Kg^{-1}) in an aqueous solution. Sterile distilled water was routinely added to each pot to maintain the soil moisture content at 33Kpa. Seeds of T. pratense cv. 'florie' were surface sterilized by standard methods (Vincent, 1970) and germinated on water agar plates for 2d at 30^{o}C. Approximately 10 seeds were sown into each pot and later thinned to 3 seedlings per pot. Plants were grown in a completely randomized design in a glass house using natural daylight supplemented with artificial illumination as described elsewhere (Dughri and Bottomley, 1983). Ten weeks

after sowing, plants were harvested and the roots were gently separated and washed with tap water to remove adhering soil. As many nodules as possible were recovered from the root systems of the plants. The nodules were surface sterilized (Vincent, 1970), crushed onto plates of a defined mannitol glutamate medium (Dughri and Bottomley, 1983) and the occupants streaked to purity. Single colonies were picked onto slants of yeast extract mannitol (YEM) agar (Vincent, 1970), incubated for 48 h and stored at 4°C.

Methods of Identification

Growth Medium - Isolates were routinely grown in a defined growth medium (Dughri and Bottomley, 1983) modified by substituting mannitol with maltose to minimize the production of exopolysaccharide (Pilacinski and Schmidt, 1981).

Intrinsic Antibiotic Resistance - Cultures of isolates from the Whobrey and Abiqua collections were challenged against a broad spectrum of antibiotics to determine their intrinsic antibiotic resistance characteristics. The antibiotics used were as follows (milligrams per liter): vancomycin hydrochloride (2.5, 5.0), streptomycin sulfate (1.0, 2.5), rifampicin (1.0, 3.0), kanamycin sulfate (5.0, 8.0), novobiocin sodium salt (1.0, 2.5), ampicillin (2.5, 5.0), spectinomycin dihydrochloride (1.0, 2.5), and nalidixic acid (5.0, 10.0), (Sigma Chemical Co., St. Louis, Mo.). Stock solutions of the antibiotics were made in sterile distilled water, with the exception of rifampicin and nalidixic acid which were both dissolved in

small volumes of 95% ethanol and 1 M NaOH, respectively. Stock solutions were filter sterilized and appropriate volumes were added to sterile YEM agar held at 49°C. Procedures for analysis of antibiotic resistance were as described elsewhere (Jenkins and Bottomley, 1984).

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) - Isolates were grown, harvested, and cell-free extracts were prepared as described elsewhere (Fuquay et al., 1984). The gel preparation and the electrophoresis protocol were carried out according to Dughri and Bottomley (1983).

Serological Analysis - Based upon preliminary information provided by SDS-PAGE protein profiles and intrinsic antibiotic resistance characteristics, two nodule R. trifolii isolates from Whobrey soil, WR 26 and WR 27, and two from Abiqua soil, AR 18 and AR 21, were selected for raising antisera. The two letters preceeding the number of each strain indicate the soil, Whobrey (W) or Abiqua (A), and the clover species, red (R) or subclover (S), from which they were isolated. Antisera were prepared against somatic antigens as described previously (Dughri and Bottomley, 1983) with the exception that primary immunizations were injected interdigitaly (0.4 ml) and subcutaneously (1.5 ml). Blood samples (40 ml) were drawn weekly from the marginal ear vein of each rabbit, commencing 3 weeks after the primary immunization. The rabbits immunized with isolates AR 21 and WR 27 exhibited acceptable titers (1:2560) and a booster immunization was not necessary. Rabbits immunized with isolates WR 26 and AR 18 required secondary immunization since the homologous titers

dropped to 1:40 by the third and fourth week, respectively, after the initial injection. The antigens for booster injections were emulsified in equal volumes of Freund incomplete adjuvant (Difco) and administered as described above. The final blood samples (60-70 ml) were collected from the rabbits by ear bleeding and cardiac puncture 7 weeks after the rabbits were initially immunized. Sera were processed and stored as described elsewhere (Fuquay et al., 1984).

Whole cell somatic tube agglutination and gel-immune diffusion analyses were carried out essentially as described elsewhere (Dughri and Bottomley, 1983) with the exception that cell suspensions were prepared in .086 M NaCl containing 0.5% (v/v) phenol prior to heating at 95^{o}C for 60 minutes to denature flagellar antigens. The antisera utilized were prepared to the representative isolates recovered from red clover grown in Whobrey soil (WR 26 and WR 27) and in Abiqua soil (AR 18 and AR 21). Antisera to isolates previously identified in root nodules of subclover (T.subterraneum L.) grown in either soil were also used. These isolates were WS 1-01 and WS 2-01 from Whobrey soil (Demezas and Bottomley, 1984) and AS 6, AS 16, AS 27 and AS 36 from Abiqua soil (Dughri and Bottomley, 1983).

Immunofluorescent conjugates were prepared as described elsewhere (Demezas and Bottomley, 1986a) to the six subclover isolates mentioned above. Smears used in the immunofluorescence analysis were prepared from log phase, 2 day old cultures, grown on modified defined medium. Cells were harvested, washed three times with 0.15 M phosphate buffered saline, pH 7.0, and suspended in distilled water to a density of 2.5 x 10^{8} cells ml^{-1}. Smears

were air dried, heat fixed, treated with rhodamine gelatine to suppress non-specific fluorescence (Bohlool and Schmidt, 1968) and stained with the appropriate fluorescent antibody conjugate as described elsewhere (Demezas and Bottomley, 1986a). Staining was allowed to act for 10 minutes and the destaining was carried out by flooding the smears twice with 0.02 M phosphate buffer, pH 7.2, for 10 minutes.

Determination of Symbiotic Effectiveness

Surface sterilized seeds of T. pratense cv. 'florie' and of T. subterraneum cv. "Mt. Barker" were germinated and transplanted to nitrogen-free mineral salts agar contained in large (30 x 3 cm) cotton-stoppered test tubes. The composition of the medium and the growth conditions of the plants were as described elsewhere (Dughri and Bottomley, 1983). Four replicate seedlings of each clover species were inoculated with 1 ml (5×10^8 cells) of each of the red clover isolates from Whobrey and Abiqua soils. Representatives from previously identified serogroups recovered from subclover grown in the two soils were also evaluated. Two commercial inoculant strains, P17 and P46 (obtained from the Nitragin Co., Milwaukee, Wis.), were used as symbiotic effectiveness controls for the red clover series. Strain RT 162 X 95, also obtained from the Nitragin Co., was used as the effectiveness control for the subclover isolates. Uninoculated seedlings were included to serve both as contamination controls and also for subsequent fertilization with 3 ml of 18 mM KNO_3 at 14 and 28 days after

planting. Shoots were removed 35 days after inoculation, dried at 60°C for 5 d and dry weights obtained. Analysis of variance was performed and the symbiotic effectiveness categories of the isolates were ranked according to Tukey's honest significant difference (HSD) procedure.

Enumeration of Indigenous R. trifolii in Whobrey Soil by Immunofluorescence Analysis

The procedure was as described previously (Demezas and Bottomley, 1986a) with the following modifications. After flocculation, the supernadant was recovered and lactic acid and formaldehyde were added to each of eight Whobrey soil samples to a final concentration of 0.1 and 2%, respectively. Three ml of the cleared supernatant were passed through 0.4µm pore size Irgalan-black treated polycarbonate filters (Hobbie et al., 1977). The 0.4 µm filtered solution was then passed through a 0.2 µm pore size filter. Both filters were stained with fluorescent antibody conjugates, destained and observed by immuno-fluorescence microscopy as described earlier in Materials and Methods. The rhizobia were enumerated and a correction factor for serogroups WR 27 and AS 6 was calculated to take into account those cells passing through the 0.4µm membrane filters.

RESULTS

Preliminary analysis of the root nodule isolates of R. trifolii from red clover grown in either Whobrey or Abiqua soil showed that distinct cellular protein profile patterns, determined by sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE), could be distinguished within each group of Whobrey and Abiqua isolates. Isolates with identical protein profiles were rare among the isolates from each soil type. Two representative isolates from each soil, namely WR 26 and WR 27 from Whobrey and AR 18 and AR 21 from Abiqua, were chosen for raising antisera based on distinct differences in their protein profile patterns (Fig. 1) and their intrinsic antibiotic resistance characteristics (Table 1).

The results of gel-immune-diffusion reactions of the Whobrey nodule isolates with antisera to WR 26 and WR 27 and to two previously identified serogroups from this soil, WS 1-01 and WS 2-01, are summarized in Table 2. Thirty and 47% of the isolates reacted with antisera to WR 26 and WR 27, respectively. None of the isolates were antigenically related to WS 2-01. The WR 26 group had 3 distinct serotypes: A, B, and C. Isolates in A and B reacted in a manner of complete identity with the parent, WR 26 (Fig. 2a), while those in C formed only a faint precipitin band of non-identity with the parent serum. The A and B types, with 3 and 4 isolates respectively, differed in their reaction to antiserum to isolate WS 1-01; only the isolates in the B type were cross-reactive (Fig. 2b). Two serotypes were also identified among the 14 isolates of the WR 27

group. Nine of the isolates (serotype A) cross reacted in a manner of partial identity with antiserum WR 26. The remaining 5 isolates (serotype B) showed no such interaction.

The Whobrey isolates were also challenged to antisera raised to four isolates of R. trifolii recovered originally from subclover grown in Abiqua soil, geographically isolated from the Whobrey site by 300 miles. All the isolates in serogroup WR 27 were antigenically identical to serogroup AS 16 (Table 3). The identity between these two serogroups was also confirmed from identical reciprocal titers in whole cell somatic tube agglutination tests and reactions with fluorescent antibody to AS 16 (data not shown). Two more Abiqua serogroups, AS 6 and AS 27, with one and two isolates respectively, were also identified among the 7 isolates which had not reacted previously with any of the Whobrey antisera (Table 2). Only 4 of 30 Whobrey isolates did not possess antigens identifiable with any of the 8 antisera available.

The results of the reactions of the Abiqua isolates with the 8 available antisera are presented in Table 4. One half of the isolates reacted in complete identity with antiserum AR 21. Isolate AR 18 reacted in a manner of complete identity with antiserum AS 6, as did the reciprocal test with isolate AS 6 and antiserum AR 18 (data not shown). Therefore, AR 18 was established as a unique red clover isolate within serogroup AS 6. Isolates antigenically related to the other 3 serogroups known to be present in abiqua soil, AS 16, AS 27 and AS 36, were virtually absent from the red clover nodule isolates. Only one isolate was antigenically related to serogroup AS 16 and yet antiserum to isolate WR 27

did not react with it. Three serotypes were identified within the dominant AR 21 serogroup. The three isolates in serotype A did not cross-react with antiserum AS 36 while the 2 in serotype B were cross reactive. All the isolates in serotype C formed a weak immunoprecipitin line of nonidentity with the parent antiserum. An antigenic link between the major nodule occupying groups in each soil, WR 26 and AR 21, was also established. Whole cell somatic tube agglutination analyses revealed that these two isolates reacted reciprocally to titer of 1:320 although no such interaction was observed in gel-immune-diffusion assays.

The population densities of 6 serogroups in samples of Whobrey soil, taken fresh from the field, were determined by immunofluorescence (Table 5). Highly significant ($P = 0.01$) differences in the population sizes of some of the indigenous serogroups were observed. The number of serogroup WR 27 ($1.63 \pm .68 \times 10^5$/g soil), which was recovered from almost 50% of the nodules analyzed, was significantly less ($P = 0.01$) than the population size of serogroup AS 6 and not different from WS 1-01, WS 2-01, and AS 36, despite the later 4 groups being virtually absent from the red clover nodules.

The data presented in Table 6 summarize the symbiotic effectiveness characteristics of the dominant nodule occupying groups, WR 26, WR 27 and AR 21, on both red and subclover. Three different symbiotic effectiveness characteristics, E_1, E_2, and E_3, were identified among the isolates. The relative abundance of isolates within each category varied between the two soil types. More Whobrey isolates (38%) were highly effective on red clover than Abiqua isolates (25%). When subclover was used as the host legume, almost one half

(48%) of the Whobrey isolates were ineffective although some (24%) were still capable of fixing a high amount of nitrogen. All of the Abiqua isolates were either ineffective or suboptimally effective when inoculated onto subclover.

The nitrogen fixing capabilities of representatives of the serogroups originally recovered from nodules of subclover grown in both soils were compared on red and subclover. Seventy five percent of the isolates from the Whobrey serogroups (WS 1-01 and WS 2-01) were highly effective (E_3) on subclover but all were completely ineffective (E_1) on red clover. The representative isolates of the four serogroups originally identified in Abiqua soil (AS 6, AS 16, AS 27, and AS 36) were highly effective (E_3) on the parent host, yet on red clover they were of moderate (E_2) effectiveness (data not shown).

Comparing the effectiveness performances of isolates on the two clover species permitted even further discrimination between isolates within the dominant Whobrey and Abiqua serotypes (Table 7). Subdivision of the symbiotic effectiveness categories was observed in all but one serotype (category E_3 from WR 26, serotype B). Isolates that displayed a single effectiveness rating when grown on red clover proved to be of at least 2 different effectiveness categories when evaluated on subclover. For example, the 6 isolates within WR 27, serotype A, which were moderately effective (E_2) on the parent host, expressed three traits of nitrogen fixing capability when evaluated on subclover.

DISCUSSION

The findings reported here confirm and expand upon earlier findings on host selection by Trifolium spp. from indigenous soil populations of R. trifolii. Of equal importance are the new insights revealed regarding autecology of indigenous R. trifolii.

In general, the trend in symbiotic effectiveness ratings of the isolates, as a whole, was supportive of red clover nodulating with members of the soil population capable of being effective (Table 6) on red clover and less effective, as a whole, on subclover. However, there was tremendous diversity in effectiveness status within each of the major nodulating serotypes identified.

Despite four and two serogroups of R. trifolii having been previously identified in Abiqua and Whobrey soils, respectively (Dughri and Bottomley, 1983; Demezas and Bottomley, 1984), only 4 isolates analyzed from red clover nodules grown in either soil were antigenically identical to any of those six groups. The discovery of three new antigenically distinct serotypes, occupying between 77 and 50% of red clover nodules, adds direct evidence to support the findings of Robinson (1969) and Masterson and Sherwood (1974) that Trifolium spp. can "select" different members from the same soil population of R. trifolii. The data suggest, however, that effectiveness status might be a secondary consequence in the host selection process and that the primary consequence is a more basic, as yet undefined, character of particular subpopulations.

At this time, six and seven groups of R. trifolii, each possessing a distinctly

different major immuno determinant, have been identified in Abiqua and Whobrey soils, respectively. Such diversity at the major antigen level and at one location has been reported previously only for B. japonicum (Johnson and Means, 1963; Damirgi et al., 1967; Ham et al., 1971; Caldwell and Vest, 1968; Caldwell and Weber, 1970).

The antigenic relationship between the R. trifolii populations in the two soils was not expected. The sites are isolated by 300 miles, with the Whobrey soil having no history of inoculation and neither site having been planted to any clover other than subclover. Although it has been common to find B. japonicum isolates from different regions within the U.S.A. (Johnson and Means, 1963; Caldwell and Hartwig, 1970; Keyser et al., 1984) and even from different countries (Dowdle and Bohlool, 1985) sharing major antigens, this has only been reported sporadically for other Rhizobium species. In those cases the findings were often arrived at serendipitously as a result of screening collections of inoculant strains (Kingsley and Bohlool, 1983; Robert and Schmidt, 1985) or screening isolates from various international culture collections (Sinha and Peterson, 1980). Rarely have such geographical antigenic relationships been revealed as a result of a systematic ecological approach (Mahler and Bezdicek, 1980). We were able to confirm the antigenic relationship between the Abiqua and Whobrey populations by immunofluorescence direct counts on soil samples taken aseptically from the field and analyzed within 24 h of sampling. Such an approach makes it highly unlikely that the results were the outcome of cross contamination of soils occurring during the prolonged clover growth period in the

greenhouse. It cannot be proven unequivocally, however, that contamination of the Whobrey site did not occur during the years when the field plots were established and worked extensively (Demezas and Bottomley, 1984).

Although the above example illustrates the power of immunofluorescence direct counts, the same observations can be used to illustrate a weakness of the technique. The organisms in group WR 27 were highly successful at occupying red clover nodules in the Whobrey soil. In contrast, its antigenic counterpart in Abiqua soil, AS 16, was recovered from only one red clover nodule. On the other hand, antigenically related AR 21 and WR 26 were successful red clover occupants in both soils. The extreme diversity within the WR 27 group at the serotype, protein profile pattern, intrinsic antibiotic resistance, and effectiveness levels make it highly unlikely that the WR 27 and AS 16 groups at the two sites are of identical composition. However, abiotic and biotic differences between the two soils cannot be overlooked as also being potential influences on nodulating success or failure. It is not surprising, therefore, that conclusions drawn from autecological studies, with immunofluorescence as the sole identification technique, have not easily been confirmed with serotype representatives under nonsoil conditions (Kosslack and Bohlool, 1985; Demezas and Bottomley, 1986b).

In the case of Whobrey soil, the five serotypes enumerated can be accounted for, within the total R. trifolii population determined, by plant infection-soil dilution to be between 1 and 5 x 10^6/g soil. Significant differences between the population sizes of indigenous serotypes of rhizobia in soil have

never been reported previously from autecological studies of rhizobia. Although the term "saprophytic competence" has been used widely in Rhizobium ecology (Chatel and Parker, 1973; Date and Brockwell, 1978; Bushby, 1982), little evidence has been forthcoming from studies in non-sterile soils to support or refute the possibility that strain differences are real in this regard. Most evidence is derived indirectly through monitoring nodule occupancy success or failure over consecutive growing seasons (Dudman and Brockwell, 1968; Roughley et al., 1976; Chatel and Parker, 1973; Jansen van Rensburg and Strijdom, 1985). Nevertheless, the issue of whether the population differences measured have any ecological significance will require more intensive study. In addition, extending the power of fluorescent antibody technology to determine the population sizes of the antigenically distinct subtypes within the serogroup populations awaits further study and probably monoclonal antibody development (Wright et al., 1986).

Although it has been recognized for many years that micro- organisms in soil can be extremely small (Bae et al., 1972; Casida, 1971; Lundgren, 1984; Jenkinson et al., 1976; Balkwill et al., 1975; Bakken, 1985), this paper presents the first evidence for size class differences within a species and even within a specific serotype of a species. Evidence was obtained that the populations within certain serotypes (AS 6) were of different size classes. In other instances (AS 36) small cells were not observed. To our knowledge there are no reports of soil microbiologists routinely using 0.2μm pore size filters, which contrasts with their common usage in aquatic microbiology literature (Hobbie et al., 1977;

Zimmerman and Meyer-Reil, 1974). Our findings suggest that soil microbiologists should be aware of potential losses occuring during studies where extractions, filtrations, and enumerations are carried out. Obviously, much more work is needed to determine if there is any biological significance to these findings.

Fig.1 Representative protein profiles, displayed on a 10% wt/vol polyacrylamide gel of the two Abiqua isolates, AR 21 (a) and AR 18 (b), and of the two Whobrey isolates, WR 26 (c) and WR 27 (d). Protein standards (ps) are displayed in the far right lane. These are identified in order of decreasing molecular weight in kilodaltons, from top to bottom of gel: phosphorylase, 92.5; bovine serum albumin, 66.2; ovalbumin, 45.0; carbonic anhydrase, 31.0; soybean trypsin inhibitor, 21.5 and lysozyme, 14.4.

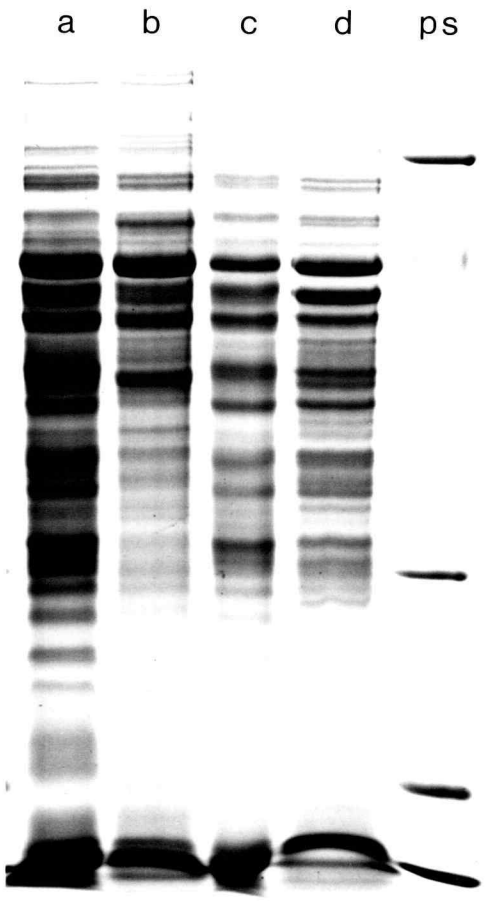

Fig. 1.

Table 1. Intrinsic antibiotic-resistance characteristics of the representative Whobrey (WR) and Abiqua (AR) soil isolates recovered from red clover and used to raise antisera.

	Antibiotic concentrations (mg l^{-1})															
	Van		Str		Rif		Kan		Nov		Amp		Spe		Nal	
Isolates	2.5	5.0	1.0	2.5	1.0	3.0	5.0	8.0	1.0	2.5	2.5	5.0	1.0	2.5	5.0	10.0
WR 26	-[a]	-	-	-	+[b]	-	+	-	-	-	-	-	-	-	+	+
WR 27	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+	+
AR 18	+	+	-	-	+	-	+	+	-	-	-	-	+	-	+	+
AR 21	+	+	+	+	+	+	-	-	-	-	-	-	-	-	+	+

[a]Designates no visible growth after 48h incubation at 30°C.

[b]Designates visible growth after 48h incubation at 30°C.

Fig. 2. Gel-immune-diffusion characteristics illustrating the antigenic reactions of different isolates representing serotypes WR 26-A (A_1-A_3) and WR 26-B (B_1-B_4). Antisera WR 26 and WS 1-01 are in the center wells of (a) and (b), respectively. Antigens are in the outer wells with the parent antigens represented by A_1 and 1-01, respectively.

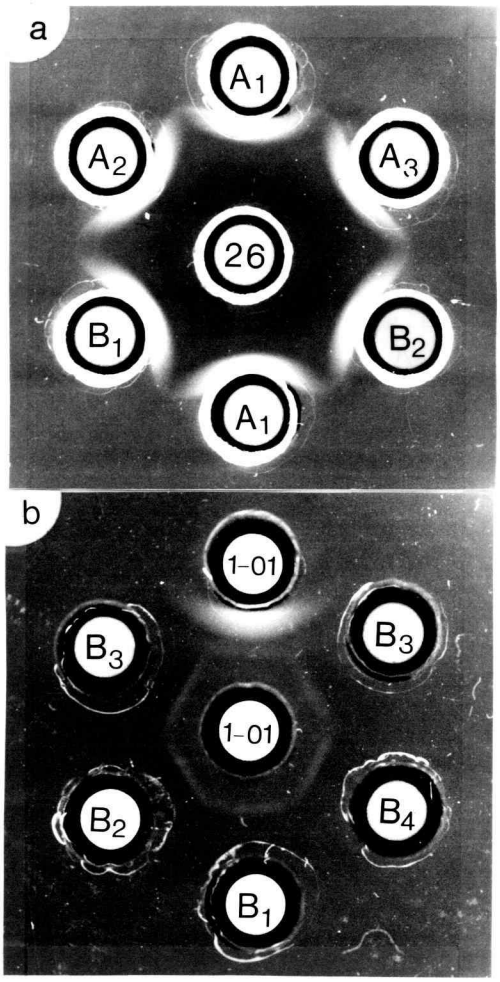

Fig. 2.

Table 2. Gel-immune-diffusion characteristics of the Whobrey isolates with antisera raised to R. trifolii from this soil.

% of total isolates	Sero-group	Sero-type	No. of isolates	Antisera[a]			
				WR 26	WR 27	WS 1-01	WS 2-01
				Precipitin reaction			
30.0	WR 26	A[e]	3	I[b]	-[d]	-	-
		B	4	I	-	NI	-
		C	2	NI[c]	-	-	-
47.0	WR 27	A[e]	9	NI	I	-	-
		B	5	-	I	-	-
23.0	Others[f]		7	-	-	-	-

a The antisera utilized were raised to R. trifolii isolates recovered either (1) in this study from root nodules of red clover (WR), or (2) previously from root nodules of subclover (WS) grown in Whobrey soil (Demezas and Bottomley, 1984).

b Precipitin line is identical to the homologous interaction.

c Precipitin line is nonidentical to the homologous interaction.

d No precipitin lines are visible under these conditions.

e Designates the serotype containing the respective parent isolate.

f Designates isolates which showed no antigenic reaction with the antisera tested.

Table 3. Gel-immune-diffusion characteristics of the Whobrey isolates with antisera raised to R. trifolii from Abiqua soil.

% of total isolates	Sero-group	Sero-type	No. of isolates	Antisera[a]			
				AS 16	AS 6	AS 27	AS 36
				Precipitin reactions[b]			
30.0	WR 26	A	3	-	-	-	-
		B	4	-	-	-	-
		C	2	-	-	-	-
47.0	WR 27	A	9	I	-	-	-
		B	5	I	-	-	-
3.0	AS 6		1	-	I	NI	-
7.0	AS 27	A	1	-	-	I	-
		B	1	-	-	NI	-
13.0	Others		4	-	-	-	-

a Antisera raised to R. trifolii isolates recovered previously from root nodules of subclover grown in Abiqua soil (Dughri and Bottomley, 1983).

b Refer to Table 2 for definitions of precipitin reactions.

Table 4. Gel-immune-diffusion characteristics of the Abiqua soil isolates.

% of total isolates	Sero-group	Sero-type	No. of isolates	Antisera[a]							
				AR21	AR18	AS6	AS16	AS27	AS36	WR26	WR27
				Precipitin reactions[b]							
50.0	AR 21	A[c]	3	I	-	-	-	-	-	-(+)[d]	-
		B	2	I	-	-	-	-	NI	-	-
		C	7	NI	-	-	-	-	-	-	-
8.0	AR 18	A[c]	1	-	I	I	-	NI	-	-	-
		B	1	-	NI	-	-	-	-	-	-
4.0	AS 16		1	-	-	-	I	-	-	-	-
38.0	Others		9	-	-	-	-	-	-	-	-

a AR 18 and AR 21 are R. trifolii isolates recovered from root nodules of red clover grown in Abiqua soil in this study. See Tables 2 and 3 for information on other antisera.

b Refer to Table 2 for definitions of precipitin reactions.

c Designates the serotype containing the respective parent isolate.

d Isolates AR 21 and WR 26 and their antisera cross-reacted reciprocally in whole cell somatic tube agglutination tests to titers of 1/320th dilution of antisera. No precipitin reactions were observed.

Table 5. Population densities of six indigenous R. trifolii serogroups in Whobrey soil in relation to their presence in nodules of red clover.

Sero-group	Mean population size determined by immuno-fluorescence direct count† (cells x 10^5/g soil)	Proportion (%) of nodules occupied
AS 27	0.02c‡	3.0
WR 27	1.63b	47.0
WS 2-01	2.00b	0
AS 36	2.06b	0
WS 1-01	2.68b	0
AS 6	6.52a	3.0

† Mean population size from 8 replicate soil samples.

‡ Values not followed by the same letter differ significantly from each other (P = 0.05) according to Duncan's multiple range test procedure.

Table 6. Symbiotic effectiveness characteristics on red clover and subclover of the R. trifolii isolates within the dominant serogroups.

Soil	Sero-group	Sero-type	No. of isolates tested	Host plant: Red clover — Effectiveness Categories[a] E_1	E_2	E_3	Host plant: Sub clover — Effectiveness Categories[a] E_1	E_2	E_3
				No. of isolates					
Whobrey	WR 26	A	3	0	1	2	2	1	0
		B	4	0	2	2	3	1	0
		C	1	0	1	0	0	0	1
	WR 27	A	8	1	6	1	4	3	1
		B	5	0	2	3	1	1	3
				Proportion of isolates (%)[b]					
				5	57	38	48	28	24
Abiqua	AR 21	A	3	0	2	1	1	2	0
		B	2	1	1	0	1	1	0
		C	7	2	3	2	3	4	0
				Proportion of isolates (%)[b]					
				25	50	25	42	58	0

[a] E_1 = Isolates producing shoot dry weights not significantly different from uninoculated plants according to Tukey's w-procedure at P = 0.05. Mean shoot dry wt of uninoculated red and subclover plants = 6.5 mg and 12.4 mg, respectively.

E_2 = Isolates producing shoot dry weights significantly greater than uninoculated plants and less than nitrate supplemented plants according to Tukey's w-procedure at P = 0.05.

E_3 = Isolates producing shoot dry weights not significantly different from nitrate supplemented plants according to Tukey's w-procedure at P = 0.05. Mean shoot dry wt of red and subclover plants = 28.7 mg and 32.6 mg, respectively.

[b] Designates proportion of the total isolates within each effectiveness category on each species of clover.

Table 7. Host plant influences on the symbiotic effectiveness of R. trifolii isolates within the serotypes.

Soil	Sero-group	Sero-type	Host Plant: Red clover — Effectiveness[a] category	Red clover — No. of isolates	Subclover — Effectiveness category
Whobrey	WR 26	A	E_3	2	$E_1(1)$[b]; $E_2(1)$
		B	E_2	2	$E_1(1)$; $E_2(1)$
		B	E_3	2	$E_1(2)$
	WR 27	A	E_2	6	$E_1(2)$; $E_2(3)$; $E_3(1)$
		B	E_2	2	$E_1(1)$; $E_3(1)$;
		B	E_3	3	$E_2(1)$; $E_3(2)$
Abiqua	AR 21	A	E_2	2	$E_1(1)$; $E_2(1)$
		C	E_1	2	$E_1(1)$; $E_2(1)$
		C	E_2	3	$E_1(1)$; $E_2(2)$
		C	E_3	2	$E_1(1)$; $E_2(1)$

a Refer to Table 6 for the definition of the symbiotic effectiveness categories.

b Values in parentheses refer to the number of isolates with the designated effectiveness trait.

BIBLIOGRAPHY

Amarger, N. 1984. Evaluation of competition in Rhizobium spp. In: Current Prospectives in Microbial Ecology. Proc. of the Third International Symposium on Microbial Ecology. M. J. Klug and C. A. Reddy, ed. pp. 300-305.

Bae, H. C., E. H. Cota-Robles, and L. E. Casida. 1972. Microflora of soil as viewed by transmission electron microscopy. Appl. Microbiol. 23:637-648.

Bakken, L. R. 1985. Separation and purification of bacteria from soil. Appl. Environ. Microbiol. 49:1482-1487.

Balkwill, D. L., D. P. Labeda, and L. E. Casida, Jr. 1975. Simplified procedures for releasing and concentrating microorganisms from soil for transmission electron microscopy viewing as thin-sectioned and frozen-etched preparations. Can. J. Microbiol. 21:252-262.

Beynon, J. L., and D. P. Josey, 1980. Demonstration of heterogeneity in a natural population of Rhizobium phaseoli using variation in intrinsic antibiotic resistance. J. Gen. Microbiol. 118: 437- 442.

Bohlool, B. B., and E. L. Schmidt. 1968. Nonspecific staining: Its control in immunofluorescence examination of soil. Science 162: 1012-1014.

Bohlool, B. B., and E. L. Schmidt. 1972. Fluorescent antibody for determination of growth rates of bacteria in soil. Abs. Ann. Meet. Am. Soc. Microbiol. 72:13-15.

Brockwell, J., W. G. Bryant, and R. R. Gault. 1972. Ecological studies of root

nodule bacteria introduced into field environments. 3. Persistence of Rhizobium trifolii in association with white clover at high elevations. Aust. J. Exp. Agric. Anim. Husb. 12:407-413.

Brockwell, J., E. A. Schwinghamer, and R. R. Gault. 1977. Ecological studies of root-nodule bacteria introduced into field environments. V. A critical examination of the stability of antigenic and streptomycin-resistance markers for identification of strains of Rhizobium trifolii. Soil Biol. Biochem. 9:19-24.

Brockwell, J., R. R. Gault, M. Zorin, and M. J. Roberts. 1982. Effects of environmental variables on the competition between inoculum strains and naturalized populations of Rhizobium trifolii for nodulation of Trifolium subterraneum L. and on Rhizobia persistence in the soil. Aust. J. Agric. Res. 33:803-815.

Burns, R. C. and R. W. F. Hardy. 1975. Nitrogen fixation in bacteria and higher plants. Springer-Verlag, Berlin.

Bushby, H. V. A. 1982. Ecology. In: Nitrogen Fixation, Vol. 2: Rhizobia. W. J. Broughton, ed. Clarendon Press, Oxford, U.K. pp. 35-75.

Caldwell, B. E., and G. Vest. 1968. Nodulation interactions between soybean and serogroups of Rhizobium japonicum. Crop. Sci. 8:680-682.

Caldwell, B. E., and E. E. Hartwig. 1970. Serological distribution of soybean root nodule bacteria in soils of Southeastern U.S.A. Agron. J. 62:621-622.

Caldwell, B. E., and D. F. Weber. 1970. Distribution of Rhizobium japonicum

serogroups in soybean nodules as affected by planting dates. Agron. J. 62:12-14.

Casida, L. E., Jr. 1971. Microorganisms in unamended soil as observed by various forms of microscopy and staining. Appl. Microbiol. 21:1040-1045.

Chatel, D. L., and R. M. Greenwood. 1973. II. Strain differences in the species Rhizobium trifolii. Soil Biol. Biochem. 5:433-440.

Chatel, D. L., and C. A. Parker. 1973. The colonization of host-root and soil by rhizobia. I. Species and strain difference in the field. Soil. Biol. Biochem. 5:425-432.

Cole, M. A., and G. H. Elkan. 1979. Multiple antibiotic resistance in Rhizobium japonicum. Appl. Environ. Microbiol. 37:867-870.

Damirgi, S. M., L. R. Frederick, and I. C. Anderson. 1967. Serogroups of Rhizobium japonicum in soybean nodules as affected by soil types. Agron. J. 59:10-12.

Date, R. A. and J. Brockwell. 1978. Rhizobium strain competition and host interaction for nodulation, p. 206-216. In J. R. Wilson (ed.), Plant relations in pastures. Commonwealth Scientific and industrial Research Organization, East Melbourne, Australia.

Demezas, D. H. and P. J. Bottomley. 1984. Identification of two dominant serotypes of Rhizobium trifolii in root nodules of uninoculated field-grown subclover. Soil Sci. Soc. Am. J. 48:1067-1071.

Demezas, D. H. and P. J. Bottomley. 1986a. Autecology in rhizospheres and

nodulating behavior of indigenous Rhizobium trifolii. Appl. Environ. Microbiol. 52:1014-1019.

Demezas, D. H. and P. J. Bottomley. 1986b. Interstrain competition between representatives of indigenous serotypes of Rhizobium trifolii. Appl. Environ. Microbiol. 52:1020-1025.

Dobereiner, J., R. H. Burris, A. Hollaender, A. A. Franco, A. C. Neyra, D. B. Scott (ed.). 1978. Limitations and potentials for biological nitrogen fixation in the tropics. Plenum, New York.

Dowdle, S. F. and B. Bohlool. 1985. Predominance of fast-growing Rhizobium japonicum in a soybean field in the People's Republic of China. Appl. Environ. Microbiol. 50:1171-1176.

Dudman, W. F. 1964. Immune diffusion analysis of the extracellular soluble antigens of two strains of Rhizobium meliloti. J. Bacteriol. 88:782-794.

Dudman, W. F., and J. Brockwell. 1968. Ecological studies of root-nodule bacteria introduced into field environments. 1. A survey of field performance of clover inoculants by gel immune diffusion serology. Aust. J. Agric. Res. 9:729-747.

Dughri, M. H. and P. J. Bottomley. 1983. Complementary methodologies to delineate the composition of Rhizobium trifolii populations in root nodules. Soil. Sci. Soc. Am. J. 47:939-945.

Dughri, M. H., and P. J. Bottomley. 1984. Soil acidity and the composition of an indigenous population of Rhizobium trifolii in nodules of different cultivars of Trifolium subterraneum L. Soil Biol. Biochem. 16:405-411.

Evans, H. J., P. J. Bottomley, and W. E. Newton (ed.) 1985. Nitrogen fixation research progress. Martinus Nijhoff Publishers, Dordecht, Netherlands.

Fuquay, J. I., P. J. Bottomley, and M. B. Jenkins. 1984. Complementary methods for the differentiation of Rhizobium meliloti isolates. Appl. Environ. Microbiol. 47:663-669.

Gibson, A. H., R. A. Date, and J. Brockwell. 1976. A comparison of competitiveness and persistence amongst five strains of Rhizobium trifolii. Soil Biol. Biochem. 8:395-401

Gibson, A. H., and W. E. Newton (ed.). 1981. Current perspectives in nitrogen fixation. Australian Academy of Science, Canberra, Australia.

Graham, P. H. 1963. Antigenic affinities of the root-nodule bacteria of legumes. Antonie Van Leeuwenhoek. 29:281-291.

Hagedorn, C. 1979. Nodulation of Trifolium subterraneum L. by introduced Rhizobia in southwest Oregon soils. Soil Sci. Soc. Am. J. 43:515-518.

Ham, G. E., L. R. Frederick, and I. C. Anderson. 1971. Serogroups of Rhizobium japonicum in soybean nodules sampled in Iowa. Agron. J. 63:69-72.

Hardy, R. W. F., F. Bottomly, and R. C. Burns (ed.), 1977. A treatise on dinitrogen fixation. Sections I and II. Inorganic and physical chemistry and biochemistry. Wiley-Interscience, New York.

Hardy, R. W. F., and A. H. Gibson, (ed.), 1977. A treatise on dinitrogen fixation. Section IV. Agronomy and ecology. Wiley-Interscience, New York.

Hardy R. W. F., and W. S. Silver (ed.), 1977. A treatise on dinitrogen fixation. Section III. Blology. Wiley-Interscience, New York.

Hellriegel, H., and H. Wilfarth. 1888. Beil, Z. Ver. Ruben-Zucker Ind. Dtsch. Reichs.

Hobbie, J. E., R. J. Daley, and S. Jasper. 1977. Use of nucleopore filters for counting bacteria by fluorescence microscopy. Appl. Environ. Microbiol. 33:1225-1228.

Hollaender, A., R. H. Burris, P. R. Day, R. W. F. Hardy, D. R. Helinski, M. R. Lamburg, L. Owens, R. C. Valentine (ed.). 1977. Genetic engineering for nitrogen fixation. Plenum, New York.

Holland, A. A. 1966. Serological characteristics of certain root-nodule bacteria of legumes. Antonie van Leeuwenhoek 32:410-418.

Hughes, D. Q., and J. M. Vincent. 1942. Serological studies of the root nodule bacteria. III. Tests of neighboring strains of the same species. Proc. Linn. Soc. N. S.W. 67:142-152.

Jansen van Rensburg, H., and B. W. Strijdom. 1982. Competitive abilities of Rhizobium meliloti strains considerd to have potential as inoculants. Appl. Environ. Microbiol. 44:98-106.

Jansen van Rensburg, H. and B. W. Strijdom. 1985. Effectiveness of Rhizobium strains used in inoculants after their introduction into soil. Appl. Environ. Microbiol. 49:127-131.

Jenkins, M. B. and P. J. Bottomley. 1984. Composition and field distribution of the population of Rhizobium meliloti in root nodules of uninoculated field-grown alfalfa. Soil Biol. Biochem. 17:173-179.

Jenkinson, D. S., D. S. Powlson, and R. W. M. Wedderburn. 1976. The effects

of biocidal treatments on metabolism in soil. III. The relationship between soil biovolume, measured by optical microscopy, and the flush of decomposition caused by fumigation. Soil Biol. Biochem. 8:189-202.

Johnson, H. W. and U. M. Means. 1963. Serological groups of Rhizobium japonicum recovered from nodules of soybeans (Glycine max) in field soils. Agron. J. 55:269-271.

Johnson, H. W., and U. M. Means. 1964. Selection of competitive strains of soybean nodulating bacteria. Agron. J. 56:60-62.

Johnson, H. W., V. M. Means, and C. R. Weber. 1965. Competition for nodule sites between strains of Rhizobium japonicum applied as inoculum and strains in the soil. Agron. J. 57:179-185.

Jones, D. G., and P. S. E. Bromfield. 1978. A study of the competitive ability of streptomycin mutants of Rhizobium trifolii using various marker techniques. Ann. Appl. Biol. 46:445-487.

Jones, D. G., and S. Morley. 1981. The effect of pH on host plant "preference" for strains of Rhizobium trifolii using fluorescent ELISA for strain identification. Ann. Appl. Biol. 97:183-190.

Josey, D. P., J. L. Beynon, B. W. Johnson, and J. E. Beringer. 1979. Strain identification in Rhizobium using intrinsic antibiotic resistance. J. Appl. Bact. 46:343-350.

Keyser, H. H., D. F. Weber, and S. L. Uratsu. 1984. Rhizobium japonicum serogroups and hydrogenase phenotype distribution in 12 states. Appl. Environ. Microbiol. 47:613-615.

Kingsley, M. T. and B. B. Bohlool. 1981. Release of Rhizobium spp. from tropical soils and recovery for immunofluorescence enumeration. Appl. Environ. Microbiol. 42:241-248.

Kingsley, M. T. and B. Bohlool. 1983. Characterization of Rhizobium spp. (Cicer arietinum L.) by immunofluorescence, immunodiffusion, and intrinsic antibiotic resistance. Can. J. Microbiol. 29:518-526.

Kosslack, R. M., and B. B. Bohlool. 1985. Influence of environmental factors on interstrain competition in Rhozobium japonicum. Appl. Environ. Microbiol. 49:1128-1153.

Kremer, R. J., and H. L. Peterson. 1982. Nodulation efficiency of legume inoculation as determined by intrinsic antibiotic resistance. Appl. Environ. Microbiol. 43:636-642.

Lundgren, B. 1984. Size classification of soil bacteria: Effects on microscopically-estimated biovolumes. Soil Biol. Biochem. 16:283-284.

Mahler, R. L., and D. F. Bezdicek. 1980. Serogroup distribution of Rhizobium leguminosarum in peas in the palouse of Eastern Washington. Soil. Sci. Soc. Am. J. 44:292-295.

Masterson, C. L., and M. T. Sherwood. 1974. Selection of Rhizobium trifolii strains by white and subterranean clovers. Ir. J. Agric. Res. 20:827-841.

Materon, L. A., and C. Hagedorn. 1983. Competitiveness and symbiotic effectiveness of five strains of Rhizobium trifolii on Red Clover. Soil Sci. Soc. Am. J. 47:491-495.

Means, U. M., H. W. Johnson, and R. A. Date. 1964. Quick serological method

of classifying strains of Rhizobium japonicum in nodules. J. Bacteriol. 87:547-553.

Moawad, H. A., W. R. Ellis and E. L. Schmidt. 1984. Rhizosphere response as a factor in competition among three serogroups of indigenous Rhizobium japonicum for nodulation of field-grown soybeans. Appl. Environ. Microbiol. 47:607-612.

Mulder, E. G., and W. L. van Veen. 1960. Effect of pH and organic compounds on nitrogen fixation by red clover. Plant and Soil 13:91-112.

Newton, W. E., and C. J. Nyman (ed.). 1975. Nitrogen fixation, Vols. 1 and 2. Washington State Univ. Press, Pullman.

Newton, W. E., J. R. Postgate, and C. Rodriguez-Barrenco (ed.). 1977. Recent developments in nitrogen fixation. Academic, New York.

Newton, W. E., and W. H. Orme-Johnson (ed.). 1980. Nitrogen fixation, Vols. 1 and 2. Univ. Park, Baltimore.

Nicol, H., and H. G. Thornton. 1941. Competition between related strains of nodule bacteria and its influence on infection of the legume host. Proc. R. Soc. (London) Ser. B. 130:32-59.

Noel, K. D., and W. J. Brill. 1980. Diversity and dynamics of indigenous Rhizobium japonicum population. Appl. Environ. Microbiol. 40:931-938.

Nutman, P. S. 1965. The relation between nodule bacteria and the legume host in the rhizosphere and in the process of infection, p. 231-247. In K. F. Baker and W. C. Snyder (ed.), Ecology of soil-borne plant pathogens. Prelude to biological control. Berkeley, Univ. Calif. Press.

O'Gara, F., and K. T. Shanmugan. 1976. Regulation of nitrogen fixation by rhizobia: export of fixed N_2 as NH_4. Biochem. Biophys. Acta. 437:313-321.

Pankhurst, C. E. 1977. Symbiotic effectiveness of antibiotic resistant mutants of fast and slow growing strains of Rhizobium nodulating Lotus species. Can. J. Microbiol. 23:1-26-1033.

Peters, N. K., J. W. Frost., and S. R. Long. 1986. A plant flavone, luteolin, induces expression of Rhizobium meliloti modulation genes. Science, 233:977-980.

Pilacinski, W. P., and E. L. Schmidt. 1981. Plasid transfer within and between serologically distinct strains of Rhizobium japonicum using antibiotic resistance and auxotrophs. J. Bacteriol. 145:1025-1030.

Pinto, C. M., P. Y. Yao, and J. M. Vincent. 1974. Nodulating competitiveness amongst strains of Rhizobium meliloti and R. trifolii. Aust. J. Agric. Res. 25:317-319.

Purchase, H. F. 1958. Restriction of infection threads in nodulation of clover and lucerne. Aust. J. Biol. Sci. 2:155-161.

Purchase, H. F., and J. M. Vincent. 1949. A detailed study of the field distribution of strains of clover nodule bacteria. Proc. Linn. Soc. N.S.W. 74:227-236.

Purchase, H. F., J. M. Vincent, and L. M. Ward. 1951. Serological studies of the root nodule bacteria. IV. Further analysis of isolates from Trifolium and Medicago. Proc. Linn. Soc. N.S.W. 76:1-6.

Purchase, H. F., and P. S. Nutman. 1957. Studies on the physiology of nodule formation. VI. The influence of bacterial numbers in the rhizosphere on nodule initiation. Ann. Bot. 21:439-454.

Quispel, A. (ed.). 1974. The biology of nitrogen fixation. Amsterdam, North Holland.

Rawsthorne, S., F. R. Minchin, R. J. Summerfield, C. Cookson, and J. Coombs. 1980. Carbon and nitrogen metabolism in legume root nodules. Phytochem. 19:341-355.

Read, M. P. 1953. The establishment of serologically identifiable strains of Rhizobium trifolii in field soils in competition with native microflora. J. Gen. Microbiol. 1:1-4.

Rennie, R. J. 1985. Nitrogen fixation in agriculture in temperate regions. In H. J. Evans, P. J. Bottomley and W. E. Newton (ed.) Nitrogen fixation research progress, p.659-665. Martinus Nijhoff Publishers, Dordrecht, Netherlands.

Reyes, V. G., and E. L. Schmidt. 1979. Population densities of Rhizobium japonicum strain 123 estimated directly in soil and rhizospheres. Appl. Environ. Microbiol. 37:854-858.

Reyes, V. G., and E. L. Schmidt. 1981. Populations of Rhizobium japonicum associated wtih the surfaces of soil-grown roots. Plant Soil 61:71-80

Robert, F. M., and E. L. Schmidt. 1985. Somatic serogroups among 55 strains of Rhizobium phaseoli. Can. J. Microbiol. 31:519-523.

Robinson, A. C. 1969. Host selection for effective Rhizobium trifolii by red

clover and subterranean clover in the field. Aust. J. Agric. Res. 20:1052-1060.

Roughley, R. J., W. M. Blowes, and D. F. Herridge. 1976. Nodulation of Trifolium subterraneum by introduced Rhizobia in competition with naturalized strains. Soil Biol. Biochem. 8:403-407.

Rovira, A. D. 1961. Rhizobium numbers in rhizospheres of red clover and paspalum in relation to soil treatment and the numbers of bacteria and fungi. Aust. J. Agric. Res. 12:77-83.

Russell, P. E., and D. G. Jones. 1975. Variation in the selection of Rhizobium trifolii by varieties of red and white clover. Soil Biol. Biochem. 7:15-18.

Schmidt, E. L., R. O. Bankole, and B. B. Bohlool. 1968. Fluorescent-antibody approach to study rhizobia in soil. J. Bacteriol. 95:1987-1992.

Schmidt, E. L. 1974. Quantitative autecological study of microorganisms in soil by immunofluorescence. Soil Sci. 118:141-149.

Schwinghamer, E. A., and W. F. Dudman. 1973. Evaluation of spectinomycin resistance as a marker for ecological studies with Rhizobium spp. J. Appl. Bacteriol. 36:263-272.

Schwinghamer, E. A., and W. F. Dudman. 1980. Methods for identifying strains of diazotrophs, p. 337-365. In F. J. Bergersen (ed.), Methods for evaluating biological nitrogen fixation. John Wiley and Sons Ltd., New York.

Sinha, R. C. and E. A. Peterson. 1980. Homologous serological analysis of Rhizobium meliloti strains by immunodiffusion. Can. J. Microbiol.

26:1157-1161.

Trinick, M. J. 1969. Identification of legume nodule bacteroids by the fluorescent antibody reaction. J. Appl. Bacteriol. 32:181-186.

Veeger, C., and W. E. Newton (ed.). 1984. Advances in nitrogen fixation research. Martinus Nijhoff, Dordecht, Netherlands.

Vincent, J. M. 1942. Serological studies of the root nodule bacteria. II. Strains of Rhizobium trifolii. Proc. Linn. Soc. N.S.W. 67:82-86.

Vincent, J. M. 1954. The root-nodule bacteria of pasture legumes. Proc. Linn. Soc. N.S.W. 87:8-38.

Vincent, J. M. 1962. Australian studies of the root-nodule bacteria. Proc. Linn. Soc. N.S.W. 87:8-38.

Vincent, J. M. 1965. Environmental factors in the fixation of nitrogen by the legume. p. 384-385. In W. V. Bartholomew and F. E. Clarke (ed.), Soil nitrogen, Agronomy, Vol. 10. American Society of Agronomy, Madison, Wisconsin.

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

Vincent, J. M., and L. M. Waters. 1953. The influence of host on competition amongst clover root-nodule bacteria. J. Gen. Microbiol. 9:357-370.

Wright, W. H., W. B. Sarles, and E. G. Holst. 1930. A study of Rhizobium japonicum isolated from various soils. J. Bacteriol. 19:39.

Wright, S. F., J. G. Foster, and O. L. Bennet. 1986. Production and use of

monoclonal antibodies for identification of strains of Rhizobium trifolii. Appl. Environ. Microbiol. 52:119-123.

Zimmerman, R., and L. A. Meyer-Reil. 1974. A new method for fluorescence staining of bacterial populations on membrane filters. Kieler Meeresforschungen 30:24-27.