Chapter 8

LEGUME SYMBIOSIS

A. ECOLOGY OF THE LEGUME ROOT NODULE BACTERIA

E. L. SCHMIDT

1. INTRODUCTION

Legume root nodule bacteria comprising the genus *Rhizobium* initiate, with the help of an appropriate legume plant partner, a symbiosis of immense global importance to plant succession, soil fertility, agriculture, and general biological productivity. A vast, diverse, and currently greatly expanding literature attests to the significance vested in this plant—microbe interaction. The overwhelming focus of the literature has been on the symbiotic structure itself and its nature and function in relation to nitrogen fixation, the most important expression of the symbiosis. This review will be concerned with events that precede the very earliest evidence of the microbe—legume root interaction. These are key events in the establishment of the symbiosis but they have been relatively inaccessible to direct experimentation.

For the greater part of their existence, rhizobia survive as free-living bacteria in that most complex of natural environments, the soil. As soil bacteria, the rhizobia are virtually unknown; they exist nondescriptly in the midst of the myriad biota of the soil, respond to the complicated interplay of abiotic soil factors at the microenvironment level, and somehow survive, perhaps even grow, in the absence of a host legume. A remarkable phenomenon follows when the root of an appropriate legume is interposed into the environment of the free-living rhizobia. The phenomenon is that of mutual recognition between the two compatible partners: the legume plant root recognizes just the right kind of *Rhizobium* among all other bacteria including other rhizobia in the vicinity; the *Rhizobium* in turn recognizes just the right kind of legume root among all other roots that may occur in that environment. The molecular base or bases for selective interaction between the partners is not known, nor is it known at what stage or stages in the interaction selectivity is expressed. Evidence that the interaction is underway is generally said to be found first in the elongation and deformation of the legume root hairs. Long before that, many of the events that comprise the ecology of the rhizobia in the soil and especially in the rhizosphere must be prerequisite to the successful interaction of symbiont and host.

There are compelling reasons to focus more of the attention given the legume symbiosis on the ecology of the root nodule bacteria. Pragmatic considerations alone would demand this. Much of man's capacity to manage biological nitrogen fixation for food production has, after all, been achieved at the ecological level with the practice of legume seed inoculation. Yet the bases for legume inoculation necessarily have been highly arbitrary. The objective of establishing in the soil the most desirable strains of rhizobia for a given legume crop can be attained only when the free-living ecology of rhizobia is better understood. Questions of survival, population densities, growth responses, interactions with other microorganisms, nutritional substrates, host specificity, and competition between strains for host sites must be asked about the rhizobia in their natural environment. A wealth of hypotheses are to be found in the decades of experimentation with axenic legumes and pure cultures of rhizobia, but their relevance if any will remain vague until the natural environment is confronted. To place this emphasis on the ecology of the rhizobia in order to obtain a theoretical base for understanding and managing the legume nodule symbiosis is not to ignore the higher plant component. The plant, obviously, exerts fine controls that are crucial to the association, but these, too, are addressed when the behavior of the rhizobia is followed in the normal rhizosphere.

2. TECHNIQUES FOR ECOLOGICAL STUDY OF RHIZOBIA

Neglect of the ecology of the free-living rhizobia has not been due to lack of recognition of its importance. Several reviews of the past decade (Nutman, 1965; Brown et al., 1968; Fåhraeus and Ljunggren, 1968; Dixon, 1969; Vest et al., 1973) have noted many significant problems to be resolved concerning rhizobia in the soil and in the legume rhizosphere. The neglect stems instead from the inadequacies of the techniques available for ecological studies.

Rhizobia that are added to a soil deliberately through inoculation or fortuitously through the growth of legumes, are lost in the general soil population. As bacteria in the soil, the rhizobia lack any distinguishing features that would permit their selective study; they fix no nitrogen, have no unusual nutritional requirements, and look like any other medium-sized rod-shaped bacterium. Retrieval from the general soil population has until recently been possible only by virtue of the ability of the rhizobia to induce a nodule on an appropriate legume. Because the methodology imposes such severe limitations on ecological research, brief attention should be given to the more important techniques.

2.1. Soil dilution plating

Need for a selective medium that would facilitate the isolation and enumeration of soil rhizobia has long been recognized, but prospects for the

development of such a medium are very poor. Inherent difficulties are posed by the physiological diversity of both the rhizobia and the soil microflora, so that a medium that would inhibit all other bacteria, yet be completely selective for a wide range of rhizobia is most unlikely. Some media are sufficiently selective for limited application as in the case of enumerating rhizobia present in high abundance in previously sterilized peat (Date and Vincent, 1962) and for differentiating strains isolated from nodules (Davis, 1962).

A medium containing four antibiotics, pentachloronitrobenzene and sulfafurazole was claimed as a selective medium for soil isolation by Graham (1969a) in a brief paper with little documentation. Careful and extensive work by Pattison and Skinner (1973) demonstrates very clearly the difficulties in formulating a selective medium for the rhizobia. They tested 47 strains representing 6 species of *Rhizobium* and the cowpea group for sensitivity to 6 antibiotics. Each strain was inhibited by at least one antibiotic and no consistent patterns of inhibition were found within a species. The only antibiotic that had little effect on rhizobia was penicillin at a low concentration. When that concentration of penicillin was incorporated into a selective medium which by itself inhibited many soil microorganisms but not the rhizobia, the selective medium plus penicillin strongly inhibited most strains of rhizobia. These same authors tried 19 of their strains on the medium of Graham (1969a) and none grew. Selective media adequate for the isolation and enumeration of rhizobia in soil or rhizosphere apparently are out of the question except as a limited possibility for specific strains.

2.2. Plant-dilution (plant infection) techniques

The selective medium most widely used to detect the presence of rhizobia in soil has been the legume host itself. The method is indirect as it depends on the eventual appearance of a nodule on the roots of a legume test plant as the indicator of an appropriate *Rhizobium* in the soil. Numbers of rhizobia usually are estimated by preparing 10-fold dilutions as inocula for replicate rhizobia-free seedlings. This "plant-dilution" technique is thus a variation of the statistical, most probable number (MPN) assay. Since the method is expensive of time, materials, and space, the number of replicate tubes (plants) per dilution is commonly reduced from the usual MPN protocol of 5 or 10 to 2 or 4, with corresponding decrease in accuracy. Calculations based on statistical tables are made with the assumption that a single *Rhizobium* can initiate nodulation. Most recent workers seem to have been guided by the plant-dilution protocols of Date and Vincent (1962).

Enumeration by the plant-dilution technique has been the basis of virtually all ecological studies dealing with the persistence of the legume nodule bacteria in soil, and their response to rhizosphere conditions. Since other methods were not available it is unfortunate to find, as Date and

Vincent (1962) have observed, that there is little published evidence on which to assess the validity of the method. These authors are among the few to report the validity of the MPN determination for the pure culture system involving just the diluted *Rhizobium* culture and the assay plants. Whereas Date and Vincent (1962) and Brockwell (1963) found that a number of different kinds of rhizobia gave plant-dilution counts in pure culture trials that were in good agreement with plate counts, similar controls in a study by Tuzimura and Watanabe (1961a) were much more variable. More attention should be given to calibration of the assay system as a prelude to studies that involve estimation by plant-dilution techniques.

Thompson and Vincent (1967) made further evaluations of plant-dilution estimations in the light of unexpected "skips" encountered in the dilution series when low numbers of rhizobia demanded that the dilutions include relatively large amounts of soil. They concluded that the plant infection method is applicable to soils with rhizobia at population densities greater than 100/gram, but not for more sparsely populated soils. Should this be the case it is difficult to appreciate why the method has not been applied more vigorously (and rigorously) to *Rhizobium* ecology at the few hundred or more per gram level where perhaps the more significant questions arise. Applicability, however, seems to be conditioned by more than a threshold population density. The impact of the plant in the nodulation process is recognized increasingly as a factor in the plant-dilution technique. As pointed out by Vest et al. (1973) the host plant screens and selects certain strains of rhizobia from among those present in a soil population. Certain soybean genotypes, for example, are selective for particular strains of *R. japonicum* (Caldwell and Vest, 1968). Weber et al. (1971) reported that inoculant strains apparently survived in the root zone of soybean in sufficient numbers to initiate nodulation, but that recovery of the inoculant strain in the nodules was low, attesting to plant preference for indigenous rhizobia. Legumes other than soybean also are highly selective of their rhizobia. Masterson and Sherwood (1974) and Sherwood and Masterson (1974) used effectiveness as the strain marker to show clearly that white clover and subterranean clover were nodulated with different populations of *R. trifolii* when inoculated with each of 8 different soils; they also demonstrated the host selection phenomenon in test tubes when clover plants were inoculated with a 50:50 mixture of strains isolated from both the white and subterranean clovers. A study by Russell and Jones (1975) showed that two serologically distinct strains of *R. trifolii* were not only selected for quite differently by six varieties of red and white clover, but also were selected for quite variably within any one variety. The plant dilution assay is a measure of the legume symbiosis rather than a direct index of the free-living ecology of rhizobia. Not only must the selectivity of the test legume be recognized but also the likelihood that this selectivity probably will be variable in response to the environmental conditions of the assay.

2.3. Immunological approaches

If not the widely used plant-dilution technique, or not that alone at least, what are the alternatives in microbial ecology methodology? The most promising alternative has emerged only in recent years in the form of the fluorescent antibody (FA) technique. The technique is a general one for autecological study of microorganisms directly in natural environments. Antibodies are prepared against the microorganism to be studied, and after the addition of a fluorochrome the antibody is used as a highly selective stain for the direct microscopic examination of the natural environment. The antigen—antibody reaction is visualized by fluorescence microscopy to allow for the simultaneous detection and recognition of the microorganisms of interest. A discussion of the technique may be found in Schmidt (1973), and a representative microscope field illustrating the specific detection of *R. japonicum* USDA 110 in normal nonsterile soil is shown in Fig. 1.

For autecology of rhizobia, two features of FA are particularly pertinent: detection is specific at the strain level (only *R. japonicum* has been studied), and the technique does apply to the normal nonsterile soil environment

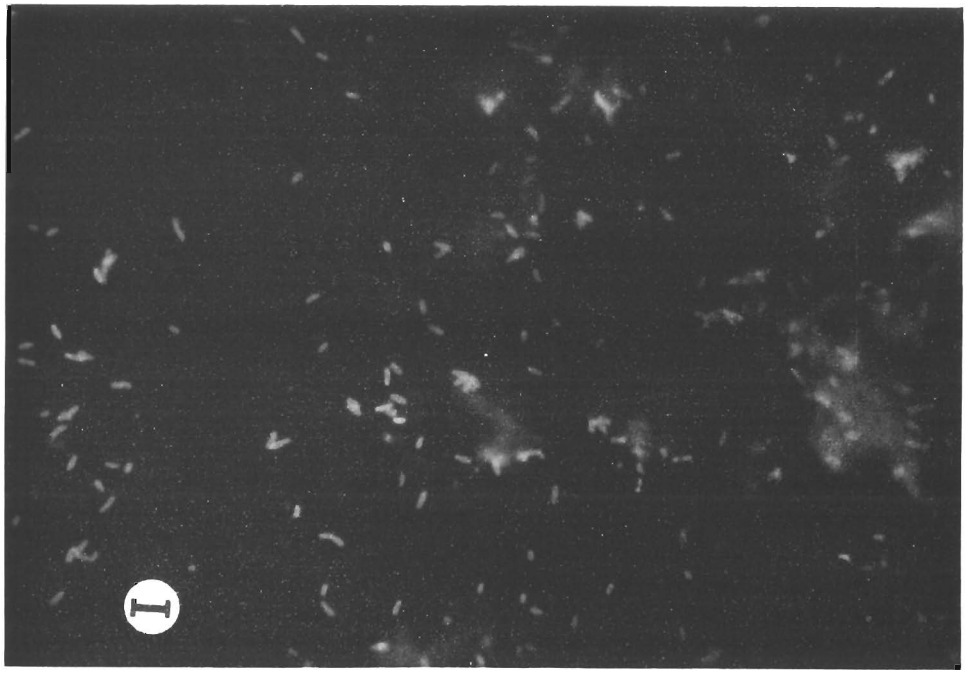


Fig. 1. *R. japonicum* USDA 110, specifically stained by immunofluorescence in the midst of other microorganisms on a contact slide. Slide had been buried in field soil inoculated with *R. japonicum* 110. Cells appear yellow-green against an orange-brown background of soil particles. Scale line indicates five μm. From Schmidt, 1974, p. 143, fig. 2.

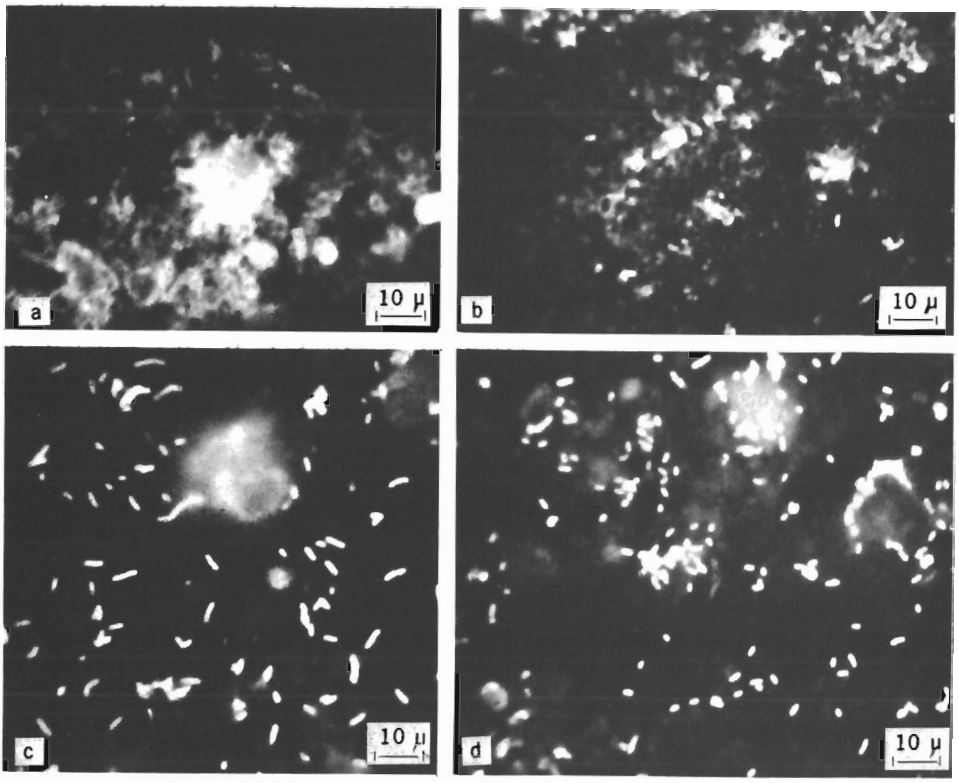


Fig. 2. Representative fields from slides that were in contact with soil and prepared for detection of *Rhizobium japonicum* by immunofluorescence. (a) and (b) — Without prior treatment; soil particles that appear as diffuse white areas adsorbed the fluorescent antibody to *R. japonicum* and fluoresced dull green to yellow-green; bacteria can be distinguished in some areas away from soil particles. (c) and (d) — Prior treatment with gelatin-rhodamine isothiocyanate conjugate; bacteria with specific yellow-green fluorescence were seen clearly, while soil particles (gray areas) fluoresced dull orange-brown. From Bohlool and Schmidt, 1968, p. 1013, fig. 1.

(Schmidt et al., 1968; Bohlool and Schmidt, 1970). A major early problem in the application of immunofluorescence techniques to soil environments was the nonspecific adsorption of labelled antibody to soil particles and films, often obscuring the specific staining reaction. Problems due to nonspecific staining were solved with the development of a gelatin preparation used to treat specimens prior to addition of the fluorescent antibody (Bohlool and Schmidt, 1968). Detection of a strain of *R. japonicum* (61A72) on contact slides following its inoculation into soil is illustrated in Fig. 2, with and without the gelatin-rhodamine preparation to control nonspecific staining. *R. japonicum* cells when present are seen clearly and specifically on buried slides following gelatin treatment and FA staining (Figures 2c and 2d), and

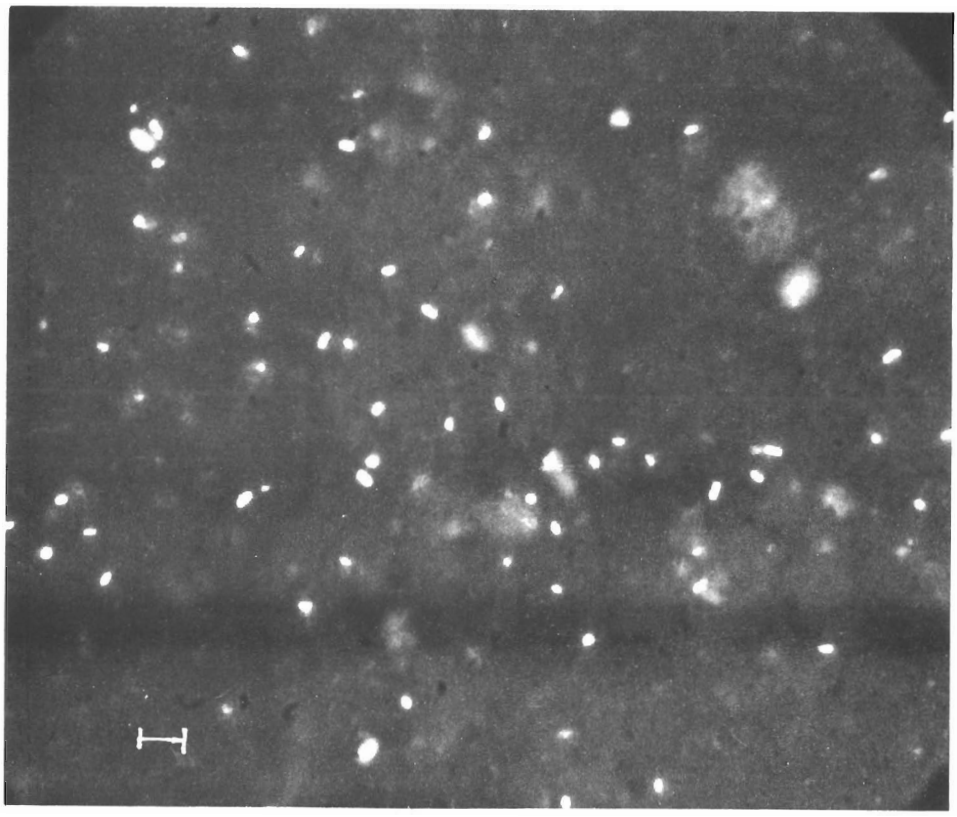


Fig. 3. *R. japonicum* 110 as enumerated in a Clarion loam field soil by the quantitative FA-membrane filter technique. Photomicrograph shows specifically stained cells against the background of the membrane filter surface. Scale line indicates 10 μm. From Schmidt, 1974, p. 146, fig. 3.

localization of the rhizobia on soil particles is evident. Thus, along with specific detection and recognition of a particular rhizobium in soil by immunofluorescence is the capacity to observe some qualitative interrelationships of that strain with its environment.

While restricted to qualitative applications, FA was not adequate to probe any of a host of autecological questions that involve population changes. The main difficulty with quantification by direct microscopy stems from the limited area of the microscope field at the magnification to see bacteria. With a conventional Breed slide count (Pramer and Schmidt, 1967) a density of about 10^6 cells per gram of soil is necessary to encounter but 1 cell per 10 microscope fields. In order to work with natural populations and to observe population changes it is necessary to remove the bacteria from interfering soil and concentrate them for enumeration. Suitable techniques for release

of rhizobia from soil and their concentration on black membrane filters for enumeration of FA stained cells have been outlined (Bohlool and Schmidt, 1973a; Schmidt, 1974). The lower limits for enumeration by quantitative FA are 10^3 to 10^4 depending on the dispersion and flocculation properties of the soil. Fig. 3 is a photomicrograph of a representative microscope field observed during enumeration of *R. japonicum*, strain USDA 110 in nonsterile loam soil. Fluorescent antibody techniques require specialized facilities not yet widely available, but on the other hand stand alone in their potential for direct investigation of rhizobia as soil and rhizosphere bacteria; as such, these techniques should provide insight into some of the intriguing events leading to symbiosis.

Fluorescent antibody techniques are an adaptation of serology, and their application to autecology with *R. japonicum* as a model system made use of an extensive literature on the serology of the rhizobia. That literature has been reviewed recently with respect to taxonomic and ecologic considerations (Graham, 1969b; Vest et al., 1973). Serology has been used in ecological studies primarily to identify different strains of rhizobia as to the survival of inoculant strains, competition among inoculant strains, and the nature of indigenous soil rhizobia. These objectives are usually accomplished by exposing the experimental system to a legume host plant, isolating rhizobia from the nodules that are formed, and studying the occurrence of the added serotype(s) among the nodule isolates. The agglutination technique has been used extensively to identify nodule bacteria, and especially so for those of the *R. japonicum* group in recent years (Purchase et al., 1951; Škrdleta, 1965). Rather than isolate rhizobia prior to serological analysis, Means et al. (1964) used antisera directly on crushed nodule suspensions to type the bacteroids. Agglutination tests carried out in this manner have allowed for large-scale field studies of competition between members of different serogroups.

Agglutination techniques fail to give adequately detailed analysis of certain cross-reacting nodule isolates. Some investigators have turned to immunodiffusion for better identification of such isolates. Dudman (1971) examined 20 isolates from soybean nodules by immunodiffusion after all were determined to belong to *R. japonicum* strain 123, 127, or 129 by the agglutination technique. The 20 proved to comprise 13 unknown cross-reacting strains that were distinguishable by immunodiffusion.

All serological techniques other than immunofluorescence depend on host nodules for the rhizobia that are to be analyzed in ecological experiments. Mention has been made earlier of the selectivity of the host, so that the rhizobia sampled by the plant may be strongly biased by the host species, or the variety of that species. In competition studies in particular, that involve a host plant, the competitive attributes of the strains studied should be assessed only in relation to that particular host plant.

2.4. Mutant markers and phage typing

Remarks just made in relation to the impact of host selectivity on experiments that deal with the ecology of rhizobia apply as well when the experimental strains are identified by methods other than serology. Two such methods have had limited use: identification of mutant strains by virtue of a particular genetic marker (resistence to antibiotics) and use of bacteriophage typing. Both demand intervention by a host plant to recover the rhizobia from the soil.

Mutants resistant to the antibiotic streptomycin were first proposed for ecological studies by Obaton (1971). Streptomycin resistance mutations are readily obtained spontaneously at adequately high levels in a single step and are stable. Rhizobia so marked must first be tested to insure that the mutant has not lost symbiotic effectiveness (some may, Schwinghamer, 1967). After inoculation of soil or seed the mutant may be identified by recovery from nodules plated on appropriate media containing the antibiotic. Schwinghamer and Dudman (1973) found that mutants to the antibiotic spectinomycin were suitable as alternatives to supplements to streptomycin-resistant mutants, or possibly together with streptomycin resistance as doubly marked strains. As pointed out by Schwinghamer and Dudman (1973), the practicability of such markers for ecological studies can only be confirmed by extended field trials. There is little indication that such trials are indeed underway.

Bacteriophages that are specific for *Rhizobium* spp. occur commonly in nodules and in field soils cropped to legumes. It has been of interest to determine if the specificity is adequate to permit the use of bacteriophage sensitivity (phage typing) as a means of distinguishing among closely related strains. Should this be the case phage typing might be used together with or as an alternative to immunodiffusion, immunoelectrophoresis, and antibiotic mutant markers for more precise identification of distinctive strains within a given serogroup. Lytic bacteriophages are isolated by first using chloroform-treated or filter-sterilized suspensions prepared from soil or nodules as inoculum for a dense "lawn" of a pure culture of a *Rhizobium* sp. The presence of the bacteriophage is indicated by plaques, which may then be passed through serial transfer for purification. A collection of bacteriophages is obtained in this manner from a variety of different isolates of a given species of *Rhizobium*. Those phages that prove to be distinctive as to host range possibly may serve as a suite of reagents to characterize bacteria isolated from legume nodules according to infectivity patterns. An example of phage isolation and phage typing procedures is provided by Kowalski et al (1974).

Attempts to evaluate phage typing for the rhizobia have been so limited as to demonstrate only that the possibility exists. The most promising findings were those of Kowalski et al. (1974) for *R. japonicum*. They found that 30

of 51 phage isolates lysed only the strain from which they were isolated initially, indicating the availability of a suite of phages likely to differentiate *R. japonicum* isolates at the serogroup level. Differentiation of strains within a serogroup also appeared promising, since not all isolates of a given serogroup were uniformly susceptible to the phages isolated from some members of the group. Of 20 isolates that fell in serogroup 123 by agglutination, for example, only 4 reacted with phage, suggesting at least that the 4 differed from the unreactive ones, and that further work might result in additional phages that could characterize others in the group. Less promising were the data of Staniewski (1970) who studied 230 strains of *Rhizobium* representing several species and found only 157 reactive to phage; among those that reacted a relatively high proportion proved to be multivalent. Should phage typing eventually prove to be as successful for some rhizobia as it has been for strain identification in *Staphylococcus* and *Salmonella*, applications to ecological studies again will be for those that deal with the symbiosis, since the plant is involved in the selection of the rhizobial strains from the environment.

3. RHIZOBIA AND SOIL PHYSICAL–CHEMICAL FEATURES

The physical parameters of the soil environment have been considered extensively but nearly always in relation either to the performance of the symbiosis, or as inferred from the growth response of inoculant cultures to laboratory manipulations. Variables that influence the processes involved in the functioning of the nodule have little relevance to the impact of soil environmental factors on the free-living rhizobia. Those factors that affect the initiation of nodulation are probably more pertinent to the ecology of the rhizobia, but still represent the interaction of both plant root and microorganism responses. Only by studying the rhizobia directly in the soil can the responses be attributed to effects on the rhizobia, to effects on the plant, or to effects on the early symbiosis. Meanwhile, the response of the symbiont to environmental factors imposed on the pure culture will probably prove to be a rough but unreliable indicator of its response in the soil, but there is as yet no information on this point.

3.1. Temperature

Rhizobia are mesophilic bacteria that function in subarctic, temperate, and tropical regions. Various kinds of rhizobia differ markedly in their tolerance to elevated temperatures (around 40°C), with no correlation between the strains' heat tolerance and the geographic latitude of their origin (Vincent, 1965). Rhizobia isolated from alfalfa evidenced considerable resistance to high temperatures, while those isolated from tropical hosts were quite variable in responses above their optimal growth temperatures (Vincent, 1965). The question of high temperature tolerance on the part of

inoculant strains is particularly important to the introduction of forage legumes such as medic in semi-arid sub-tropical regions, and to the introduction of soybeans for irrigation cultivation in arid regions. Semi-arid soils reach high temperatures and desiccated conditions due to exposure to radiant heating; survival and die-off studies concerned with the behavior of temperature-tolerant strains in such soils would be very worthwhile.

A good illustration of the need to include soil features in temperature response studies comes from the work of Marshall (1964). He observed that *R. meliloti* and *R. trifolii* were sensitive to high temperature in sandy soils, but not to the same temperature in heavier textured soil. The same author (Marshall, 1967) suggested that the surface charge properties of the rhizobia may lead to the formation of a montmorillonite clay envelope around the cell to confer resistance to high temperature and desiccation.

Gibson (1967) reported on the effect of root temperature on infection and nodulation in one of the few attempts to separate nodule initiation events from nodule function. Possible effects due to soil colloids were excluded as the test plants were cultured on agar slants. The maximum temperature at which *R. trifolii* would form nodules on subterranean clover was 33° C and the minimum was about 7° C with most rapid initial nodulation taking place around 30°C. Initiation of nodulation slowed markedly below 22°C, and root hair infections were severely retarded above 33°.

Low temperatures may have adverse effects on infection and leghaemoglobin in early nodules (Gibson, 1967) for *R. trifolii*, but there are indications of low temperature tolerance and low temperature adaptations in other symbiotic systems. *R. japonicum* is comparatively resistant to high temperatures (Marshall, 1964), but considerable variation must occur among strains with respect to both high and low temperature tolerance (Weber and Miller, 1972). The serogroups found by Weber and Miller (1972) to form the majority of the nodules on soybeans at 30°C formed very few nodules at 10 or 15°C, while the serogroups dominant at the low temperatures were poorly represented in nodules developed at 30°C. Adaptation to low temperatures by *R. trifolii* was clearly evident in work by Ek-Jander and Fåhraeus (1971). Isolates taken from sub-arctic regions of northern Scandinavia grew faster and nodulated clover earlier when tested at 10°C than did isolates from southern Scandinavia. When tested at 20°C there was little difference between the two groups.

3.2. Moisture

The importance of water as an environmental factor in the ecology of soil rhizobia has been considered almost solely from the point of view of survival. As is the case for all soil bacteria, rhizobia must be surrounded by a water film in which the solutes are not so concentrated as to pose osmotic

problems for the cell, so that too little water rather than too much is the threat to their survival and function. Extreme drying is accompanied by increased osmotic pressure in the soil solution, and if the desiccation is due to elevated temperatures this factor interacts as well.

Because of the interplay of soil factors and biotic effects during desiccation, studies made on drying relative to the survival of rhizobia in sterile soil or in culture probably have no significance to the ecology of the rhizobia.

Masterson and Sherwood (1974) found that isolates of *R. trifolii* obtained from wet soils were poorly effective on test legumes as compared to those from dry sites. Any influence of high soil moisture on the effectiveness pattern was probably more a function of plant selection than of rhizobia modification, since the isolates from acid soils proved to be effective when tested on clover native to the wet soils (Sherwood and Masterson, 1974). Moisture effects are amenable to laboratory study even though relatively little has been done. Foulds (1971) for example, examined survival of rhizobia during air drying of 3 soils by plant infection techniques. *R. trifolii* proved to be more tolerant of drought than *R. meliloti* and *Rhizobium* of the "Lotus" group. Studies of this type could be extended to the rhizosphere, or at least to the nonlegume rhizosphere, to explore possibly significant protective effects due to plant roots.

An aspect of soil moisture that is deserving of additional attention is the impact of soil moisture content on the movement of rhizobia. Just how rhizobia get from a soil base to a host root site, or if there is any significant mobility at all on the part of the rhizobia in soil is unknown, but important. Soil moisture relative to soil pores would strongly affect any movement, active or passive, on the part of rhizobia. Hamdi (1971) did exploratory work on relationships between soil water tension and movement of *R. trifolii* from a point of inoculation into sterilized soil. With suction applied to soils of varying textures, it was demonstrated that the zones of movement were sharply decreased by increased water tensions, and movement was found to cease when "water-filled pores became discontinuous". The author noted that movement of rhizobia was restricted at soil water tensions that still allowed for the germination of host legume seed.

3.3. Soil pH

There is general agreement that soil pH has a marked effect on the survival of rhizobia. As in the case of all other parameters, a great deal of variability in pH tolerance has been found among the rhizobia. The main significance of soil pH is in the acid range, where according to Vincent (1965) the effects are on the growth and survival of the bacteria rather than on the symbiosis. In reviewing some of the research on survival of rhizobia with respect to soil

acidity, this author concluded that *R. meliloti* is acid sensitive, *R. trifolii* is less so, and *R. japonicum* is acid tolerant.

Different strains within the same *Rhizobium* sp. vary considerably in response to soil pH. Holding and King (1963) correlated the occurrence of ineffective strains of *R. trifolii* to the acidity of the soil. The addition of lime to increase the pH resulted in a higher proportion of effective strains.

Use of the quick serological testing procedure of Means et al. (1964) made it possible to serotype soybean nodules collected from a large number of sites. Damirgi et al. (1967) used the test in Iowa to identify the bacteroids found in nodules of field-grown plants and examined the occurrence of serogroups with respect to soil properties. Serogroups 123, 135, 31, and 3 included nearly all *R. japonicum* bacteroids found. Serogroup 123 dominated in soils of pH 5.5—7.5, while serogroup 135 was dominant in the highly alkaline soils. While other soil factors appeared likely to influence the predominance of a serogroup, pH seemed to be the most important. More detailed work by Ham et al. (1971) confirmed the overall importance of soil pH in the dominance of these same serogroups, but indicated that other soil factors need to be considered.

In the Iowa studies mentioned, numerous serogroups were represented in each of the soils, and although 123 occupied most of the nodules on most of the soils, the proportion of sub-dominants varied considerably from soil to soil. The extent to which soil pH or other soil properties dictated the distribution of nodule serotypes through effects on the indigenous rhizobia as compared to effects on the host plant, cannot be evaluated. It is surely worthwhile to determine the part played by the population dynamics of the rhizobia because it might lead to some control of strain dominance. If different strains within a natural population respond differently to changes in soil pH or related factors and such changes are reflected in nodulation, soil management by liming or fertilization may prove a practical means to foster the more efficient strains.

3.4. Carbon and energy bases in soil

The practice of introducing appropriate rhizobia into soil by way of the inoculated seeds of host legumes has been common since the beginning of the century. With the development of the nodulated crop, large numbers of the newly introduced rhizobia are released into the soil to join the complex indigenous microbiota already there and in equilibrium with the soil environment and each other. It is particularly significant that the rhizobia, alone among bacteria added to soil for a specific agricultural purpose, successfully adapt to the rigorously competitive, energy-limited life of soil bacteria. The transition from luxurious, competition-free existence in the nodule apparently is not successful for all rhizobia, nor necessarily for all

time for those that do effect the transition. Some of the more intriguing and certainly some of the most practically important questions in microbial ecology center on this adaptation of rhizobia to a soil base. The example with respect to *R. japonicum* is illustrative. Soybeans and *R. japonicum* were first introduced to soils of midwestern USA perhaps 50 years ago; both are now extremely widespread in the best soils of the region. When Ham et al. (1971) examined some of these soils they found consistently many different strains of *R. japonicum* representative usually of several serogroups. Among the numerous strains of *R. japonicum*, those comprising serogroup 123 appeared most frequently in field-nodulated plants. If 123 predominated by virtue of superior adaptation to a saprophytic soil existence, which is a distinct possiblity, how has this strain become so successful? Can the elements of success for 123, if once discovered, be applied to the introduction of other strains, other *Rhizobium* sp. or for that matter, other bacteria? Though none of the many possible ingredients of eminent success as a saprophyte have been resolved for even one strain, an obvious essential is an adequate carbon and energy substrate in the soil. What the substrates are and what level of growth the rhizobia attain in competition with all other microbiota is an important mystery.

An indication of the saprophytic carbon and energy substrate for free-living rhizobia could possibly be gained through study of host excretions. A dogma has developed to the effect that legumes stimulate the growth of their homologous rhizobia. This concept will be explored in more detail on p. 287 since rhizosphere studies show poor agreement and some workers claim little or no specific stimulation. The possibility that rhizobia respond specifically to a host legume root exudate was examined in considerable detail by Egeraat (1972). Sterile pea seedlings were grown in liquid culture and concentrates of the root environment were analyzed for ninhydrin-positive compounds. A considerable amount of amino nitrogen was released from the main root during the formation of the first lateral root, and approximately 70% consisted of homoserine. Further experiments showed that homoserine as the sole carbon and nitrogen (and energy) source in a culture medium favored the growth of rhizobia capable of nodulating peas, whereas rhizobia of several other cross-inoculation groups were either inhibited by the homoserine or utilized it only in the presence of mannitol. Other legumes were analyzed similarly for the occurrence of specific compounds but nothing of comparable specificity was noted. Miller and Schmidt (1965) analyzed the free amino acids excreted by the garden bean (*Phaseolus vulgaris*) into sterile soil in a special plant culture unit. Threonine, valine, and phenylalanine were the amino acids excreted in largest amounts, but quantities varied considerably among replicate plants. The possibility that compounds are present in the soil to serve as carbon and energy source available to the rhizobia apart from the rhizosphere has not been accessible to direct study. Mulder and van Veen (1960) reported that treatment of acid

soils with yeast extract or a suspension of effective or ineffective *R. trifolii* promoted nodulation of red clover in most experiments. Stable manure or an extract of it behaved similarly but less consistently. They postulated that the stable manure may have had an affect on the rhizobia by adding some organic or inorganic nutrient, by modifying the pH in soil pockets for the bacteria, or that it may have improved the CO_2 content of the soil. In work with *R. japonicum*, Bezdicek (1972) correlated a prevalence of serogroup 110 with organic matter in one soil, and serogroup 94 with organic nitrogen in another. Although very indirect, some work by Hussien et al. (1974) at least suggests that rhizobia may use phenolic compounds akin to some soil constituents. The work was with a suite of seven *Rhizobium* spp. in pure culture with catechol, protocatechuic acid, p-hydroxybenzoic acid and salicylic acid added in separate experiments to sodium glutamate medium. Under varying conditions, all of which included high substrate concentration, co-metabolism, and lack of competition, nearly all strains degraded at least some of the phenolic compounds. These results are interesting but too remote from the soil situation to permit extrapolation. Rhizobia as soil bacteria share with all other soil bacteria an absolute dependence on the factor that is nearly always limiting in the soil — the supply of available carbon and energy. The nutrition of the rhizobia within the perspective of the soil environment has received far too little attention.

3.5. Nitrogen

The effects of combined nitrogen on the symbiotic nitrogen fixation process have been studied only in relation to host response, and such studies have placed increasing emphasis on nitrate nitrogen. Nitrate and its reduction product nitrite are of particular interest because they inhibit nodule formation at low concentrations not only in legumes but in nonlegume nitrogen fixing symbiosis as well. Responses such as those noted by Darbyshire (1966) are reasonably typical; 10 micrograms of nitrate or nitrite nitrogen per clover plant on agar slant had no effect on the plant but did delay nodulation by *R. trifolii*. Various hypotheses have been suggested to account for a specific inhibitory mechanism for nitrate; these have been summarized by Dixon (1969).

Nitrate inhibition of nodulation has been examined in relation to effects on root hair curling, infection thread formation and infection thread development, rather than in relation to possible effects on the rhizobia. This is reasonable perhaps in view of the difficulties involved in observing the rhizobia prior to nodulation and the lack of a rationale for a nitrate effect on the rhizobia. The possibility of an indirect effect due to the rhizobia is suggested by the work of Tanner and Anderson (1964). They showed that nitrite will oxidize indoleacetic acid, an auxin thought by many to be essential to invasion of root hairs by rhizobia. Since rhizobia can reduce

nitrate to nitrite they could conceivably mediate the nitrate inhibition by forming the nitrite that inactivates the auxin. Other bacteria in the rhizosphere presumably would reduce nitrate to nitrite just as effectively as the rhizobia so it is difficult to anticipate a specific role for them in the indoleacetic acid destruction hypothesis. Raggio et al. (1965) examined the effects of nitrate with respect to the hypothesis that the carbohydrate—nitrogen ratio of the plant is concerned with nitrate inhibition. They found no evidence for the hypothesis and suggested instead that it may be the rhizobia that are affected by nitrate. The possible nature of effects on the rhizobia that would render them incompetent or slow to nodulate are obscure.

3.6. Soil management

Nitrogen fertilization is not a normal management practice in legume culture, but phosphate fertilization and liming can be a prerequisite for successful nodulation (Vincent, 1965). Other management practices that might be expected to influence the soil rhizobia include the use of pesticides and trace elements. Little emphasis has been given to the impact of these practices on the soil as a habitat for rhizobia, but rather to the effects on inoculants. Some fungicides (Hofer, 1958) and insecticides (Braithwaite et al., 1958) were shown to be detrimental to rhizobia. Undesirable effects of specific pesticidal chemicals on soil rhizobia are still poorly documented. However, when added to soil at rates that do not have adverse effects on the host, pesticides probably have little effect on the rhizobia (Kapusta and Rouwenhorst, 1973; Kulkarni et al., 1974). Among the minor elements sometimes used for soil application, cobalt is of special interest from the standpoint of the rhizobia. Lowe and Evans (1962) showed clearly a requirement for cobalt in the rhizobia in connection with the synthesis of vitamin B_{12}. In view of the extremely small requirement of the cell it is difficult to envisage cobalt as a limiting factor in the microenvironment of soil rhizobia.

4. RHIZOBIA AND SOIL BIOLOGICAL FEATURES

A very wide range of biological interactions both in terms of mechanisms and in terms of biotic participants has been postulated in the literature as of potential importance to rhizobia in the soil or in the rhizosphere. Because of the limitations imposed by techniques available to deal with the vast complexities of the soil, little work relates directly to the natural environment and few specific interactions have been resolved. Interest in soil rhizobia as participants in biological interactions has stemmed largely from practical problems such as nodulation failures in some soils and widely reported difficulties in the introduction of favored strains into an

established population of indigenous rhizobia. In view of this it is not surprising that the emphasis has been on inhibitory and antagonistic effects.

4.1. Microbial interactions

Microbial antagonism by fungi and bacteria has been demonstrated repeatedly against the rhizobia in laboratory culture or in sterile soil. Experiments of this type have established only that a great many pure culture isolates taken from soil or rhizosphere and grown on rich culture media can produce antagonistic (frequently antibiotic) effects against rhizobia also grown on rich media. Extrapolation of these data to the complex, competitive, low-nutrient soil situation is so tenuous as to be without value, and few investigations have gone beyond mere conjecture.

Holland and Parker (1966) reported an extensive study dealing with inhibition of *Rhizobium*. They tested 286 bacteria and fungi for antagonistic effects on *R. trifolii*. *Phoma* and *Aspergillus* were inhibitory while *Penicillium* and 2 phycomycetes were bactericidal in laboratory assays. All but 2 of the penicillia were isolated from areas where nodulation failures were common. The isolates were grown aseptically in tubes of sterilized sand together with inoculated subterranean clover. Of the 31 cultures that inhibited nodulation in sand culture, 29 proved to be species or strains of *Penicillium*. Even in these artificial conditions of a non-competitive environment the penicillia interfered with nodule formation only when sucrose and straw were present, and these at relatively high concentrations. Holland and Parker nevertheless considered it likely that *Penicillium* was responsible for the failure of nodulation in some areas. Interactions that may occur in soil between rhizobia and common soil fungi such as *Penicillium* are further complicated by the need to demonstrate active fungal development. Sporulating fungi appear readily on dilution plates and are easily isolated, but the dilution plate colony may represent merely a spore that was quiescent in the soil and hence incapable of significant antagonistic influence. It is necessary to demonstrate that the laboratory antagonist grows in the problem soil at the time the rhizobia are inhibited.

In the work cited above, Holland and Parker made the interesting observation that aqueous extracts from the soils with nodulation problems were inhibitory to *R. trifolii* on seeded agar plates on two occasions. This was taken to indicate the presence of "antibiotics", but unfortunately, the activity of the extracts was lost before the antibiotic nature of the antagonism was demonstrated. Antibiotics are generally considered to be specific metabolites of a microorganism that selectively inhibit a spectrum of certain other microorganisms, and as such are but one of numerous expressions of antagonism. The difficulties involved in demonstrating the occurrence of antibiotics in normal soil are vast in any case; those instances of success have been for bacteria other than rhizobia, and almost always

were dependent on the addition of fresh organic amendments at high rates.

A microbial interaction of a novel nature is suggested by the work of Parker and Grove (1970). The predatory bacterium *Bdellovibrio bacteriovorus* was found in 6 of 10 soils in the vicinity of Perth, Western Australia. This investigation constituted the first report of *Bdellovibrio* parasitizing certain rhizobia; those lysed actively were *R. meliloti* and *R. trifolii*, while *R. lupini* was not lysed by any strain of *Bdellovibrio*. Two species usually considered as close relatives of the rhizobia, *Agrobacterium tumefaciens* and *A. radiobacter*, also were lysed. The authors felt that the significance of *B. bacteriovorus* as a factor in the ecology of *Rhizobium* remained to be determined.

4.2. Bacteriophage and bacteriocins

Bacteriophages specific for rhizobia apparently are of common occurrence in soils and in nodules. Kowalski et al. (1974) for example, found bacteriophages lytic to several host strains of *R. japonicum* in nearly all samples of soil and nodules examined. The possibility that attack by bacteriophage could have deleterious effects on the survival of rhizobia was first mentioned more than 40 years ago. Work in the interim has called attention to the possibility of more subtle effects such as the natural selection of rhizobial strains resistant to phage but less effective in nitrogen fixation, or that mutation to phage resistance might be accompanied by loss of nitrogen fixing ability. In his review of the literature, Vincent (1965) concluded that despite a relatively voluminous literature, the practical significance of bacteriophage as a factor militating against the survival and functioning of rhizobia in the soil has yet to be unequivocally demonstrated. Interesting possibilities for transfers of genetic information in the form of plasmids to rhizobia by means of a bacteriophage carrier are probably offered by modern capabilities in microbial genetics. If strains of rhizobia are specially tailored as to certain desirable genetic features it becomes critically important to somehow trace them in the soil and rhizosphere to evaluate their practical potential.

Bacteriocins are a diverse group of substances, composed largely of proteins, which are produced by many bacteria and which act to kill certain other bacteria on a highly selective basis. They are strain specific and are thus distinguished from antibiotics which have a wider spectrum of activity. The ability to produce bacteriocins is often inherited via plasmids. Roslycky (1967) was the first to observe bacteriocins in rhizobia, and noted their occurrence in several cross-inoculation groups. Bacteriocins of *R. lupini* were characterized by Lotz and Mayer (1972) as defective bacteriophage particles since they resembled the tails of certain *E. coli* phages, but had no nucleic acid. An inducible bacteriocin of *R. trifolii* was studied in detail by Schwinghamer et al. (1973) as to its molecular properties. They concluded

that this particle, designated bacteriocin 37, was a very defective temperate phage, morphologically intact but retaining only the bactericidal function of a phage. Schwinghamer et al. (1973) were the only workers to conjecture on the significance of bacteriocins as an ecological factor. They stated that from the standpoint of competition between strains of rhizobia in field environments, the known bactericidal function of the bacteriocin could be significant in providing the bacteriocinogenic strain with some competitive advantage over other rhizobia. Further work with the bacteriocin system may serve to renew interest in the general question of bacteriophage as a factor in the ecology of rhizobia.

4.3. Persistence of rhizobia in soils

The persistence of strains of *Rhizobium* following their introduction into soil has long been recognized as an important practical problem in microbial ecology. As is so generally the case in study of the free-living rhizobia of the soil, the methodological approaches are sharply limiting. Where rhizobia-free soils are available, the investigator can work with one defined strain and an appropriate host legume for its detection. If the soil has an indigenous population of rhizobia or if it is necessary to use a mixed inoculum it is essential to differentiate the strain to be enumerated from other strains that may appear in the nodule. It has not been possible to investigate the persistence of a given strain relative to one or more competing strains in the same environment because of the limitations imposed by the selectivity of the plant infection technique of enumeration.

Dudman and Brockwell (1968) used immunodiffusion techniques to examine the persistence in the field of two strains of *R. trifolii* applied as commercial peat inoculants to clover seed. They looked for the strains at intervals of 3 to 42 months. One strain dominated the other but both diminished greatly with time. At one site, one inoculum strain was recovered after 30 months, but at another it had disappeared by 18 months. The presence of a natural population had little to do with the ability of the inoculant strain to establish in soil. The survival of field-grown rhizobia in certain problem soils of Western Australia was studied by Chatel and Parker (1973a). Their findings suggested that colonization during the growing period was critical in order to build up sufficient numbers to withstand a long hot dry summer. In further experiments designed to examine colonization during crop development, Chatel and Parker (1973b) observed marked differences between *R. lupini* and *R. trifolii*. These differences carried over in the survival pattern evidenced in the second year. As may have been anticipated, *R. trifolii* had no difficulties in maintaining high populations in permanent clover pastures that had been established for 6 years.

The site of colonization in the soil is of central importance to the

persistence of rhizobia, and so long as host legume cultivation is continuous, that colonization site is provided in and around the host roots. The interesting question of the dependence of rhizobia on particular micro-environments in the soil mass was examined by Chatel and Greenwood (1973) who looked at soil cores under senesced legume pastures. They divided soil cores into soil, host plant roots, nodules, and extraneous material fractions for estimates of populations of *R. trifolii* and *R. lupini*. The former was found to be predominately associated with the nodule fraction of soil cores from subterranean clover sites, while the latter was distributed more uniformly in cores from beneath senesced stands of serradella. Unfortunately, the subsequent persistence of these rhizobia relative to the respective fractions was not included in the study. Because commercial inoculant strains did not colonize soil and persist there, Chatel et al. (1973) included fresh field isolates with inoculant strains to evaluate persistence. The recent isolates originally were selected for superiority in efficiency as well as persistence, and in particular they showed much improved persistence over the older inoculant strains in this study.

The only work on the persistence of rhizobia in soil that did not make use of the plant infection technique is that of Bohlool and Schmidt (1973b). They used the fluorescent antibody technique for a time course study of the persistence of *R. japonicum* strain 110 introduced into normal soil by burying glass microscope slides with the rhizobia attached. In each of the 4 soils studied, live cells persisted for the duration of the experiment (2 or 4 months) even though numbers were reduced drastically as a function of time. Although preliminary, this study demonstrated the applicability of the fluorescent antibody technique to direct microscopic evaluation of the persistence of a particular strain of rhizobium in soil amid other rhizobia and other microorganisms. Also evident was some adjustment on the part of a small portion of the introduced rhizobial population to soil devoid of a host legume. The above study was only semi-quantitative, as based on comparative counts of buried slides, since methods for quantitative fluorescent antibody examination of soil were still under development. The first report on the fluorescent antibody method for enumeration of soil bacteria was illustrated by data on *R. japonicum* 110 (Bohlool and Schmidt, 1973a), and a subsequent one (Schmidt, 1974), included short term persistence of the same organism in two soils. Strain 110 approximately doubled as its initial response to inoculation in each of the 2 soils examined. But the generation time was extremely slow: 241 hours in the Clarion soil and 361 hours in the Ulen soil as compared to 14 hours in autoclaved, sterile Clarion soil. Die-off followed the initial doubling and proceeded at a faster rate and to a much lower level after 80 days in the nonsterile Ulen soil as compared to the nonsterile Clarion soil.

The literature reflects an increasingly strong awareness of the importance of soil colonization and persistence capabilities as essential attributes of

desirable rhizobia. These parameters are at the heart of ecological studies of the rhizobia in the soil habitat.

5. RHIZOBIA IN THE RHIZOSPHERE

Interactions between plants and soil microorganisms exhibit increased intensity close to the plant root in a zone called the rhizosphere. The rhizosphere is ill-defined as to its boundaries, but always reflects the dominant influence of the root. The basis for the influence is universally accepted to reside in the exudates and cellular debris contributed by the plant root; its expression is found in the heightened activity by a quantitatively greater and a qualitatively different population of microorganisms as compared to soil outside the rhizosphere zone. This heightened activity that defines the rhizosphere zone is known as the "rhizosphere effect".

The legume rhizosphere is nearly always agreed to be particularly rich in the amount and diversity of substrate materials contributed by the root. As Rovira and Stern (1961) have pointed out however, the rhizosphere effect varies greatly according to the manner in which data are expressed. Legumes exhibit an impressive rhizosphere effect when the microbial population is expressed on the basis of weight of rhizosphere soil, but are greatly outstripped by grasses when the rhizosphere count is expressed on the basis of root surface. The rhizosphere effect is nonetheless real in the legume, and the rhizosphere zone is overwhelmingly important to the legume root nodule bacteria and to all practical considerations of the legume—rhizobia symbiosis. Factors that influence rhizobia in the soil are present and likely greatly intensified in the rhizosphere. Those additional factors that are brought into play in the rhizosphere by the normal legume root unquestionably exert influences that are central to an understanding of the ecology of the rhizobia and to the initiation of the symbiosis. It is both trite and inadequate to state that the behavior of rhizobia in the legume rhizosphere is poorly understood—the subject is virtually untouched.

5.1. Occurrence of rhizobia in the root zone

Once an indigenous population of rhizobia establishes in a soil, it can be induced from practical legume culture that many of these rhizobia make the transition from soil bacteria to legume rhizosphere bacteria in the course of becoming legume nodule bacteria. That the transition to a rhizosphere is not made by all soil bacteria is evidenced by the wealth of data that document the qualitative differences between the population of the rhizosphere and that of the soil at large just outside the rhizosphere (Katznelson, 1965). Just what is involved in the microbe/microbe and the microbe/plant interactions that are set in motion when the legume seed germinates and rhizosphere

development begins is unknown, but clearly the rhizobia find suitable substrates and compete effectively for them. The possibility that establishment in the vicinity of the germinating seed may be delayed by inhibitory diffusates from the seed coat is suggested by several studies that were cited by Vincent (1965). Seed coat effects vary with the legume and their significance and duration under other than highly artificial conditions have not been established.

Development of the legume rhizosphere apparently provides an environment wherein rhizobia not only adapt but thrive. This is evident from experiments in which rhizosphere soil was compared with nonrhizosphere soil nearby as to numbers of rhizobia capable of nodulating a test legume. Tuzimura and Watanabe (1961b) found rhizobia that nodulate *Astragalus* present in the rhizosphere of that legume at about 10^3—10^4 per gram of soil, but adjacent nonrhizosphere soil in the same pot had less than 10^1 per gram of soil. Rovira (1961) counted *R. trifolii* using rhizosphere and nonrhizosphere soil dilutions and red clover as the assay legume. Rhizobia in soil unaffected by plant roots numbered about 2×10^5 per gram, whereas numbers in soil adhering to the clover roots increased 8 to 200-fold. Further work by Tuzimura and Watanabe (1962) demonstrated that *R. trifolii* increased 10 to 100-fold in the rhizosphere of legumes, and Nutman (1963) cited unpublished data on trefoil and beans that described substantial rhizosphere effects for *R. meliloti* and *R. leguminosarum*. These data and earlier Russian studies cited by Krasil'nikov (1958) attest to the ability of rhizobia to become established in the rhizosphere of legumes.

Data cited above have been interpreted as "specific stimulation" by a number of authors (Nutman, 1962, 1965; Brown et al., 1968) with the implication that rhizobia multiply extensively in the legume rhizosphere as one of the events preliminary to symbiosis. Moreover, the host legume rhizosphere is thought somehow to favor selectively the particular kind of *Rhizobium* that can enter into the symbiosis. The hypothesis is an appealing one, but there are no data in support of it. A rhizosphere effect for the rhizobia appears to be real even though the question has not been addressed extensively. A rhizosphere effect, however, merely indicates that the rhizobia like many, but by no means all, of the other soil bacteria find the rhizosphere a more favorable environment than that of the soil at large. Only three investigations (Rovira, 1961; Tuzimura and Watanabe, 1961b, 1962) have followed rhizobia counts in the rhizosphere in relation to total bacteria counts; in these it was found that the rhizosphere effect for rhizobia was proportionally greater than for that of total aerobic bacteria. In this sense there is evidence of "selective stimulation" in that rhizobia appear to be better rhizosphere bacteria than the average soil bacterium.

The concept of specific stimulation of a particular *Rhizobium* by the rhizosphere of its host legume seems to have been deduced from rhizosphere effects. Nutman (1962) for example, cited studies of Krasil'nikov that

indicated a greater density of *R. trifolii* in the rhizosphere of clover than in the rhizospheres of other legumes, and similar studies by others, and then concluded that there was a large and specific stimulation, and that "a given legume tends to promote the multiplication of bacteria able to infect it more than others". The critical experiments designed to compare populations of rhizobia of different inoculation groups in the same legume rhizosphere, or to compare different strains of the same *Rhizobium* in the rhizosphere of a compatible host have not been carried out. That this is necessary to establish the existence of a specific stimulatory effect is indicated by two aspects of the data at hand from the few rhizosphere studies that have been carried out in natural soil. On the one hand legumes other than the host legume provide rhizospheres conducive to the growth of a given *Rhizobium*. This is seen in the experiments of Tuzimura and Watanabe (1962) wherein *R. trifolii* was only slightly enhanced in the rhizosphere of its host ladino clover, but exhibited a large rhizosphere effect with alfalfa and soybean, neither of which can be nodulated by *R. trifolii*. On the other hand there is consistent evidence that rhizobia display definite rhizosphere effects with a wide range of nonlegume plants. Rovira (1961) demonstrated enhanced development of *R. trifolii* in the rhizosphere of the grass *Paspalum*. Tuzimura and Watanabe (1962) reported marked rhizosphere effects for *R. trifolii* with rape and tomato and smaller effects for rice, wheat, and Sudan grass. Both *R. trifolii* and *R. meliloti* established in the rhizospheres of a native Australian grass (Robinson, 1967). Rhizobia do so well in the rhizosphere of certain nonlegumes in fact that Rovira (1961) suggested that nonlegumes could be a factor favoring the survival of rhizobia in the absence of host plants, and Diatloff (1969) investigated the possibility of introducing *R. japonicum* into certain soils by inoculating seed of cereals as well as non-host legumes. The latter approach was successful in the greenhouse, but much less so in the field trials. The question of specific stimulation of a given *Rhizobium* in its host's rhizosphere is one of the more important ones to be answered in studies of the rhizosphere. As of now, the question resolves itself as merely a hypothesis that is unsupported by any direct experimental evidence.

Egeraat (1972) is among those who have accepted the "specific stimulation" hypothesis. This investigator studied the root zone of sterile pea plants in solution culture in attempts to identify root exudates that influence the pea symbiont *R. leguminosarum*. Egeraat conducted detailed analyses of ninhydrin-positive compounds excreted by pea plants under the above conditions, to find that homoserine was quantitatively most important. In testing growth responses to homoserine in pure culture it was found that *R. leguminosarum* grew very well with that amino acid as the only carbon and nitrogen source, whereas strains of *R. trifolii* and *R. phaseoli* behaved quite differently in that homoserine apparently was toxic (Fig. 4). The work thus suggests an appealing mechanism whereby appropriate pea rhizobia are favored while at the same time rhizobia of other inoculation groups are

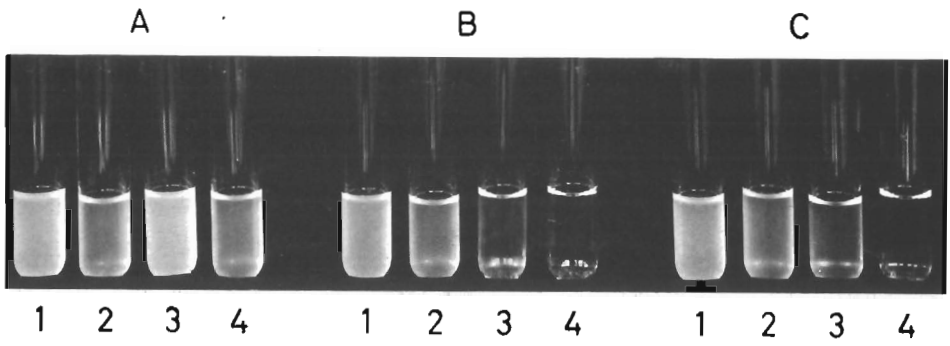


Fig. 4. The influence of homoserine on growth of rhizobia belonging to different cross-inoculation groups. A. *Rhizobium leguminosarum*, strain PF_2; B. *Rhizobium phaseoli*, strain Zijlma; C. *Rhizobium trifolii*, strain CLF.
1. Basal medium (B) supplemented with glutamic acid and mannitol; 2. B + glutamic acid; 3. B + homoserine + mannitol; 4. B + homoserine.
From Egeraat, 1972, p. 73, fig. 8.2.

repressed. Conditions of experimentation were necessarily highly artifical in order to identify components of the exudate. Should the pea follow a similar pattern of exudation in non-sterile culture in a particulate root environment, and should rhizobia respond similarly to homoserine even in the midst of a host of other rhizosphere bacteria, the lead of Egeraat will prove to be a break-through of the first magnitude. The steps from culture tube to normal rhizosphere are, however, giant steps.

5.2. Mobility of rhizobia in the rhizosphere

The manner in which given rhizobia make contact with the appropriate part of an appropriate legume root system to commence a symbiosis is still conjectural. The only mechanism given serious consideration is that just discussed — the possibility of enhanced growth in the rhizosphere and consequent multiple contact with a growing root system. Growth is perhaps the most reasonable explanation to account for data such as provided by Rovira (1961) where clover rhizobia were found 10—20 mm from the clover root, but much larger numbers occurred in soil adhering to the roots. Growth rates of microorganisms in natural environments are virtually unknown and rhizobia in the rhizosphere present no exception. Distances covered by rhizobia by growth or whatever mechanism had not been a subject of study for about 50 years when Hamdi (1971) noted effects of moisture tension on rhizobial migration in sterilized soil (page 280). Along with the needed studies of movement of rhizobia in the rhizosphere from a point inoculum as a function of growth rates, consideration should also be

given to means alternative to growth. Chemotaxis is one such biological mechanism that could conceivably account for the traverse of a specific bacterium from outer rhizosphere to a specific site on the host root, and might account as well for the selectivity of the host in choosing one strain among many. Another possibility, but probably a remote one, is that of transport by a soil animal or by colonization of fungus hyphae as they may extend in the rhizosphere.

Interest in the occurrence of highly motile, dwarf forms of rhizobia referred to in early literature as "swarmers" was rekindled with the electron microscope study of Dart and Mercer (1964). Barrel medic seedlings were grown in sterile solution culture and inoculated with *R. meliloti*. Under these highly artificial "rhizosphere" conditions the authors obtained beautiful electron micrographs some of which showed "swarmer" cells among the microfibrils of the root hairs. These cells were coccoid and multi-flagellate, some as small as 0.1 μm in diameter. The possibility that such cells were the infective stage of the *Rhizobium*, and capable of reaching nodulation sites by virtue of extreme mobility was suggested; this is an intriguing possibility that unfortunately has not been pursued.

5.3. Rhizobium/host root specific recognition

Rhizobia, including those that ultimately successfully nodulate, probably are always outnumbered by other legume rhizosphere bacteria given even the relatively high densities indicated by some of the studies previously mentioned. Just how the recognition event takes place between a host legume root and one specific kind of *Rhizobium* amid all the activity of the rhizosphere is a question of immense interest. An answer to this question for example, could be a key to the realization of the old but honorable dream of extending the nitrogen fixing symbiosis to nonlegumes of agricultural interest. Extensive study of root hair infection has led Nutman (1962) to conclude that infection occurs at specific sites on the root system. A recognition mechanism of some sort must be called into play at the root hair infection site or rather soon after infection thread formation begins.

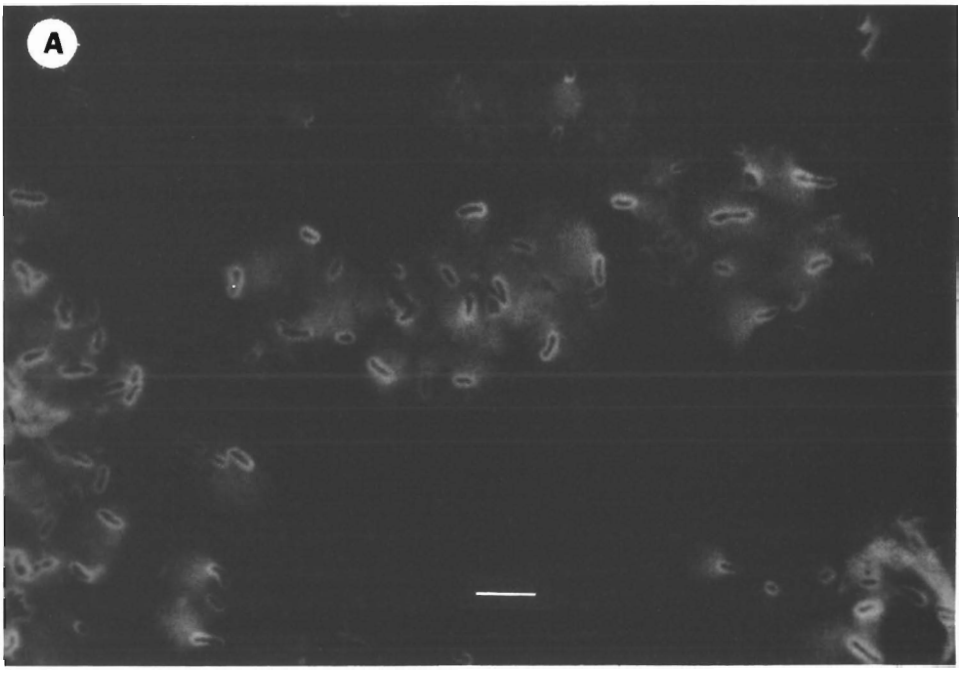
Few hypotheses have been advanced to account for the specificity of the *Rhizobium*—legume association and only one has generated data at the biochemical and physiological level. Starting in 1959, Ljunggren and Fåhraeus (see Fåhraeus and Ljunggren, 1968, for summary) presented evidence that the specificity is governed by the ability of the bacterium to induce the enzyme polygalacturonase in roots of compatible hosts. This enzyme was suggested to cause softening of the cell walls of root hairs preliminary to invasion by the *Rhizobium*. In the decade that followed some scattered support appeared at first, followed by a number of papers with strong evidence countering the theory (Solheim and Raa, 1971, and references cited). The hypothesis seems not to hold, nor did it account for

the specificities expressed at the strain level to explain the manner in which a particular strain induces the mobilization of a relatively nonspecific enzyme.

With a large number and perhaps numerous kinds of rhizobia in a legume rhizosphere it would seem more likely that recognition of an appropriate *Rhizobium* partner would occur at the infection site and not after an infection thread had started. Should this be the case, the appropriate *Rhizobium* must carry some recognition factor as a rhizosphere bacterium that differentiates it from other rhizobia and from other bacteria to make it recognizable to the plant. Hubbell (1970) looked for such a marker in the form of a strain-specific "curling factor" that could be responsible for the curling and deformation of root hairs that characteristically precede infection. He obtained a crude extracellular polysaccharide preparation from a *Rhizobium* sp. that mimicked the root hair curling involved in infection in strawberry clover. Further developments on the nature and function of specific components of a *Rhizobium* cell relative to effects on legume root hairs should be of considerable interest.

A new hypothesis that legume—rhizobia specificity is mediated by lectins produced by the legume has been advanced (Hamblin and Kent, 1973; Bohlool and Schmidt, 1974). Lectins, sometimes referred to as phytohaemagglutinins, are cell agglutinating, sugar-specific glycoproteins found widely in legume seeds. The hypothesis is that lectins present a site on the legume root surface which interacts specifically with a distinctive polysaccharide on the surface of the appropriate *Rhizobium* cell as a prelude to nodulation. Hamblin and Kent (1973) worked on a single strain of *R. phaseoli* and found that lectin derived from the bean *Phaseolus vulgaris* bound to bacteria of that strain. Evidence for lectin binding was that the lectin-treated bacteria were capable of agglutinating red blood cells — a characteristic property of lectins. Control cells of *R. phaseoli* had no haemagglutinating activity. Whereas these experiments did not address the question of specificity, those of Bohlool and Schmidt (1974) sought to examine this point. They devised techniques whereby reactions between soybean lectin and *R. japonicum* could be observed by direct microscopy. It was found that soybean lectin labelled with fluorescein isothiocyanate as a marker combined specifically with all but three of 25 strains of *R. japonicum*. The lectin did not bind to any of 23 other strains representative of rhizobia that do not nodulate soybeans. The microscopic appearance of two strains of *R. japonicum* after reaction with the fluorescent labelled soybean lectin is shown by fluorescence microscopy in Fig. 5.

Fig. 5. Microscopic appearance of the specific reaction between *R. japonicum* cells and soybean lectin labelled with fluorescein isothiocyanate. (A) *R. japonicum* strain USDA 110. Note the heavy concentrations of diffuse lectin bound to nearly every cell. (B) *R. japonicum* strain USDA 138. Lectin was bound by most cells, but in a compact, well-defined zone. Cells which bound no lectin can be seen in the field. From Bohlool and Schmidt, 1974, p. 269, fig. 1.


A


B

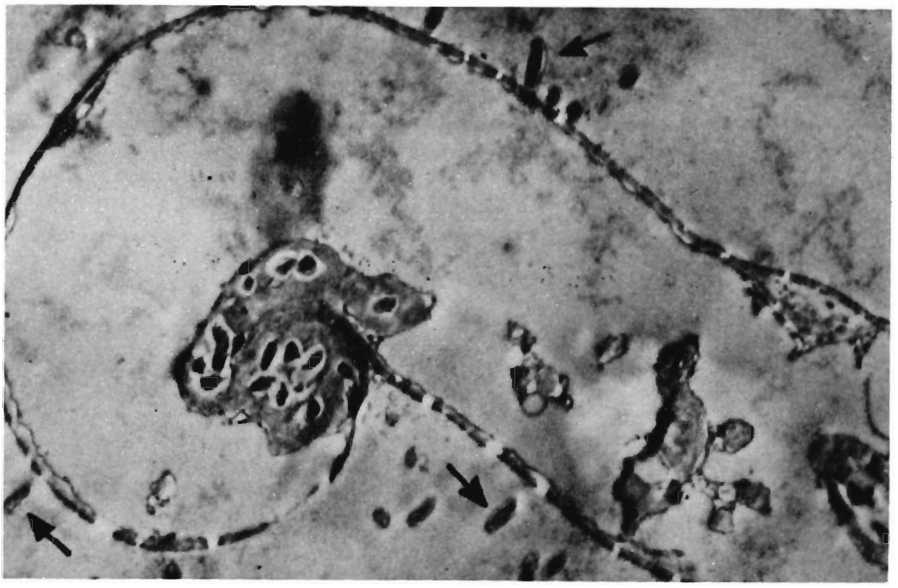


Fig. 6. Thin section of infected root hair of *Trifolium parviflorum* seen on electron micrograph at x7000. *Rhizobium* cells are attached to the outside of the root hair with an endwise orientation. From Sahlman and Fåhraeus, 1963, facing p. 427, plate 1, fig. 1.

Lectins have been known for many years by virtue of an ability to react with erythrocytes and cause their agglutination, but the role of lectins in nature has been a mystery. Haemagglutination activity, as well as certain other biological properties of the lectins, perhaps has nothing to do with their function in nature; still, two properties fit well with a hypothetical role for lectins as the determinant of specificity in the legume—rhizobia interaction. One property is that of binding specifically to saccharides on the surface of cells; this has provided a new tool for the investigation of the architecture of cell surfaces (Sharon and Lis, 1972) and makes it entirely reasonable to conceive of legume lectins combining specifically with small saccharide-binding sites on the surface of appropriate *Rhizobium* cells. The second property, that of the mitogenic action of lectins, is less obvious but worthy of consideration nonetheless. If lectins are localized at actively growing sites of root extension — the nodulation sites — they may function there as mitogens to increase the rate of cell division; the same lectins, if bound to the rhizobia during infection could conceivably be carried to the cortical cells and there be a stimulus to the cell division that initiates nodule primordia.

The hypothesis that legume lectins mediate the specificity of the *Rhizobium*—legume interaction is most attractive although the evidence in its support is still highly preliminary. It may be anticipated that considerable

physiological and biochemical work will be carried out on the place of lectins in the relationship. Ecological studies bearing on the lectin hypothesis will be difficult unless the complexities of the natural environment are avoided as has been done so frequently in "rhizosphere" studies. The availability of fluorescent antibodies for the detection of specific rhizobia in the natural soil environment provides the expectation that localization and perhaps even orientation of rhizobia at hypothetical lectin-binding sites on roots may be visualized. That such localization and orientation may exist under artificial culture conditions is suggested by the low magnification electron micrographs of Sahlman and Fåhraeus (1963) shown here as Fig. 6. Cells of *R. trifolii* are seen outside the longitudinal section of a root hair of clover, attached with one end to the mucilaginous cover of the root hair wall.

5.4. Competition

The same factors that enter into the capacity of a *Rhizobium* to establish in a soil in competition with the biota already present come into play more intensively in the rhizosphere, and are augmented there by additional factors. Away from the root in the nonrhizosphere soil, competition is likely to resolve around the ability of a *Rhizobium* to utilize a given substrate in rivalry with other bacteria. In the rhizosphere, competition for substrate seems to go at least somewhat in favor of the rhizobia, for as noted earlier, they frequently are reported to establish well in the rhizospheres of many legume and nonlegume plants. Apart from competition with other organisms in the rhizosphere, rhizobia must compete with other rhizobia not only for available substrate but for nodulation sites as well. This latter consideration has dominated studies of competition because of the practical importance of evaluating a good inoculant strain in terms of its ability to form nodules in competition with nodulating strains already established in the soil. In the competition for nodulation sites, the legume plant plays a selective role that is not understood but which needs to be given much greater recognition as a factor in competition studies.

A strain that is to be studied in a normal rhizosphere with soil, other microorganisms, and usually other rhizobia must be identifiable. Identification has usually been by immunological techniques, but the work of Means et al. (1961) is an interesting departure. They used a *R. japonicum* strain marked by its ability to induce a chlorosis in certain soybean cultivars and found that it was highly competitive in mixtures with other strains. Mixtures containing as little as 1% of the chlorosis-inducing strain resulted in that strain forming 85% of the nodules. Host selectivity may have been a factor since chlorosis was readily induced in the Hawkeye and Lee varieties used as test plants.

A good deal of the work on strain competition in the past 15 years has centered on *R. japonicum*. A thorough review by Vest et al. (1973) of the

soybean rhizobia work points to some consistent patterns with respect to the behavior of inoculant strains. In fields with a small population of naturally occurring strains to compete for infection sites, and sometimes with a population of largely ineffective strains, the more desirable inoculant strain could be deemed competitive in that it appeared in a high proportion of the nodules. Introduction of new inoculant strains in the face of a competent naturalized population results in a surprisingly low percentage of nodules formed from the applied inocula. The situation is improved somewhat by early inoculation, and by inoculum populations that are very high relative to the indigenous population, but the gains are frequently minimal. Weaver and Frederick (1974) again proved this point by using a very heavy inoculum to obtain modest increases of 10—50% in nodules formed by inoculum when the native population they sought to replace was 100,000 or more per gram.

The extent to which an inoculant strain can compete with resident rhizobia undoubtedly is a function of soil conditions, the naturalized competitors, the legume, and the soil colonizing ability of the introduced strain. Bohlool and Schmidt (1973b) plotted the percent nodules due to an inoculant strain versus the log of the numbers of the inoculant over a range of inoculant densities. A sigmoidal curve resulted which reflected little gain in inoculant-derived nodules until the resident soybean rhizobia were apparently overwhelmed; then subsequent small increases in inoculant population led to large increases in inoculant-derived nodules. Such a "competition curve" was proposed as a possibly useful means to evaluate the complex of interacting factors for a particular soil and legume combination.

The competitive ability of a strain of *Rhizobium* has been evaluated only in terms of a contest for nodulation sites, since the nodule was a prerequisite to strain recognition. In order to evaluate the selective barrier effect of the host and to gain an understanding of rhizosphere factors that influence competition it is necessary to augment the nodulation site approach with more direct study of the behavior of a strain in the rhizosphere. A possible further complication in the dependence on nodules for strain identification was posed with the recent evidence by Lindemann et al. (1974), that the dogma of one nodule — one strain may not hold. They reported the unequivocal occurrence of two competing strains of *R. japonicum* in the same nodule in 12 to 32% of the nodules analyzed.

6. ANALYSIS AND PROJECTION

Vest et al. (1973) speaking of soybeans stated that the overriding problem now and in the future is establishing a desired *R. japonicum* genotype in the nodules of the variety one chooses to plant in a given field. The problem is no less important for legumes other than the soybean. Solution can come only by focusing attention on the ecology of the rhizobia with questions that address the behavior of specific strains as free-living bacteria in the soil

and rhizosphere. Progress to date has been slow, due basically to the complexity of these natural environments and the limitations of available techniques.

New techniques for the detection, recognition and quantification of specific rhizobial serotypes directly in the natural environment are available. Advances now have been made in distinguishing between members of the same serotype. Application of the long established plant infection technique should be more effective now that the selectivity of the test plant is becoming recognized and the implications of this selectivity can be appreciated. Thus there are promising approaches to be taken, with techniques old and new to be combined as necessary against an array of significant questions.

One of the pressing problems involves a close examination of those strains that have adapted successfully to the soil and apparently out-compete inoculant strains with disconcerting regularity. Obviously it is important to know as much as possible of the attributes behind such success. Exactly how abundant are such strains in the absence and in the presence of a suitable host rhizosphere? What are the properties of the soil strains that lead to widespread distribution, and to what soil factors are they responsive? What is the substrate away from the plant? Again why, in certain locations or at certain times does it happen that the normally successful strain loses out to lesser rivals in the competition for nodule sites?

There is evidence that it is sometimes possible to overwhelm the successful soil residents if sufficiently high numbers of inoculant rhizobia are introduced. It should be significant to know the quantitative relationships between the resident and introduced strains at this point, and how they interact in the rhizosphere after inoculation. Much more specific consideration of rhizobia in interaction with other microorganisms, and especially fungi, in the rhizosphere is merited. Some of the data suggest the means to do this, in the form of fungi to introduce, fungicides to alter fungal populations, antibiotics to achieve temporarily selective effects, and herbicides to alter the plant root and probably the microbial equilibrium as well.

It is clearly worthwhile to exploit all clues as to the possible biochemical basis of the specific recognition between *Rhizobium* and host root. In a sense all other events in the rhizosphere may be a prelude to the recognition event. This could be the case if the rhizosphere were to provide the precise nutritional environment required by the correct rhizobia for synthesis of a "recognition factor". Elaboration of a recognition mechanism could provide a powerful tool for better management of the legume symbiosis.

Rhizobia as they exist and respond in the soil and in the vicinity of a host legume root are obviously worthwhile objects of scientific interest in relation to the vastly important process of symbiotic nitrogen fixation. The details of this existence are difficult to expose but the rewards should be considerable. For whatever information is eventually yielded will in all likelihood prove

useful beyond nitrogen fixation as a general model for soil microorganism—plant root interaction.

7. REFERENCES

Bezdicek, D. F., 1972. Effect of soil factors on the distribution of *Rhizobium japonicum* serogroups. Soil Sci. Soc. Am. Proc., 36: 305—307.

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

Bohlool, B. B. and Schmidt, E. L., 1970. Immunofluorescent detection of *Rhizobium japonicum* in soils. Soil Sci., 110: 229—236.

Bohlool, B. B. and Schmidt, E. L., 1973a. A fluorescent antibody technique for determination of growth rates of bacteria in soil. In: T. Rosswall (Editor), Modern Methods in the Study of Microbial Ecology. Bull. Ecol. Res. Comm. (Stockholm), 17: 336—338.

Bohlool, B. B. and Schmidt, E. L., 1973b. Persistence and competition aspects of *Rhizobium japonicum* observed in soil by immunofluorescence microscopy. Soil Sci. Soc. Am. Proc., 37: 561—564.

Bohlool, B. B. and Schmidt, E. L., 1974. Lectins: a possible basis for specificity in the *Rhizobium*—legume root nodules symbiosis. Science, 185: 269—271.

Braithwaite, B. M., Jane, A. and Swain, F. G., 1958. Effect of insecticides in sod sown sub-clovers. J. Aust. Inst. Agric. Sci., 24: 155—157.

Brockwell, J., 1963. Accuracy of a plant-infection technique for counting populations of *Rhizobium trifolii*. Appl. Microbiol., 11: 377—383.

Brown, M. E., Jackson, R. M. and Burlingham, S. K., 1968. Growth and effects of bacteria introduced into soil. In: T. R. G. Gray and D. Parkinson (Editors), The Ecology of Soil Bacteria. Liverpool University Press, Liverpool, pp. 531—551.

Caldwell, B. E. and Vest, G., 1968. Nodulation interactions between soybean genotypes and serogroups of *Rhizobium japonicum*. Crop Sci., 8: 680—688.

Chatel, D. L. and Greenwood, R. M., 1973. The location and distribution in soil of rhizobia under senesced annual legume pastures. Soil Biol. Biochem., 5: 799—808.

Chatel, D. L. and Parker, C. A., 1973a. Survival of field-grown rhizobia over the dry summer period in Western Australia. Soil Biol. Biochem., 5: 415—423.

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

Chatel, D. L., Shipton, W. A. and Parker, C. A., 1973. Establishment and persistence of *Rhizobium trifolii* in Western Australia soils. Soil Biol. Biochem., 5: 815—824.

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

Darbyshire, J. F., 1966. Studies on the physiology of nodule formation. IX. The influence of combined nitrogen, glucose, light intensity and day length on root hair infection in clover. Ann. Bot. N. S., 30: 623—638.

Dart, P. J. and Mercer, F. V., 1964. The legume rhizosphere. Archiv. Mikrobiol., 47: 344—378.

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

Davis, R. J., 1962. Resistance of rhizobia to antimicrobial agents. J. Bact., 84: 187—188.

Diatloff, A., 1969. The introduction of *Rhizobium japonicum* to soil by seed inoculation of non-host legumes and cereals. Aust. J. Exp. Agric. Anim. Husb., 9: 357—360.

Dixon, R. O. D., 1969. Rhizobia (with particular reference to relationships with host plants). Ann. Rev. Microbiol., 23: 137—158.

Dudman, W. F., 1971. Antigenic analyses of *Rhizobium japonicum* by immunodiffusion. Appl. Microbiol., 21: 973—985.

Dudman, W. F. and Brockwell, J., 1968. Ecological studies of root nodule bacteria introduced into field environments. I. A survey of field performance of clover inoculants by gel immune diffusion serology. Aust. J. Agric. Res., 19: 739—747.

Egeraat, A. W. S. M. van, 1972. Pea root exudates and their effect upon root nodule bacteria. Meded. Landbouwhogesch. Wageningen, 72-27, H. Veenman en Zonen N. V., Wageningen, The Netherlands, 90 pp.

Ek-Jander, J. and Fåhraeus, G., 1971. Adaption of *Rhizobium* to sub-arctic environment in Scandinavia. Plant Soil, Spec. Vol.: 129—137.

Fåhraeus, G. and Ljunggren, H., 1968. Pre-infection phases of the legume symbiosis. In: T. R. G. Gray and D. Parkinson (Editors), The Ecology of Soil Bacteria. Liverpool University Press, Liverpool, pp. 396—421.

Foulds, W., 1971. Effect of drought on three species of *Rhizobium*. Plant Soil, 35: 665—667.

Gibson, A. H., 1967. Physical environment and symbiotic nitrogen fixation. IV. Factors affecting the early stages of nodulation. Aust. J. Biol. Sci., 20: 1087—1104.

Graham, P. H., 1969a. Selective medium for growth of *Rhizobium*. Appl. Microbiol., 17: 769—770.

Graham, P. H., 1969b. Analytical serology of the Rhizobiaceae. In: J. B. G. Kwapinski (Editor), Analytical Serology of Microorganisms. Vol. 2. Interscience Publishers, New York, pp. 353—378.

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

Hamblin, J. and Kent, S. P., 1973. Possible role of phytohaemagglutinin in *Phaseolus vulgaris* L. Nature New Biol., 245: 28—30.

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

Hofer, A. W., 1958. Selective action of fungicides on *Rhizobium*. Soil Sci., 86: 282—286.

Holding, A. J. and King, J., 1963. The effectiveness of indigenous populations of *Rhizobium trifolii* in relation to soil factors. Plant Soil, 18: 191—198.

Holland, A. A. and Parker, C. A., 1966. Studies on microbial antagonism in the establishment of clover pasture. II. The effect of saprophytic soil fungi upon *Rhizobium trifolii* and the growth of subterranean clover. Plant Soil, 25: 329—340.

Hubbell, D. H., 1970. Studies on the root hair "curling factor" of *Rhizobium*. Bot. Gaz., 131: 337—342.

Hussein, Y. A., Tewfik, M. S. and Hamdi, Y. A., 1974. Degradation of certain aromatic compounds by rhizobia. Soil Biol. Biochem., 6: 377—381.

Kapusta, G. and Rouwenhorst, D. L., 1973. Interaction of selected pesticides on *Rhizobium japonicum* in pure culture and under field conditions. Agron. J., 65: 112—115.

Katznelson, H., 1965. Nature and importance of the rhizosphere. In: K. F. Baker and W. C. Snyder (Editors), Ecology of Soil-Borne Plant Pathogens. University of California Press, Berkeley, pp. 187—209.

Kowalski, M., Ham, G. E., Frederick, L. R. and Anderson, I. C., 1974. Relationship between strains of *Rhizobium japonicum* and their bacteriophages from soil and nodules of field-grown soybeans. Soil Sci., 118: 221—228.

Krasil'nikov, N. A., 1958. Soil Microorganisms and Higher Plants. Acad. Sci., USSR. Y. Halperin (Translator). English edition published for the National Science Foundation and Department of Agriculture, USA. 474 pp.

Kulkarni, J. H., Sardeshpande, J. S. and Bagyara, D. J., 1974. Effect of four soil-applied insecticides on symbiosis of *Rhizobium* sp. with *Arachis hypogaea* Linn. Plant Soil, 40: 169—172.

Lindemann, W. C., Schmidt, E. L. and Ham, G. E., 1974. Evidence for double infection within soybean nodules. Soil Sci., 118: 274—279.

Lotz, W. and Mayer, F., 1972. Isolation and characterization of a bacteriophage tail-like bacteriocidin from a strain of *Rhizobium*. J. Virol., 9: 160—173.

Lowe, R. H. and Evans, H. J., 1962. Cobalt requirement for the growth of rhizobia. J. Bact., 83: 210—211.

Marshall, K. C., 1964. Survival of root nodule bacteria in dry soils exposed to high temperatures. Aust. J. Agric. Res., 15: 273—281.

Marshall, K. C., 1967. Methods of study and ecological significance of rhizobium—clay interactions. In: J. Phillipson (Editor), Methods of Study in Soil Ecology. Proc. UNESCO/IBP Symposium, Paris, pp. 107—110.

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

Means, U. M., Johnson, H. W. and Erdman, L. W., 1961. Competition between bacterial strains effecting nodulation in soybeans. Soil Sci. Soc. Am. Proc., 25: 105—108.

Means, U. M., Johnson, H. W. and Date, R. A., 1964.Quick serological method of classifying strains of *Rhizobium japonicum* in nodules. J. Bacteriol., 87: 547—553.

Miller, R. H. and Schmidt, E. L., 1965. A technique for maintaining a sterile soil:plant root environment and its application to the study of amino acids in the rhizosphere. Soil Sci., 100: 267—273.

Mulder, E. G. and Veen, W. L. van, 1960. Effect of pH and organic compounds on nitrogen fixation by red clover. Plant Soil, 13: 91—113.

Nutman, P. S., 1962. The relation between root hair infection by *Rhizobium* and nodulation in *Trifolium* and *Vicia*. Proc. Roy. Soc. Lond., Ser. B., 156: 122—137.

Nutman, P. S., 1965. The relation between nodule bacteria and the legume host in the rhizosphere and in the process of infection. In: K. F. Baker and W.C. Snyder (Editors), Ecology of Soil-Borne Plant Pathogens. University of California Press, Berkeley, pp. 231—247.

Obaton, M., 1971. Utilisation de mutants spontanes résistants aux antibiotiques pour l'étude écologique des *Rhizobium*. C. R. Acad. Sci. Paris, Série D, 272: 2630—2633.

Parker, C. A. and Grove, P. L., 1970. *Bdellovibrio bacteriovorus* parasitizing *Rhizobia* in Western Australia. J. Appl. Bact., 33: 253—255.

Pattison, A. C. and Skinner, F. A., 1973. The effects of antimicrobial substances on *Rhizobium* spp. and their use in selective media. J. Appl. Bact., 37: 239—250.

Pramer, D. and Schmidt,, E. L., 1967. Experimental Soil Microbiology. Burgess Publ. Co., Minneapolis, 107 pp.

Purchase, H. F., Vincent, J. M. and Ward, L. M., 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.

Raggio, M., Raggio, N. and Torrey, J. G., 1965. The interaction of nitrate and carbohydrate in rhizobial root nodule formation. Plant Physiol., 40: 601—606.

Robinson, A. C., 1967. The influence of host on soil and rhizosphere populations of clover and lucerne nodule bacteria in the field. J. Aust. Inst. Agric. Sci., 33: 207—209.

Roslycky, E. B., 1967. Bacteriocin production in the rhizobia bacteria. Can. J. Microbiol., 13: 431—432.

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

Rovira, A. D. and Stern, W. R., 1961. The rhizosphere bacteria in grass—clover associations. Aust. J. Agric. Res., 12: 1108—1118.

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

Sahlman, K. and Fåhraeus, G., 1963. An electron microscope study of root hair infection by *Rhizobium*. J. Gen. Microbiol., 33: 425—427.

Schmidt, E. L., 1973. Fluorescent antibody techniques for the study of microbial ecology. In: T. Rosswall (Editor), Modern Methods for the Study of Microbial Ecology. Bull. Ecol. Res. Comm. (Stockholm), 17: 67—76.

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

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

Schwinghamer, E. A., 1967. Effectiveness of *Rhizobium* as modified by mutation for resistance to antibiotics. Antonie van Leeuwenhoek; J. Microbiol. Serol., 33: 121—136.

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

Schwinghamer, E. A., Pankhurst, C. E. and Whitfield, P. R., 1973. A phage-like bacteriocin of *Rhizobium trifolii*. Can. J. Microbiol., 19: 359—368.

Sharon, N. and Lis, H., 1972. Lectins: cell-agglutinating and sugar-specific proteins. Science, 177: 949—959.

Sherwood, M. T. and Masterson, C. L., 1974. Importance of using the correct test host in assessing the effectiveness of indigenous populations of *Rhizobium trifolii*.Ir. J. Agric. Res., 13: 101—108.

Škrdleta, V., 1965. Somatic serogroups of *Rhizobium japonicum*. Plant Soil, 23: 43—48.

Solheim, B. and Raa, J., 1971. Evidence countering the theory of specific induction of pectin-degrading enzymes as basis for specificity in *Rhizobium*—Leguminosae associations. Plant Soil, 35: 275—280.

Staniewski, R., 1970. Typing of *Rhizobium* by phages. Can. J. Microbiol., 16: 1003—1009.

Tanner, J. W. and Anderson, I. C., 1964. External effect of combined nitrogen on nodulation. Plant Physiol., 39: 1039—1043.

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

Tuzimura, K. and Watanabe, I., 1961a. Estimation of number of root nodule bacteria by a nodulation-dilution frequency method. Ecological studies of *Rhizobium* in soils (Part 1). Soil Sci. Plant Nutr. (Tokyo), 7: 61—65.

Tuzimura, K. and Watanabe, I., 1961b. The growth of *Rhizobium* in the rhizosphere of the host plant. Ecological studies of root nodule bacteria (Part 2). Soil Sci. Plant Nutr. (Tokyo), 8: 19—24.

Tuzimura, K. and Watanabe, I., 1962. The effect of rhizosphere of various plants on the growth of *Rhizobium*. Ecological studies of root nodule bacteria (Part 3). Soil Sci. Plant Nutr. (Tokyo), 8: 13—17.

Vest, G., Weber, D. F. and Sloger, C., 1973. In: B. E. Caldwell (Editor), Soybeans: Improvement, Production, and Uses. Am. Soc. Agron., Monograph 16. Madison, Wisc., pp. 353—390.

Vincent, J. M., 1965. Environmental factors in the fixation of nitrogen by the legume. In: W. V. Bartholomew and F. E. Clark (Editors), Soil Nitrogen. Am. Soc. Agron., Madison, Wisc., pp. 384—435.

Weaver, R. W., and Frederick, L. R., 1974. Effect of inoculum rate on competitive nodulation of *Glycine max* L. Merrill. II. Field studies. Agron. J., 66: 233—236.

Weber, D. F. and Miller, V. L., 1972. Effect of soil temperature on *Rhizobium japonicum* serogroup distribution in soybean nodules. Agron. J., 64: 796—798.

Weber, D. F., Caldwell, B. E., Sloger, C. and Vest, H. G., 1971. Some USDA studies on the soybean—*Rhizobium* symbiosis. Plant Soil, Spec. Vol: 293—304.

Schmidt E.L. (1978)

Legume symbiosis : A-Ecology of the legume root nodule bacteria

In : Dommergues Yvon (ed.), Krupa S.V. (ed.). *Interactions between non-pathogenic soil microorganisms and plants*

Amsterdam : Elsevier, p. 269-303. (Development in Agricultural and Managed Forest Ecology ; 4)

ISBN 0-444-41638-2