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Factors Affecting Survival of Rhizobium in Soil

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1. Introduction

Legumes are already widely used as an extremely important source of protein for human and animal consumption and of nitrogen to maintain soil fertility. Cowpeas, lentils, gram (usually chick-peas or mung beans), and pigeon peas are major crops used for edible seeds (pulses); beans, peas, peanuts (groundnuts), and soybeans are important vegetable or industrial crops. Clovers, alfalfa (lucerne), and lupines stand out as common pasture plants, whereas *Centrosema*, *Desmodium*, and *Stylosanthes* are becoming increasingly important in the tropics. Enormous land areas are devoted to production of pulses and oils. In protein-poor localities, legumes are especially important because they are excellent sources of protein, and the crude protein content often ranges as high as 34% (as in winged bean; see National Academy of Sciences, 1975) compared to 5% in grasses.

Because of their symbiotic relationship with *Rhizobium*, the root-nodule bacterium which fixes atmospheric nitrogen, legumes are often able to fulfill their own nitrogen requirements and therefore require the addition of little or no nitrogen fertilizers. Where these fertilizers are too costly for the farmer, especially the small farmer, legumes are of substantial benefit both in producing food and also in adding usable nitrogen to the soil, thus benefiting the next crop.

Despite the actual and potential advantages of legumes to tropical agriculture, there are many serious problems in growing them. Often excellent nitrogen fixation and crop yields are found in areas adjacent to farms getting poor yields. Large acreages produce small yields, and often farmers apply nitrogen fertilizers to legumes rather than depend on symbiotic nitrogen fixation.

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Many of these problems can be resolved by employing proper farm management or by using improved legume varieties. Other problems are specifically related to the bacterial symbiont. Among legume-production problems related to *Rhizobium* are the following: (1) there are few or no rhizobia in the soil, those in the soil do not nodulate the crop that is planted, or the indigenous bacterial strains are not effective nitrogen fixers with the particular legume under cultivation; (2) the commercial *Rhizobium* inoculum provided does not contain the proper symbiont or, if proper, then there are insufficient live bacteria to bring about satisfactory rates of infection; (3) any of a number of difficulties with the handling of the inoculum by the farmer; (4) the inoculated rhizobia do not survive on the seeds or in the soil long enough to infect the first crop; or (5) the rhizobia infect the first crop but do not survive to infect subsequent plantings.

Some of these problems can be solved by establishing regional programs to train indigenous technicians. Once trained, these individuals could determine which problems are of significance in the region. They would know how to handle and test commercial inocula, and they could verify that the commercial inocula are prepared properly (Schall *et al.*, 1970).

A more difficult task will be to deal with those field problems arising because the required rhizobia do not persist in the soil. In such instances, the many soil types, climates, and legume (and therefore rhizobial) species are each variables and potential causes of poor survival. Poor survival may be a problem even where the proper inoculum is available since the farmer may not continue to use inoculum because of variable results, presumed lack of time, or even cost considerations. Thus, assuring survival of rhizobia in the soil, along with other required characteristics of the symbiosis, is critical for producing high yields of legumes, guaranteeing a satisfactory supply of edible protein, and raising the fertility of many problem soils.

Although books and reviews on the subject of legumes, the legume *Rhizobium* symbiosis, and nitrogen fixation are numerous, there is no comprehensive review of work specifically relating to rhizobial survival in soil. Nutman (1975) and Alexander (1977a) have dealt briefly with selected topics regarding rhizobial ecology, and there are related reviews touching on or covering certain aspects of the subject (Vincent, 1965, 1974; Chowdhury, 1977; Parker *et al.*, 1977). The objectives of this report are to describe the research done on rhizobial survival, cite the major results obtained, determine if generalizations can be made, and make recommendations ultimately for appropriate farm practice.

2. Terminology and Methods Used for Enumerating and Distinguishing Strains of *Rhizobium* in the Soil

2.1. Terminology

In the initial studies of *Rhizobium*, the only certain identification of members of that genus was to assess their capacity to cause nodulation of legume

roots. Species identification was then made according to which genus of legume the bacteria infected. The two symbionts (the plant and bacterium) were considered a *homologous pair*. Scientists constructed *cross-inoculation groups* for intergeneric homology, but the validity of these groups was severly questioned because both bacteria and plants were sometimes found to be promiscuous in their relationships (Wilson, 1944).

Effectiveness refers to a successful symbiosis in which the host plant provides nutrients that the microsymbiont requires, the bacteria in return provide fixed nitrogen i.e., assimilate or "fix" N_2, converting it to a form the plant uses. Effectiveness is measured by assessing the weight of inoculated plants, total nitrogen uptake by the plants (compared to uninoculated legumes or to plants grown on nitrate or ammonium), or by determinations of nitrogen-fixation activity using ^{15}N-N_2 or the acetylene-reduction technique. Effectiveness is a relative term because plant vigor is quite variable, because there is a continuous spectrum of symbiotic efficiencies, and because a given variety of legume (or strain of *Rhizobium*) forms an effective symbiosis with some but not necessarily all of its homologous rhizobia (or legume hosts) (Vincent, 1954; Bjalfve, 1963).

2.2. Methods

Detailed methods for studying the ecology of *Rhizobium* are provided in Vincent's (1970) manual. Rhizobia are generally grown on yeast extract-mannitol medium (YEM), and their numbers, either in culture or from sterile soil, may be counted on YEM agar plates. Dilution-nodulation is a method devised by Wilson (1926) to assess the number of rhizobia in nonsterile soil. Serial dilutions of the soil are made and inoculated into sterile soil. After 10-14 days to allow for colonization of the soil, surface sterilized host-legume seeds are planted. At the appropriate time, the legume roots are observed for nodulation. Presence of nodulation is presumed to indicate at least one viable *Rhizobium* in the original sample, and from the greatest dilution leading to nodulation, the approximate number of rhizobia in the soil can be obtained. This method has been simplified by growing the test plants on agar or in sand or gravel culture, and most-probable-number (MPN) tables can be used to improve the precision of numerical estimates (Tuzimura and Watanabe, 1961; Date and Vincent, 1962; Brockwell, 1963, Weaver and Frederick, 1974). These same analyses have shown that one or two rhizobia present in the tube receiving a soil dilution are sufficient to initiate nodulation. Thompson and Vincent (1967) have, however, pointed out that this method may fail when large populations of other organisms interfere with nodulation. The MPN technique also suffers from being time consuming and laborious.

A second method, which is much quicker and easier to carry out than the above, has been devised to count rhizobia that have been introduced into nonsterile soils. It is based upon the selection of antibiotic-resistant mutants (Obaton, 1971; Danso *et al.*, 1973; Schwinghamer and Dudman, 1973), adding

them to the natural soil, and then following changes in population density by plating serial dilutions of the soil onto YEM agar containing the antibiotic and counting the colonies that appear. This method cannot be used to do field surveys unless the resistant rhizobia were earlier introduced. It can potentially be used to count each in a mixture of strains of bacteria if various markers (e.g., mutations to resistance to different inhibitors) are used to distinguish between strains. Because fast-growing rhizobia (in general, *R. trifolii*, *R. meliloti*, *R. leguminosarum*, and *R. phaseoli*) develop to visible colonies on agar plates within about 2 days, the growth of fungi usually presents no problem in enumerating the bacteria, provided the rhizobia are present in large numbers. However, with slow-growing species (*R. lupini*, *R. japonicum*, and cowpea-type rhizobia), or with soils containing few rhizobia, fungi may overgrow the plates before bacterial colonies appear. under these conditions, an antifungal compound such as cycloheximide is used. It may prove important to include a more potent fungicide (or several antifungal compounds) because cycloheximide is not always completely effective in suppressing the growth of fungi. Also it is important to test antibiotic-resistant mutants to assure that infectivity and effectiveness are not impaired (Schwinghamer and Dudman, 1973).

Serological methods may be used for identifying strains of *Rhizobium*. One of these methods, the fluorescent-antibody technique (Schmidt *et al.*, 1968; Jones and Russell, 1972) can serve for counting bacteria and visualizing them in soil. The problem of nonspecific adsorption and autofluorescence associated with this method in soil (Nutman, 1975) seems to have been overcome with proper counterstaining (Bohlool and Schmidt, 1968). However, the instrumentation and supporting facilities are often not available in the poorer countries (Alexander, 1977a).

A second serological method is immunogel diffusion (Vincent, 1970; Dudman, 1971). Both of the serological techniques are used primarily to identify isolates, particularly from root nodules. Because of antigen-antibody specificity, serological techniques are well suited to distinguish between many different strains. Thus, the serological techniques and the antibiotic resistant techniques complement each other. Brockwell *et al.* (1977) have compared the antibiotic resistance and gel-diffusion procedures in field studies over 3 years and demonstrated that both were equally and highly reliable for identifying strains recovered from soil.

3. Factors Affecting Rhizobial Survival

3.1. Observed Persistence in the Field

Although legumes have been recognized for thousands of years as crops which improve the soil, only since the late 19th century has *Rhizobium* been known as the agent of the improvement and of nitrogen fixation (Fred *et al.*,

1932). It is obvious that these bacteria have survived in the soil for untold ages, yet direct evidence of field persistence has come, of course, only in about the last half century. For example, in several studies, *R. leguminosarum*, *R. lupini*, *R. japonicum*, *R. meliloti*, and *R. trifolii* have been found to survive at least 10-125 years after the cultivation of their respective homologous host (Norman, 1942; Jensen, 1969; de Escuder, 1972; Nutman, 1975). In particular, Nutman (1969) tested samples of soil taken in 1968 that had grown wheat continuously since 1843. Following serial dilutions of the soil, he checked nodulation on representative host plants. He found *R. leguminosarum* to be the most abundant, averaging 28,000/g dry soil, and all were effective fixers. *R. trifolii* was the next most abundant, averaging 320/g, but 10% were poor fixers. *R. lupini* occurred sparsely and sporadically, averaging 310/g in 13 of 20 plots in which it was found, and all but one strain were effective. *R. meliloti* was the most sparse, being absent in nearly half the plots, and the average was 13/g in the rest, more than half of the strains were fully effective.

Nutman and Ross (1970) found roughly the same survival in fields under continuous root crops since 1843. They also determined the numbers of rhizobia in fields maintained fallow for 8 years; here, the numbers of *R. trifolii* fluctuated between 1000 and 100,000/g, *R. leguminosarum* fluctuated between 10 and 1000, and *R. meliloti* fluctuated considerably but seemed to be declining to zero. These results were obtained at two separate sites in Great Britain.

Norman (1942) observed soybean yield and nodulation in fields that had not been planted to soybeans for over 24 years. His results with a less fertile and partly eroded sandy loam and a more fertile loam showed no significant difference between numbers of nodules on the plants or yields, whether or not the seeds had been inoculated, but he did not look at numbers of *Rhizobium*. Thus, *R. japonicum* had persisted in these soils for over 24 years in numbers high enough to be agronomically significant. Weaver *et al.* (1972) determined MPN counts of *R. japonicum* in 52 Iowa fields and reported that numbers were largely correlated with whether or not soybeans had been grown at the site (within the previous 13 years). 55% of the fields which had at any time been cropped to soybeans had over 100,000 *R. japonicum*/g soil, whereas only 5% of the fields which had not been cropped to soybeans had that many root-nodule bacteria. However, no significant correlation was found between rhizobial numbers and the length of time since the last crop had been grown (even if present at the time of sampling) or between rhizobial population and the number of times soybeans were grown. Elkins *et al.* (1976) reported comparable results. Tuzimura and Watanabe (1959a), on the other hand, found that *R. japonicum* numbers decreased in the absence of the host.

It thus seems that *R. japonicum* can survive quite well in the absence of its host legume. The contrary has been reported with certain strains of *R. trifolii* (see below), which in many regions of Australia were incapable of persisting in high enough numbers to reinfect a clover crop seeded in the second year. Thus, it is possible that some rhizobia persist in cropped land for periods long enough

to get food infection of a crop without reinoculation, but it is not yet possible to predict survival for specific fields and specific strains.

3.2. Stimulation of *Rhizobium* in the Rhizosphere

It has long been known that a given species of *Rhizobium* may be found in significantly higher numbers under plants than in unplanted soil (Wilson and Lyon, 1926; Wilson, 1934a). The question of the influence of the growth of the legume host, nonhost legume, and nonlegume on the numbers and persistence of *Rhizobium* has been studied in an extensive series of experiments by Tuzimura and Watanabe (1962a,b) and Tuzimura *et al.* (1966).

In one set of experiments, Tuzimura *et al.* (1966) placed nonsterile volcanic ash soil in porcelain pots, planted either ladino clover, alfalfa, soybeans, tomatoes, or Sudan grass, and inoculated each with soil containing both alfalfa and clover rhizobia. They then tested samples of inner-rhizosphere (root zone) soil and soil without plants using ladino clover or alfalfa to provide MPN values. Their results revealed that clover rhizobia were stimulated by clover, alfalfa, and tomato plants, whereas alfalfa rhizobia were stimulated only by alfalfa plants. They also counted numbers of rhizobia in the soils 2 months after the plant tops had been removed and noted that the abundance of clover bacteria was higher in pots which had grown clover, alfalfa, or soybeans than in soil without plants. The numbers of alfalfa rhizobia were higher only in soil which had grown alfalfa. Thus, the authors concluded that certain hosts, nonhost legumes, and nonlegumes can be stimulatory to the growth of some but not all species of *Rhizobium* and that the soil under inquiry was more favorable to the clover than the alfalfa rhizobia.

In a second set of experiments, Tuzimura *et al.* (1966) observed the effects of the presence or absence of plants on rhizobial numbers in two different soils, the volcanic ash soil used in the previously described experiment (pH 5.5), which initially had counts of less than 10 clover rhizobia per gram and no detectable alfalfa rhizobia, and an alluvial clay loam from a well-drained paddy field (pH 6.2), which initially had 180 clover rhizobia per gram and also no detectable alfalfa rhizobia. The soils were inoculated with strains of *R. trifolii* and *R. meliloti* and left fallow or planted separately with ladino clover, alfalfa, wheat, red radish (*Raphanus sativus*), or common vetch (*Vicia sativa*). In both fallow soils, counts of clover rhizobia were nearly constant for the 3.5-month experiment, fluctuating between 2 and 80/g; although counts of alfalfa rhizobia varied in the fallow clay loam between 7 and 300/g, they seemed to increase in the volcanic soil from 340 to 7000/g. The authors found that alfalfa and clover stimulated both *Rhizobium* species in the two soils, but the populations were stimulated more dramatically under the homologous host, often to greater than 10^5 or 10^6/g. Vetch stimulated both species in the two soils. Wheat, however, promoted the development of alfalfa rhizobia in both soils (4 to 7 X 10^4/g, 62 days after seeding) but stimulated clover rhizobia only in the clay loam (1.4 X 10^4

compared to 8/g in the volcanic ash). Thus, the stimulation by wheat depended both on *Rhizobium* species and soil type. Radish favored the two species of *Rhizobium* but only in the clay loam. It is interesting that in the first experiment, clover did not enhance the proliferation of alfalfa rhizobia in the ash soil, but in the second experiment it did; however, the background counts in unplanted soil were both high and variable, and the *Rhizobium* strains may well have been different.

Robinson (1967) reported field results similar to those of Tuzimura *et al.* (1966). Thus *R. trifolii* was found in high numbers (up to 100,000/g) in soil near the host plant (*Trifolium subterraneum*), nonhost legume (*Medicago sativa*), and nonlegume (*Poa australis*). *R. meliloti*, on the other hand, was found in high numbers only near roots of its host (*M. sativa*) and the nonlegume (*P. australis*) and was not detected under *T. subterraneum*. Jensen (1969) found *R. trifolii* densities of 100 000/g of soil in many clover-free fields, but similar densities of *R. meliloti* were confined to existing alfalfa fields. According to the results of Hely and Brockwell (1962), the apparent numbers of *R. meliloti* at different sites in Australia depended on the species of *Medicago* used as test host. This indicated that the population density varied with particular strain and soil. The bacteria were often found in appreciable numbers in the absence of the host, and in some cases the presence of the host plant did not lead to an increased population. Grasses also usually stimulated the proliferation of *R. meliloti*.

It would seem from the above discussion that *R. trifolii* is more generally stimulated by nonhost plants than *R. meliloti*, although strain and soil differences seem to play a major role in their responses to plants. The reasons for these differences are unknown.

Wilson (1934b) found no relationship between the numbers of *R. trifolii*, *R. leguminosarum*, or *R. japonicum* and the crop on the soil. Elkan (1962) reported that there are differences in rhizosphere stimulation between a nodulating and nonnodulating line of soybean, and he noted that detecting a stimulation or inhibition of *R. japonicum* depended on when after germination the samples were collected.

Peters and Alexander (1966) found no specificity regarding the stimulation of various species of *Rhizobium* when testing legumes growing in solution culture. Vincent (1967) made similar observations. Hence, while rhizobia are favored by the presence of plant roots, the degree of stimulation depends on plant variety, soil type, *Rhizobium* species, and even strain. Furthermore, there is still controversy concerning the extent of specificity of the stimulation.

The effect of plant species on the density of rhizobia in the soil is obviously important to the farmer. It also has some important consequences for the investigator studying survival in the field. For example, de Escuder (1972) noted that in fields that had grown barley for 9 years and also in pastures of perennial rye grass, the numbers of *R. trifolii* were 10^6/g or more. However, she also noted that white clover was a widespread weed, approaching 15% of the flora in the pasture. Thus, a conclusion from her results that *R. trifolii* persisted in high

numbers under rye grass and barley must be considerably tempered because many host plants were also present. It is worth pointing out that Nutman (1969) and Nutman and Ross (1970), in the studies previously mentioned, looked for the presence of leguminous weeds in the fields that had grown wheat or root crops for 120 years. They reported that there was no record of leguminous weeds in the fields with root crops, and the distribution of rhizobia in the wheat fields did not correspond with that of the leguminous weeds. Thus, their results are not complicated by the presence of the host legume. However, in a survey of a meadowland cut for hay for over 100 years, Nutman and Ross (1970) found a "striking correspondence" between the occurrence of *Rhizobium* species in the soil of each plot and the presence of its host legume.

3.3. Soil Factors

In the preceding section, consideration was given to ways in which various plant species influence numbers and persistence of rhizobia. In this section are discussed the effects on *Rhizobium* of general and specific soil factors.

3.3.1. Soil Management

Walker and Brown (1935) investigated the influence of soil-management practices on rhizobia. Iowa fields (original soil pH 5.7) which were cropped in 3-year rotations (corn, oats, and red clover and alfalfa) were compared to fields cropped to corn and oats in 2-year rotations. The fields either were not fertilized or were treated with various combinations of manure, limestone, rock phosphate, and crop residues. Rhizobial density was determined using Wilson's dilution-nodulation technique. The results showed that the numbers of *R. trifolii* and *R. meliloti* were approximately 10-fold greater in the 3-year rotation (samples were taken during growth of a nonlegume crop) than in the 2-year rotation. The authors also noted that soil amended with manure, lime, and rock phosphate (pH 6.9) contained 100- to 1000-fold more rhizobia than fields without these amendments regardless of the kind of rotation. They therefore concluded that, although the kind of rotation affected the root-nodule bacteria, soil management was more important in promoting high rhizobial numbers than the frequency of the host crop.

Numerous other reports, on the other hand, have indicated no significant differences in rhizobial populations as a result of various types of soil-management practices (Wilson, 1931, 1934b; Weaver *et al.*, 1972). However, the effect of soil management on rhizobial survival has not been systematically studied.

3.3.2. Physical and Chemical Stresses

3.3.2a. Acidity. Because many agricultural soils are naturally somewhat acidic and because large tracts of tropical soils are often highly leached and

acidic, the effect of low pH on the survival of *Rhizobium* is of considerable importance.

Bryan (1923) has done the most comprehensive experiments in this regard. He treated five acid soils (one sand, one fine sandy loam, and three silt loams) with different amounts of lime, placed portions in test tubes, and inoculated these soils with suspensions of alfalfa, clover, or soybean rhizobia. The tubes were incubated for 75 days at room temperature, at which time suspensions of the soil were poured over sterile sand planted to alfalfa, red clover, or soybeans. After 5 to 7 weeks, the presence of nodules was assessed. The study disclosed that no nodules appeared on alfalfa inoculated with soils below pH 4.6-5.0, on red clover from soils below pH 4.5-4.9, and on soybeans from soils below pH 3.5-4.2. Above these pH values, nodulation occurred. Bryan (1923) tested alfalfa rhizobia further on fine sand, peat, and silt loam and recorded the same results. He then concluded that the above pH ranges were critical for survival of the respective rhizobia (Table I). Since Bryan (1923) made no bacterial counts, the initial rhizobial densities, the rate of decline or increase in population, and whether nodulation reflected the presence of one or many rhizobia are unknown.

The critical pH values are similar to those found for pure cultures by Fred and Davenport (1918), however. These investigators inoculated a defined, mannitol-salts medium with 35,000 cells/ml, and they observed that different strains did not grow below a critical pH value. For alfalfa and sweet clover rhizobia, the critical pH was 4.9; for garden pea, field pea, and vetch, pH 4.7; for red clover and common bean, pH 4.1; for soybean and velvet bean, pH 3.3; for lupines, pH 3.2. Thus Fred and Davenport's (1918) results for alfalfa rhizobia in pure culture correspond to those of Bryan (1923) in soil, and their results for red clover and soybean give critical pH values slightly below the respective values found by Bryan (1923). On the other hand, Graham and Parker (1964) showed that sensitivity of a given species of *Rhizobium* to pH in culture varied up to 1 pH unit according to individual strain.

Additional values for the lower pH limit to rhizobial growth or survival are given in Table I. All but Bryan (1923) and Vincent and Waters (1954) derived the limit for growth by using culture media. The latter authors inoculated sterile soils and observed growth of *R. trifolii* between pH 5.5 and 8.0 and loss of viability below pH 5.5.

Jensen (1969) carried out laboratory studies on survival in soils of varying (and somewhat variable) pH for up to 159 days. At pH values of about 6.1 and above, the numbers of both *R. trifolii* and *R. meliloti* appeared stable (3000-100,000/g) for this period. At about pH 5.5 and below, the abundance of both species decreased with time, but *R. meliloti* appeared to decline more quickly than *R. trifolii*; the lower the pH, the faster the decline. Had Jensen used Bryan's nodulation-or-not technique and stopped the experiment at 76 days, his results would have been similar to Bryan's (1923): the "critical" pH for *R. meliloti* would be 4.5-4.9 and, for *R. trifolii*, just below pH 4.5. Jensen (1969) actually

Table I. pH Below Which *Rhizobium* Does Not Grow or Survive

	Lower pH for growth or survival						
Bacterium	Bryan (1923)	Fred and Davenport (1918)	Holding and Lowe (1971)	Jensen (1942)	Mendez-Castro and Alexander (1976)	Vincent and Waters (1954)	Graham and Parker (1964)
R. meliloti	4.6–5.0	4.9		5.3–5.5			4.5–5.0
R. trifolii	4.5–4.9	4.2	4.5	5.1–5.3	4.6	5.5	4.5–5.0
R. leguminosarum		4.7					4.0–5.0
R. phaseoli		4.2					4.0–5.0
R. lupini		3.15					4.0–5.0
R. japonicum	3.5–4.2	3.3			4.2–4.6		3.5–4.5
Rhizobium sp. (velvet bean)[a]		3.3					
Rhizobium spp.							4.0–5.0

[a]*Rhizobium* from the cowpea-type cross-inoculation group is designated for clarity as *Rhizobium* sp. In parentheses is given the plant species from which the strain was isolated.

concluded that the "critical" pH for *R. meliloti* was 1 pH unit above that for *R. trifolii*.

Jensen (1969) also took samples from 214 field sites in Denmark. He reported that *R. trifolii* was almost ubiquitous in cultivated soils. In 27% of the samples, the density of *R. trifolii* was 100,000/g or more, and only a minor fraction of these samples came from clover fields. Similarly high densities of *R. meliloti*, however, were confined to existing alfalfa fields of pH above 6.0. Below pH 6.0, the occurrence of *R. meliloti* was sporadic, and its population did not seem to exceed 1000/g.

The above differences in *R. meliloti* and *R. trifolii* population densities in the field have been underscored by Rice *et al.* (1977). They found that bacterial numbers under host plants were between 100,000 and 1,000,000/g of soil at approximately neutral pH and fell off sharply below pH 6.0 for *R. meliloti* and 4.9 for *R. trifolii*.

Peterson and Goodding (1941) sampled 316 Nebraska soils to establish the effect of pH on *R. meliloti*. Samples of the soil were added to sterile sand planted to alfalfa, which was then observed for nodulation. In these studies, 40% of all samples contained rhizobia. When the percentage of samples containing rhizobia was plotted against the pH of the soil, a straight line resulted, running from pH 5.6 (10% contained rhizobia) to pH 8.6 (100% contained rhizobia). Thus, the frequency of *R. meliloti* occurring in these soils was directly related to the pH. It is worth noting that all the pH values in this study are above the critical pH of Bryan (1923) for *R. meliloti* (>pH 5.6 vs. pH 4.6–5.0). Peterson and

Goodding (1941) did not determine the numbers of rhizobia in the soil nor is it possible to establish how long the rhizobia inhabited the soil. From their results, one can conclude that the higher the soil pH, the greater is the probability of finding surviving alfalfa root-nodule bacteria.

Wilson (1934a) similarly sampled soils of different pH values, but he counted *R. japonicum* by the dilution-nodulation procedure. He found no correlation between pH and the number of soils with *R. japonicum*. As was the case with the soils used by Peterson and Goodding (1941), all of Wilson's samples were almost 2 pH units above Bryan's critical pH value (>5.0 vs. 3.5-4.2). Yet the two sets of results are quite dissimilar, possibly reflecting differences between *R. japonicum* and *R. meliloti*.

Liming of acid soils often enhances rhizobial survival. For example, Vincent (1958) failed to find *R. meliloti* in soil of pH 5.3-5.7, whereas counts of 10^4-10^5/g were found if the soil pH had been raised to 6.8. He also found that *R. trifolii* failed to spread beyond rows of lime- and superphosphate-pelleted clover seeds, presumably because the environment between the rows was too acid. From such data, it it not possible to determine if raising the pH leads to growth or just favors persistence. In other cases, adding lime or superphosphate increased nodulation (Robson and Loneragan, 1970), but again no information was obtained about survival of *Rhizobium*.

On the other hand, Mulder *et al.* (1966) found much higher numbers of *R. trifolii* under red clover in a soil at pH 5.1 than in a soil which had been limed (7800/g vs. 0) but was unplanted. Thus, the rhizosphere may protect rhizobia from the harmful effects of acidity.

Norris (1965) advanced the hypothesis that slow-growing or "tropical" rhizobia are more resistant to acidity in soils than fast-growing or temperate rhizobia. However, no supporting evidence exists apart from the results of Bryan (1923). For example, the work of Loustalot and Telford (1948) on tropical kudzu was cited by Norris (1959) as indicating that tropical rhizobia survive at low pH values; however, although kudzu grew better and contained a higher percentage of nitrogen at pH 4 than at higher pH values, Loustalot and Telford (1948) provided no evidence that rhizobia survived outside the nodules at that pH or even that nodulation can take place at pH 4 since the plants were transplanted to sand at the appropriate pH when they were 3 months old.

The results described above lead to the conclusion that strains of *R. meliloti* are less tolerant of acid soils than those of other rhizobial species, few of which have been studied in any detail. The definition of "critical" pH is not clear. One can imagine that the rhizobia either grow or at least persist in reasonably constant numbers above some acid pH value. Below this value, the bacteria probably decrease in numbers, very likely with greater rapidity as the pH decreases. It is important to establish this critical pH and to know the extent of survival in soils of lower pH values. If the soil pH is below the critical level but is high enough to allow sufficient rhizobia to persist into the next growing season, the bacteria may still bring about adequate nodulation the following years.

3.3.2b. Mn and Al. Frequent reference is made to the possibility of Mn and Al toxicity to *Rhizobium*, especially in acidic soils in which Mn and Al are usually more available (Holding and King, 1963; Vincent 1965, 1974; Franco and Dobereiner, 1971; Graham and Hubbell, 1975; Alexander, 1977), and several studies have indeed dealt with the inhibition of nodulation by Mn (Van Schreven, 1958; Franco and Dobereiner, 1971). The effect of subtoxic levels of Mn on the growth of *Rhizobium* in culture media has been tested, but the results are contradictory (Holding and King, 1963; Sherwood, 1966; Holding and Lowe, 1971).

The only study of Mn effects on rhizobial numbers in soil, albeit in the presence of a host plant, is that of Lowe and Holding (1970). They determined the numbers of several strains of *Rhizobium* in soil after harvesting 17-week-old white clover plants. Raising the concentration of Mn from 5.7 to 20 ppm resulted in a 12- to 29-fold decrease in numbers, the extent of the decline varying with the *Rhizobium* strain. It is not clear whether these decreases in density result directly from an influence of Mn on rhizobia in the soil or indirectly from changes in the plant rhizosphere or the release of bacteria from the nodules.

Thus, the direct effects of Al and Mn on survival of *Rhizobium* in soils have yet to be investigated.

3.3.2c. Salinity and Alkalinity. Saline and alkaline soils are found in arid regions which are poorly drained or in places where salts wash down through the profile because of heavy rains and are returned with water to the surface during the dry period of the year (Pillai and Sen, 1966). Such saline and alkaline soils often do not support good growth of legumes.

Bhardwaj (1972) studied dhaincha (*Sesbania cannabinea*), a salt-tolerant legume grown in saline-alkaline soils in India, and rhizobia isolated from dhaincha plants growing in normal soil or in saline-alkaline soil. The soil was sterilized, left unamended or amended with 1% gypsum or 1% manure, and inoculated with the bacterial isolates. After 7 and 14 days, the abundance of *Rhizobium* was determined. Both strains derived from saline-alkaline soil but only one of the two strains from normal soil survived in the unamended test soil. All four strains survived in the amended test soils, although the strains isolated from saline-alkaline soils persisted in approximately 100-fold higher numbers. Bhardwaj (1972) concluded that inocula for dhaincha growing in saline-alkaline soils should be derived from rhizobia isolated from like soils.

Bhardwaj (1975) subsequently showed that berseem clover, cluster bean, alfalfa, and Indian clover growing in sterile, neutral soil nodulated when inoculated with some saline-alkaline soil. Soybeans, peas, black gram, bengal gram, and cowpeas did not nodulate under these circumstances. He then tested rhizobia isolated from plants (*Trifolium alexandrinum*, *Melilotus sativum*, *M. parviflora*, *Glycine max*, *Phaseolus mungo*, and *Trifolium foenum-grecum*) growing on neutral soil to determine whether they would survive for 21 days in sterilized alkaline soils. A majority of the isolates from each species survived, although no

numbers were given. He also found no obvious differences in survival of 30 *R. leguminosarum* strains, 10 of which had been isolated from acid, 10 from neutral, and 10 from saline-alkaline soils (pH 6.3, 7.5, and 9.2 respectively). These results are not consistent with those obtained with dhaincha *Rhizobium* (Bhardwaj, 1972).

Rhizobia may in fact survive in saline-alkaline soils better than their legume host. Thus, Subba Rao *et al.* (1972) noted that most test strains of *R. meliloti* grew in YEM medium containing 1% NaCl, whereas the host *M. sativa* was considerably inhibited when grown in agar with 0.5% NaCl or less. El Essawi and Abdel-Ghaffer (1967), comparing *R. trifolii* isolated in Egypt with those from Europe and the United States, found that several Egyptian but only one foreign strain grew in culture at 5% NaCl. Mendez-Castro and Alexander (1976) were able to grow *R. japonicum* and *R. trifolii* on NaCl concentrations as high as 3-4%. On the other hand, Wilson and Norris (1970) found *R. japonicum* would not grow in 1% NaCl.

Thus, it is possible that rhizobial survival does not limit the rhizobial-legume symbiosis in saline-alkaline soils, although the data are far from convincing because none of the studies was done in nonsterile soil and none dealt with growth or survival for time periods long enough to determine the rate of decline of bacteria in these soils.

3.3.2d. Drying. In many areas of the world, a substantial part of the year passes with little or no rainfall, and in the absence of irrigation, agricultural soils dry out. This desiccation has been recognized for a long time as a potential stress on rhizobial survival, especially in the absence of the host plant. An additional and often accompanying stress, particularly in the tropics, is high temperatures. Because drying of soils and high temperatures often occur concomitantly, both have frequently been studied together. However, drying and high-temperature stress will be considered separately in order to determine what role each plays.

For purposes of this discussion, high temperatures are taken to be those above 40°C and normal temperatures to be between 20 and 30°C. This distinction is made because *Rhizobium* grows in culture at temperatures up to about 40°C and because most experiments have been done either at 20-30°C or at 40°C and above.

It is clear that some rhizobia can survive in dry soils for periods of time long enough to be agronomically useful. For example, in the 1890s, Kansas farmers could not get soybeans to nodulate, so the agricultural experiment station sent to Massachusetts for some soil on which well-nodulated soybeans had grown. It arrived "in a dry, pulverized condition, not unlike the dust in our roads during a dry season" (Cottrel *et al.*, 1900). This soil apparently served satisfactorily to nodulate soybeans from 1896 to at least 1898. Richmond (1926) stored some Illinois soils for 3.5 years in an air-dry state and was rewarded with satisfactory nodulation of soybeans and cowpeas provided the soil pH was above 5.8 and 4.8, respectively. Sen and Sen (1956) planted surface-sterilized seeds in Indian soils

which had originally shown good soybean nodulation and then had been stored in an air-dry state for 19 years. Only two of the nine soils gave nodulation. These investigators measured hygroscopic moisture, pH, P content, and percentage of sand, silt, clay, organic carbon, and organic nitrogen. The only parameter which possibly correlated with nodulation was hygroscopic moisture, the least-dry soils apparently giving the longest survival. The numbers of rhizobia were not determined in these experiments, and therefore the rates of bacterial decline are unknown.

In other studies, however, counts have been made. Thus, Jensen (1961) reported on *R. meliloti* stored in air-dried soils for 30-45 years and *R. trifolii* and *R. leguminosarum* for 10-14 years, but he found no correlation between length of storage and bacterial density. Pant and Iswaran (1970, 1972) observed that survival of *R. japonicum* and a *Rhizobium* strain for peanut was shortest in the more alkaline soils of pH 8.1 and 8.5. Danso and Alexander (1974) used a streptomycin-insensitive strain of *R. meliloti* to measure resistance to drying in a silt loam (pH 5.6) and loamy fine sand (pH 5.0), both nonsterile, and in sterile quartz sand (pH 6.5). The sampled soils were inoculated and incubated at 28°C in desiccators containing $CaCl_2$. The abundance of *R. meliloti* dropped initially from about 10^8 to 10^5 - 10^6/g in the dry soils, and the population level was then maintained for the 8-week measurement period. The results were similar in moist soils. In dry sand, however, the die-off was quite marked, and no viable cells remained at 7 weeks. In moist sand, on the other hand, no loss of viability took place in 8 weeks. Thus, rhizobia seem to resist drying in nonsterile soils but not in sterile sand.

In the examples above, the water content of the soils was not determined, so it is not clear how the numbers of *Rhizobium* vary with that parameter. This question was dealt with by Foulds (1971), who used several different Australian soils known to contain *R. trifolii*, *R. meliloti*, and *Rhizobium* sp. nodulating *Lotus*. The soils were allowed to air dry at 22°C, and soil moisture and *Rhizobium* numbers (using the MPN method) were determined. In the first soil, the water content had fallen from 50 to 5.1%, and *R. trifolii* had declined from 10^4 to 10^3/g dry soil, *R. meliloti* from 10^3 to 10^1, the lotus *Rhizobium* from 10^5 to 10 by 115 days. In these tests, there appeared to be no correlation between rhizobial numbers and water content. In a second soil, the moisture was initially 32%; this dropped to about 3% at 7 days and remained there until the final sample at day 42. *R. trifolii* counts, however, remained at their initial value (23×10^4/g) throughout the decline in soil moisture, for at least 14 days. By day 21, the numbers had declined 25-fold to 92×10^2; the cell density was slightly higher about day 42. Parallel declines, also unrelated to water content, were shown by *R. meliloti* and the lotus *Rhizobium*.

Hedlin and Newton (1948) followed numbers of *R. leguminosarum* and soil water content and reported that, in a nonsterile Canadian soil, rhizobial counts rose from 30×10^6 at the start to 287×10^6 at 38 days, but fell to 0 by 148

days. The water contents at the same times were 29, 22, and 6.6%, respectively. Comparable results were found with sterile soil. Vandecaveye (1927) also observed no clear correlation between soil water content and the number of surviving *R. meliloti*, *R. leguminosarum*, and *R. trifolii* introduced into sterilized silt loam (pH 5.5-6.5) from Washington. Results not entirely consistent with the above are those of Sen and Sen (1956) described previously and those of Peña-Cabriales (1978). In the latter was revealed a complex relationship between soil water content and survival of *Rhizobium*. Peña-Cabriales (1978) inoculated streptomycin-resistant strains of several species into 10 g of a silt loam and allowed it to air dry at 30°C. Upon determining the numbers of bacteria remaining at regular intervals, he found a fast stage of die-off followed by a slower stage, both apparently exponential. The time for half the population to die ranged from 0.4-0.8 days during the fast stage and 2-10 days during the slow stage. Although no differences in half-time for the fast stage were found among any of the species tested, a population of *R. japonicum* declined at two-thirds the rate constant of *R. leguminosarum* during the slow stage. What significance the break point between stages has or how soil parameters might influence the break point was not determined.

By holding the soil water content relatively constant at various levels of dryness, Peña-Cabriales (1978) was able to show that the exponential decline was slowest at high and low soil moisture and greatest at intermediate levels. It would be of interest if these results can be generalized to many strains and soils. Vincent *et al.* (1962) also found an exponential decline in numbers of *R. trifolii* when dried on glass beads.

Thus, in natural soils, evidence for a correlation between numbers of rhizobia and soil moisture is contradictory. However, *Rhizobium* is quite persistent when certain soils are subjected to drying. The following question is thus relevant: what soil types, amendments, or bacterial features provide resistance to drying?

Some evidence has already been cited that rhizobial resistance to desiccation differs between soils containing much clay and silt and those containing mostly sand (Danso and Alexander, 1974). Gilmer and Longworthy (1916) considered that hygroscopic water retained by colloidal substances in heavier textured soil was responsible for the resistance to drying. They added a suspension of rhizobia to sterilized sand or clay loam. After 10 days, the soil was air dry. The counts at 0, 17, and 27 days were 1.6×10^6, 25, and 0, respectively, in sand, and 1.6×10^6, 42,000, and 33,000, respectively, in the clay loam.

K. C. Marshall has done the most extensive research on the protective influence of clay in connection with his studies of "second year mortality" of annual clovers in Western Australia. In second-year mortality, nodulation and growth of inoculated clovers were satisfactory in the first year, but severe mortality was found in the second season, with greater than 80% of the plants being free of nodules (Marshall *et al.*, 1963). This high mortality was primarily found

in grey sands, was slightly less common in yellow sandy soils, and was absent from red sandy soils containing 6-8% silt and clay. MPN counts of clover rhizobia in the problem areas were less than 10/g soil, whereas in adjacent nonproblem areas the counts were 10^4 -10^5/g (Date and Vincent, 1962).

Marshall and Roberts (1963) amended some plots of these problem soils with a montmorillonite clay or fly ash from a powerhouse, the latter consisting mainly of burnt clay. They observed that the percentage nodulation in the second season was 28, 54, and 73% in unamended, montmorillonite-treated, and fly-ash-amended soils, respectively. They reasoned that "second year mortality" did not result from the pH of the soil, which was 5.8, but might result from the prolonged drought and high temperatures during summer. They found that most of the nodules on clovers in the second season were 5-8 cm below the soil surface and that MPN counts in midsummer gave no rhizobia at 2.5 cm. They concluded that montmorillonite and fly ash provided protection only at depths greater than 5 cm, where maximum temperatures are lower than at the surface. For example, in shallow sand in this region, temperatures in 1957-1961 at 3 p.m. averaged 50.5°C (maximum of 65.5°C) at 2.5 cm and 37.8°C (maximum of 4[illegible].2°C) at 10 cm. They stated that it was possible that other microorganisms, antibiotics, soil texture, or nutritional status might be altered by the clay amendments. In fact, Chatel *et al.* (1968), in a subsequent review of the problem of second-year clover mortality, concluded that it resulted from toxins produced by other microbial inhabitants of the soil.

Mechanical, chemical, and mineralogical analyses of problem (yellow sand and grey sand) and nonproblem (red sand, brown loamy sand, and brown sandy loam) soils indicated that the major differences between them were the content in the former of much less clay, potassium, calcium, and iron. In addition, the problem soils contained no illite clay, whereas the nonproblem soils contained trace to moderate amounts. All soils contained kaolinite (Marshall, 1964).

Marshall autoclaved and dried samples of the soils, inoculated them and then dried them overnight at 30°C with forced air. He noted that the strain involved in the second-year-mortality problem, *R. trifolii* TA1, survived drying slightly better in the nonproblem soils than in the problem soils. Problem sand amended with montmorillonite or fly ash also gave about 10-fold better survival. Amendment with haematite or goethite, two iron oxides, gave no protection to drying at 30°C. In addition, montmorillonite provided no distinct protection to *R. meliloti*, *R. japonicum*, or *R. lupini*.

Bushby and Marshall (1977a) reported on more detailed studies using a sandy soil from Tasmania (pH 6.7). The methods were similar to the previous study (Marshall, 1964), except that drying was carried out at 28°C instead of 30°C. For example, *R. leguminosarum* TA101 numbers dropped from an initial 10^6 to 676/g in soil without amendment, to 1410 in soil amended with wet montmorillonite, and to 14,100 in soil amended with dry montmorillonite. The reasons for the difference in protection given by wet and dry clay are not clear.

Certain clays protect a number of species of *Rhizobium* from the effects of drying at temperatures between 20 and 30°C. Other substances also give protection or alter susceptibility to drying. In the experiments of Bushby and Marshall (1977a), polyethylene glycol 6000, glucose, sucrose, and maltose (20% solutions) mixed with soil in a ratio of 1:1 (v/v) resulted in counts after drying of 6,920, 30,900, 45,700, and 41,700, respectively, compared to 676/g for unamended soil. Polyvinyl pyrrolidone 40,000 (20%) and glycerol (5%) afforded no protection, and polyethylene glycol 400 or 1500 (each 20%) were actually detrimental to survival. Vincent *et al.* (1962) also found that maltose protected rhizobia against the effects of drying on glass beads.

Other factors important in resistance to drying have also been reported. Pant and Iswaran (1972) found a correlation between soil phosphorus and ability to survive drying. It also appeared that high soil pH made a *Rhizobium* strain for peanut and *R. japonicum* more susceptible to drying (Pant and Iswaran, 1970; 1972). An earlier report indirectly implicated low pH in the susceptibility of *R. japonicum* and cowpea *Rhizobium* to desiccation (Richmond, 1926).

Some evidence exists that sterile soils present no safer haven for survival of rhizobia to drying than nonsterile soils (Hedlin and Newton, 1948), and bacterial capsules appear to provide no protection against the consequences of drying. The strain of *R. japonicum* recovered by Sen and Sen (1956) after 19 years from dry soil had no capsule. Bushby and Marshall (1977a) state that capsulated strains of *R. trifolii* were not consistently better protected against drying than their noncapsulated variants, although the data presented in that report do not uphold their statement.

Species differences to drying have also been established. Foulds (1971) found *R. trifolii* to be much more resistant to drying in soil than *R. meliloti* or the bacteria nodulating *Lotus*. The results of Peña-Cabriales (1978) described above point to no species differences during drying, but to potential differences in drought tolerance at constant low soil moisture. Bushby and Marshall (1977a) showed that many strains of slow-growing rhizobia (*R. japonicum*, *R. lupini*, and the cowpea rhizobia) survived short-term drying better than the fast growers (*R. meliloti*, *R. leguminosarum*, and *R. trifolii*), although within each group there was much variation. To the contrary, however, Delin (1971) observed that a strain of *R. meliloti* took eight-fold longer to be reduced to 1% of its initial number on glass beads than strains of *R. japonicum* or *R. lupini*.

Certain treatments have been claimed to increase resistance to drying. Delin (1971) holds a patent on a method to produce rhizobial strains resistant to desiccation. He selected strains that required seven times as long as the parent to die off to 1% of the initial number on glass beads. Chen and Alexander (1973) reported that bacterial growth in solutions of low water activity could confer drought resistance. Bushby and Marshall (1977a), on the other hand, found no relationship between the ability to grow in solutions of low water activity and resistance to desiccation, although the methods used for drying soil were quite different.

All told, the mechanism relating soil water loss to the numbers of rhizobia surviving desiccation is just beginning to come to light. The bacteria appear more susceptible to the effects of drying in sand than in heavier textured soils, and certain clays such as montmorillonite and a number of sugars can often provide protection. Susceptibility does not decline in sterile soil, so other soil microorganisms are not a factor. Evidence, not entirely consistent, suggests that the group of slow-growing rhizobia is more resistant to drying or survives better in dry soil than the fast-growing rhizobia. Bacterial capsules are reported to afford no protection to drying.

3.3.2e. High Temperature. Soil temperatures in some parts of the tropics and even in some temperate areas have been found to be quite high compared to the overlying air; e.g., 65.5°C at 2.5 cm below the surface (Marshall and Roberts, 1963). Survival of rhizobia in soil at high temperatures is strongly conditioned by whether or not the soil is wet or dry, and, whereas survival at moderate temperature is greater when soil is moist (Foulds, 1971, Danso and Alexander, 1974), survival at temperatures above 40°C is greatest when soils are dry. Poor nodulation of various legume seedlings in southeastern Queensland following mid- to late-summer rains was observed by Bowen and Kennedy (1959), although watering strains of the same bacteria onto rows several weeks later gave good nodulation. Since absence of moisture and nutrition were not problems in these trials, they decided to test survival at high temperatures, which they defined upon examining the maximum temperatures that allowed growth in culture. These maxima as well as those reported by Ishizawa (1953) and Mendez-Castro and Alexander (1976) are given in Table II.

Table II. Maximum Temperature for Growth of *Rhizobium* in Three Studies

	Bowen and Kennedy (1959)			Ishizawa (1953)		Mendez-Castro and Alexander (1976)
Cross-inoculation group	Number of strains	Maximum range (°C)	Average	Number of strains	Maximum range (°C)	Maximum range (°C)
Alfalfa	8	36.5–42.5	41.0	28	39–42.5	
Clover	9	31.0–38.4	33.2	5	35–37	38–39
Pea	2	32.0–32.7	32.3	8	35–37	
Cowpea group, etc.	68	30.0–42.0	35.5	49	32–42.5	
Lupine				9	32–33	
Soybean				10	32–35	42–43
Bean				8	35–37	

There are clear differences in maximum temperatures for growth, and to date alfalfa rhizobia show the highest average. Accordingly, Bowen and Kennedy (1959) chose 40°C at which to test survival, and they found that in moist sand only alfalfa rhizobia survived 24 hr, whereas all other strains tested had lost viability or were dying off in that time. They also found that, of two *Rhizobium* strains which nodulated *Centrosema pubescens*, the one with the higher maximum temperature for growth (38.5°C) apparently survived at 40°C better than one with a lower maximum temperature for growth (35.2°C); in this experiment, however, they measured nodulation and not numbers of bacteria.

In order to test adaptation to different climatic conditions, Wilkins (1967) took soils, all containing rhizobia, from warmer and cooler regions of New South Wales and subjected them to high temperatures. Although she measured nodulation and not counts, she concluded that rhizobia from warmer regions survived higher temperatures better than rhizobia from cooler regions; the conclusion is questionable, however, since initial rhizobial numbers in these different soils may not have been the same. On the other hand, her comparisons of survival at high temperatures under moist and dry conditions are noteworthy. In moist soil, alfalfa rhizobia were found to survive 50°C but not 55°C for 5 hr. In dry soil, by contrast, these bacteria survived over 100°C for 5 hr. *Psoralea* rhizobia survived 60°C in moist soil for 5 hr, but some cells were still viable at 100°C in dry soil for the same period of time. Moisture, which protects root-nodule bacteria at moderate temperature, is thus a deleterious factor at elevated temperatures. The same conclusion is reached by a consideration of routine sterilization procedures used in the laboratory. Means to amend moist soils to protect rhizobia from high temperatures have not been sought, but Bowen and Kennedy (1959) lowered temperatures significantly by covering soil with a plant canopy or plant residues.

Marshall has extensively studied rhizobial survival at high temperatures in dry soil and has not only found amendments that protect rhizobia, but he also tried to explain how they work. Marshall (1964) first demonstrated that *R. trifolii* TA1 was much less susceptible to high temperatures in dry, sterile, nonproblem soils than in problem ("second year mortality") soils of Western Australia, noting that after 5 hr at 70°C, rhizobial counts were 10^2-10^3 greater in the nonproblem soils. Clays such as montmorillonite and illite protected (gave 10^4-fold greater counts), but kaolinite did not. Similarly, of the iron oxides tested, haematite protected rhizobia but goethite did not. Montmorillonite also protected against four or more exposures to 50°C. In addition, Marshall showed that fast-growing *R. meliloti* SU47, like *R. trifolii* TA1, was protected by montmorillonite or fly ash, but that slow-growing strains *R. japonicum* QA372 and *R. lupini* CP1 survived in high numbers in the absence of these clays and were not protected further by their addition.

In a series of experiments, Marshall (1967, 1968a,b, 1970) and Bushby and Marshall (1977a,b) attempted to elucidate the mechanism by which montmoril-

lonite protects *Rhizobium* against heat in dry soils. Initially, Marshall (1967, 1968b) sought to determine whether montmorillonite absorbed preferentially to the fast-growing rhizobia. This was accomplished by measuring, through a microscope, the time it took suspended bacteria to drift a given distance across an electrophoretic cell with a known voltage, with and without added montmorillonite. Furthermore, by varying the pH of the bacterial suspension and noting any changes in mobility, Marshall determined at what pH values chemical groups on the bacterial surface became associated with H^+ or dissociated, thus providing information on the surface charge. He concluded that (1) two types of surface groups characterized the many rhizobia, a simple carboxyl surface found on all the fast growers tested except one (*R. trifolii* TA1) and a complex carboxyl-amino surface found on all the slow growers and *R. trifolii* TA1; and (2) at the highest montmorillonite densities, the strains with the simple carboxyl surface held about twice as much clay per unit area as the others. He then postulated that a closely packed clay envelope conserved cell water and protected the bacteria from drying out.

A number of criticisms can be raised against these conclusions. First is the anomaly of *R. trifolii* TA1. This strain–which sometimes did not survive in soil (thereby resulting in second-year clover mortality), was sensitive to drying and high temperatures, and was protected from the effects of high temperatures by montmorillonite–had a surface charge and responded to montmorillonite, just as did the slow-growing strains which resisted drying, high temperatures, and the protection of clay. Second, these experiments were conducted at 25°C, a temperature at which montmorillonite is only slightly effective. Third, no comparable experiments have been reported using kaolinite, which does not protect *R. trifolii* from high temperatures. An additional criticism relates to the previously discussed findings that rhizobia are more resistant to heat when in dry than in moist soils. One might expect that protection from the effects of heat would be greater if the protectant helped remove moisture from the cells rather than if it preserved the cell water, as Marshall proposed.

The last criticism was dealt with by Bushby and Marshall (1977a,b). The authors measured water sorption isotherms (that is, the amount of water held by bacteria, or Ca montmorillonite, at numerous different relative water vapor pressures) at 40°C. They found that at moderate and low relative vapor pressures, fast-growing bacteria absorbed more water than slow growers, although less than Ca montmorillonite. They also calculated that the water sorbed per gram of cells, and the water-absorbing surface, was twice as great for the fast growers as for the slow growers. Thus, the temperature-sensitive fast growers retained water better.

Since montmorillonite had a greater affinity for water than the root-nodule bacteria, they proposed that the clay envelope lowered the equilibrium water held by rhizobia, rather than slowing its removal as suggested by Marshall (1968b). The basis for this proposal is subject to a number of criticisms. (1)

Because the effect of montmorillonite on water absorption by bacteria was not determined, the authors have no evidence that the clay does in fact lower the amount of water retained by the organisms. (2) The authors also have no data on the effect of montmorillonite on rates of water loss by the rhizobia. (3) Furthermore, the anomalous strain, *R. trifolii* TA1, was not included in their measurements of water adsorption. It would be of interest to know whether *R. trifolii* TA1—which is susceptible to drying and high temperatures and is protected by montmorillonite (like other fast growers) but has a surface charge and affinity for montmorillonite like the slow growers—has an adsorption isotherm like the other fast growers or like the slow growers. It should be pointed out that Bushby and Marshall considered that montmorillonite primarily protects rhizobia from desiccation, but the most convincing data relate to protection against heat in dry soil.

Bushby and Marshall (1977b) also demonstrated that the intracellular osmotic pressure of the fast growers was consistently lower than in the slow growers; therefore, the greater water-holding capacity of the fast growers that they found did not result from a greater intracellular solute concentration.

Despite unresolved questions on the mechanism of protection by some materials, it is clear that certain clays and iron oxides do protect some rhizobia from high temperatures in dry soils, that fast growers are more susceptible to heat in dry soils, and that heat in moist soil is much more detrimental to the root-nodule microorganisms than in dry soil.

3.3.2f. Low Temperature. Low temperatures are of no concern in the tropics but are important to farmers in temperate regions. Rhizobia found in fields year after year in northern climates obviously survive freezing. Few studies of this question have been carried out, but Fred (1920) has shown that these bacteria do live through the winter. Furthermore, Wilson (1930) did nodulation-dilution counts of *R. trifolii* and *R. leguminosarum* during a winter in Ithaca, N. Y. fields, and he found no obvious decline. Ek-Jander and Fahraeus (1971) compared strains of *R. trifolii* isolated from northern and southern Scandinavia and observed that cultures of *R. trifolii* isolated in the north grew better at 10°C in culture and gave earlier nodulation at that temperature. These results, along with those of Wilkins (1967), Bowen and Kennedy (1959), and others, are clear indications that resistance to temperature stress is an adaptable characteristic of *Rhizobium* and should be considered in choosing inoculant strains.

3.3.2g. Flooding. Flooding of soil as a stress to rhizobia has hardly been tested, and early reports seemed to indicate that it is not a problem (Vandecaveye, 1927; Tuzimura and Watanabe, 1959b). More recent studies lead to the same conclusion: Osa-Afiana (1979) characterized two strains of *Rhizobium*, one each of *R. trifolii* and *R. japonicum*, for susceptibility to flooding in a silt loam, pH 6.6. Both strains survived well in flooded soils at 30°C. Although both strains declined over a 6-week period when inoculated at rather high densities, 10^9 cells/g of dry soil, their numbers leveled off at about 10^5/g for *R. trifolii* and

10^7/g for *R. japonicum*. These final densities were independent of initial inoculum size (down to at least 10^5/g). At 4°C, flooding was no greater stress to survival than other moisture regimes tested (10, 22, 35, and 45% water held/g of dry soil). Maximum water-holding capacity for this soil was 45%. At 40°C, die-off of both strains was more rapid for moist than for air-dry soil (see Section 3.3.2e), although *R. japonicum* seemed less sensitive to these combined stresses than did *R. trifolii*.

3.3.3. Biotic Stresses

As reported with physical and chemical stresses such as pH, drought, and high temperature, rhizobia have certain limits beyond which they survive poorly or not at all. This is true both in culture and soil, although the boundaries of survival may and often do differ between the two. In the case of stresses on *Rhizobium* brought about directly or indirectly by other organisms, however, the limits are less clear, and conflicting results have usually been obtained in culture medium and in soil (Vandecaveye *et al.*, 1940; Robison, 1945; Fogle and Allen, 1948; Anderson, 1957; Holland and Parker, 1966; Chhonkar and Subba-Rao, 1966). This is partly a consequence of the fact that the two habitats are acting both on *Rhizobium* and its living antagonists. In addition, the effects of antagonistic organisms on rhizobial survival in culture have generally been compared in the soil environment to nodulation or plant growth. Thus, attempts to establish whether antibiotics, predators, or parasites affect rhizobial survival in soil are complicated by effects on the host plant and on the rhizobia-legume symbiosis. Some investigations have pinpointed effects on rhizobia, and for the purpose of this report these studies will be emphasized.

3.3.3a. Plant-Associated Toxins. The seeds of subterranean clover, and to lesser extents white clover and alfalfa, contain a material toxic to *R. trifolii* TA1 on agar plates (Thompson, 1960). This toxin is extractable from the seed coat but not the embryo. Field tests showed that soaking seeds prior to inoculation, applying the inoculum directly to soil, or separating the rhizobia from seeds by coating the latter with an inert material eliminated the influence of the toxin.

Plants important in the pioneer stages of old-field successions inhibit growth of various species of *Rhizobium* on agar and in some cases reduce nodulation and fresh weight of plants (Rice, 1964, 1968). The inhibition of rhizobia by roots and root extracts of nonnodulating legumes has been reported by Ranga Rao *et al.* (1973); thus, *Cassia fistula* roots inhibited the rhizobia of *Cajanus cajan* on agar plates, and root extracts of *C. fistula*, *C. occidentalis*, and *Leucaena leucocephala* suppressed rhizobia in broth, although pea roots stimulated pea rhizobia.

Microorganisms in the rhizosphere of a nonnodulating line of soybeans were compared with those under a normally nodulating near-isogenic line (Elkan, 1962). During the first 40 days after seedling emergence, the rhizosphere of non-

nodulating soybeans supported more rhizobia, actinomycetes, and total microorganisms, but the stimulation disappeared until, when the soybeans reached maturity, the rhizosphere of these plants contained considerably fewer total microorganisms. Differences in nutrition of the bacteria were also observed between the two rhizosphere communities. In none of the above cases, however, have specific toxins yet been isolated and tested in soils, and thus the state of knowledge on stresses from plant toxins on *Rhizobium* is quite limited.

3.3.3b. Microbial Toxins. The "second year mortality" problems of subterranean clover in Western Australia resulted primarily from the numbers of *R. trifolii* falling off in the second and subsequent years. No such mortality was found for lupines and serradellas or for *R. lupini* (Chatel *et al.*, 1968). The major cause of *R. trifolii* TA1 die-off was traced to a water-soluble soil factor which strongly inhibited *R. trifolii* TA1 and *R. meliloti* SU47 but had no such effect on *R. lupini* WU7. The extracted factor was removed from soil by centrifuging the soil sample over a micropore filter. The toxin was found in the growing season in soils drying out after a light rain, and the inhibitory substances were quite labile, lasting usually 1 day. Of 59 microorganisms isolated from affected soils, 9 (including an actinomycete and many fungi) inhibited both *R. trifolii* and *R. lupini* on agar plates and 19 inhibited *R. trifolii* alone. No isolate inhibited *R. lupini* without also inhibiting *R. trifolii*. Based on these data, it was postulated that the "second year clover mortality" problem resulted from a microbial toxin inhibitory to *R. trifolii* TA1. The problem was solved by isolating strains of *R. trifolii* which survived in these fields and using them for the preparation of inoculants. The toxins or extracts are not known to have been tested against the presumably resistant strains or to determine their effect on the survival of bacteria in soil.

Beggs (1961, 1964) described another problem of clover establishment in certain soils in New Zealand. The problem was related to oversowing of seeds on "sun-bleached" silt loam soils, especially in the presence of danthonia plants, although clover could be established by cultivating and liming the soil or sterilizing it with formalin. Parle (1964) added nitrogen fertilizer and got good clover growth until the available nitrogen level fell. He found that the problem was related to danthonia plants present in the area; with live danthonia present, clover failed to nodulate, and no clover rhizobia could be found in the soil. When the live danthonia plants were removed, Parle (1964) observed that 23% of the test clover plants nodulated and 1670 rhizobia were present per gram of soil; with only dead plants present, 45% of the test clovers were nodulated and there were 14,300 rhizobia per gram of soil. Thus, there was apparently a potent toxin against *Rhizobium* in the danthonia rhizosphere.

Parle (1964) compared a *Rhizobium* strain from a small patch of clover established in the problem fields with the commercial strain. In pot experiments, he found 2-3 times as many nodules formed by the new isolate in the presence of danthonia. Extracts of danthonia roots and adhering soil gave a zone of in-

hibition on agar plates 50% greater with the commercial strain than with the isolate. It is not known how this toxin acts against the rhizobia or whether it is formed by the plant roots or from microorganisms associated with the roots. Because attempts to extract toxins from ground danthonia roots were unsuccessful, whereas colonies of bacteria associated with the roots were antagonistic to rhizobia, Parle (1964) suggested that the toxin was of microbiological origin.

These two examples of problems presumably caused by microbial toxins appear to have been solved by finding strains of rhizobia which survive where the originally inoculated strain did not. A third case of difficulty in establishing clover has been described by Holland (1962) and Holland and Parker (1966). On certain newly cleared fields in Western Australia, subterranean clover failed to nodulate when inoculated with *R. trifolii* strains Na30 and TA1. However, when the soil was fallowed or cropped to cereals for 3-5 years, normal nodulation took place. The soil was a grey sand (pH 6.8) over a bright-yellow clayey sand. Aqueous extracts from newly cleared fields applied to agar plates seeded with *R. trifolii* showed the presence of a toxin, but this finding was obtained only twice with certainty; however, the toxin could also be extracted from leaves of affected clover plants, which had turned purplish red.

The authors compared nodulation, growth of inoculated clover, and rhizobial counts in nonsterilized soil and soil previously treated with a fumigant. In these pot experiments, 42 of 42 plants nodulated in the treated soil but only 4 of 43 were nodulated in the untreated control. The numbers of rhizobia in the untreated and fumigated soil were 0 and 3000/g, respectively. Of a large number of microorganisms isolated from the problem and nonproblem fields, 27% from newly cleared fields and only 10% from established clover fields produced a toxin against *R. trifolii*. When these microorganisms were inoculated along with *R. trifolii* into sterile sand cultures of clover, nodulation and growth were normal, and no toxicity was observed. However, if sucrose or straw was added to the sand together with individual microorganisms, many fungi were seen to inhibit growth and nodulation and to cause purplish-red leaves to develop on the clover. None of the microorganisms from established clover fields inhibited the clover, only those from the newly cleared fields. Chatel and Parker (1972) pointed out that it remains to be shown that the soil toxins are produced and inhibit rhizobia in unamended soils (or that the problem fields are comparable to the amended experimental soils) and that the substances are microbial metabolites.

Hely *et al.* (1957) were unable to identify a presumed microbial antagonist to clover rhizobia at another location in Australia, but they obtained good results by sterilizing the soil or amending it with charcoal (100 lb/acre). It seems as if problems resulting from microbial toxins have been especially prominent with, or perhaps even limited to, newly cleared fields and fields newly cropped to legumes, at least in the case of clovers. The established microbial populations may be antagonistic to newly inoculated rhizobia.

Fungi. There are numerous studies of fungal toxins against rhizobia, but the information gained is generally not directly related to field problems. Thus, Thornton *et al.* (1949) isolated strains of *Penicillium* which inhibited *R. meliloti* on agar plates. Chhonkar and Subba-Rao (1966) found that some species of fungi isolated from nodules of common legumes produced toxins against several species of *Rhizobium* on agar, and they demonstrated that one fungus inhibited growth and nitrogen fixation of inoculated *Trifolium alexandrinum*.

Actinomycetes. Van Schreven (1964) tested 24 actinomycetes on agar against many strains and species of *Rhizobium* and reported that some of the bacteria were susceptible to antibiotics produced by the actinomycetes. Abdel-Ghaffer and Allen (1950) tested 300 strains of *Streptomyces* for antagonism to several species of *Rhizobium* in culture. However, they were unable to demonstrate the production of antibiotics in soil containing many streptomycetes. Landerkin and Lochhead (1948), Fogle and Allen (1948), Damirgi and Johnson (1966), and others also found actinomycete antagonism in culture and in some cases inhibition of nodulation in soil. Van Schreven (1964) has summarized the early literature.

Bacteria. Soil bacteria have been isolated which produce toxins or antibiotics to rhizobia in culture (Hattingh and Louw, 1969; Smith and Miller, 1974). Rhizobia themselves may synthesize bacteriocins and antibiotics (Roslycky, 1967, Schwinghamer and Belkengren, 1968; Schwinghamer, 1971). No convincing evidence exists that one species of *Rhizobium* is more susceptible to microbial antagonism than another (Landerkin and Lochhead, 1948; Fogle and Allen, 1948; Abdel-Ghaffer and Allen, 1950; Van Schreven, 1964; Chhonkar and Subba-Rau, 1966). Moreover, no evidence was given in any of these papers that microbial antagonists in question have any effect on rhizobial survival in soil.

3.3.4. Parasites and Predators

3.3.4a. Bacteriophages. Bacteriophages can rapidly decimate a host bacterial population in laboratory media, but no data exist to show convincingly that these parasites do appreciable harm to rhizobia in soil. Fatigue of alfalfa soils has been ascribed to a bacteriophage which attacks alfalfa rhizobia, and Demolon and Danez (1935, 1939) isolated a bacteriophage-resistant *Rhizobium* which in some years as much as doubled the alfalfa yields on problem soils in France. However, no evidence was offered that the beneficial effect of the resistant strain was attributable to its bacteriophage resistance. An attempt by Vandecaveye *et al.* (1940) to repeat these experiments in Washington failed; thus, inoculation of alfalfa with *R. meliloti* resistant to a bacteriophage isolated from fatigued fields did not improve yields, although nitrogen fertilizer resulted in nearly a threefold increase in yield. Bacteriophages against *R. meliloti* have been isolated in

every alfalfa field examined in New York, and these viruses appear to be common in such fields but rare in land not cropped to alfalfa (Katznelson and Wilson, 1941). Bacteriophages active against clover and pea rhizobia were isolated from soil in England maintained for 100 years in clover and yet no "clover sickness" appeared, and these viruses were like bacteriophages found in pots with "sick" clover both in numbers and host range (Kleczkowska, 1957). Bacteriophages active against the *Rhizobium* nodulating *Lotus* and other rhizobia appear equally without effect in soil (Bruch and Allen, 1955; Laird, 1932). It thus seems that bacteriophages parasitic on *Rhizobium* threaten no immediate harm to growth of legume crops in the field.

3.3.4b. Bdellovibrio. Another parasite, *Bdellovibrio*, has been found to reduce rhizobial density in culture. Despite this, however, rhizobia persist in relatively large numbers in the presence of this parasite in both culture and soil (Keya and Alexander, 1975). Furthermore, this parasite has so far not been implicated in problems of growing legumes (Parker and Grove, 1970).

3.3.4c. Protozoa. Predation is one of the many ways rhizobial populations are decreased in soil, and predation by protozoa in liquid culture and in sterile and nonsterile soils has been studied extensively at Cornell University (Danso *et al.*, 1975; Danso and Alexander, 1975; Habte and Alexander 1977, 1978).

In culture and in soil, neither of two test protozoa, *Tetrahymena pyriformis* or *Colpoda* sp., was able to reduce the initially high numbers of *R. japonicum* (10^9-10^{10} cells per milliliter or per gram) to fewer than 10^5-10^7 in 1-4 weeks (Habte and Alexander, 1978). Although these numerical reductions are significant, the remaining rhizobia are probably sufficiently abundant to nodulate soybean seedlings (Weaver and Frederick, 1974). Habte and Alexander (1978) determined that the persistence of rhizobia did not result from a decline in the rate of protozoan feeding caused by rhizobial excretions or by the lack of energy for the predators. Rhizobial persistence also did not come about because of the availability of alternate prey or because of the inedibility of the surviving bacteria. Rather, survival of the rhizobia appeared to reflect a steady state resulting from reduction of the rhizobial population by predation and replacement of the cells thus consumed through reproduction by the remaining population. This was demonstrated in sterile soil by preventing rhizobial reproduction upon addition of chloramphenicol: in the absence of predators, *R. japonicum* numbers remained high, but in their presence, the bacterial density fell to 10^2/g or less. In natural soil without inhibitor, moreover, *R. japonicum* dropped slightly from 2×10^8 to 6×10^6/g in 1 month; in the presence of chloramphenicol, the bacterial abundance dropped to 6×10^2/g because the protozoa continued to feed whereas the bacteria could no longer reproduce. Hence, rhizobia appear capable of reproducing in natural soil, and as long as this is possible, the root-nodule bacteria can remain in substantial numbers in the presence of protozoan predators.

The evidence to date points to microbial and perhaps plant toxins as being

major biotic factors governing rhizobial survival, at least in certain soils. Methods of overcoming these stresses include removal of plant parts (physically or by burning), adding charcoal to newly cleared fields (Hely *et al.*, 1957; Turner, 1955; Beggs, 1961, 1964; Holland and Parker, 1966), and especially the selection of toxin-resistant *Rhizobium* strains (Parle, 1964; Chatel *et al.*, 1968; Schwinghamer and Belkengren, 1968). The importance of selecting strains of *Rhizobium* which survive well in soils newly cropped to the host legume will be discussed in the next section.

3.4. *Rhizobium* Strain Differences

The soil and the legume host, singly and together, effect a great selective pressure on the *Rhizobium* symbiont. It is possible to select *Rhizobium* strains in the laboratory that are resistant to drying (Delin, 1971), antibiotics (Obaton, 1971; Danso *et al.*, 1973), and high salt concentrations, low pH values, and moderately high temperatures (Mendez-Castro and Alexander, 1976). Natural conditions, however, are much more complex than culture media, and in any field newly planted to a legume, it is not clear what characteristics must be selected for. The traditional criterion for choosing an inoculant strain, effectiveness, has in the past been quite narrowly defined and, as Brockwell *et al.* (1968) and Chatel *et al.* (1968) have shown, must be supplemented by several more criteria: ability to invade the roots in competition with ineffective, indigenous strains; ability to grow outside the rhizosphere; and capacity to withstand environmental extremes and persist in the soil in adequate numbers. It should be clear from the previous sections that strains meeting these criteria frequently are unavailable in commercial inoculants. An obvious question then is how to find these strains.

In consideration of the importance of the above-mentioned criteria, it is worth stressing that the inoculation of legumes with a presumed effective strain does not always lead to good nitrogen fixation. In part, this may result from a mismatch between host legume and *Rhizobium* strain. For example, Vincent (1954) found that rhizobia isolated from red and white clovers in a field survey were effective on red and white but ineffective on subterranean and crimson clovers. Effective isolates from subterranean and crimson clovers, on the other hand, showed variable effectiveness on red and white clovers. Brockwell *et al.* (1968) tested rhizobia isolated from subterranean clover grown in the Mediterranean region on several varieties of that clover used in Australia, and they observed that effectiveness varied considerably depending on variety of clover, rhizobial isolate, and temperature. It has also been shown that indigenous white clover in Ireland and Scotland gave higher yields when inoculated with rhizobia isolated from the wild ecotypes than when inoculated with commerical clover strains (Lowe and Holding, 1970; Sherwood and Masterson, 1974). Thus, crucial to high yields is proper matching of the two symbionts.

An effective *Rhizobium* strain may be unable to compete with indigenous,

ineffective strains for nodulation. In experiments with subterranean clover, Harris (1954) inoculated seeds with a combination of two effective and two ineffective strains. One effective strain in all combinations gave good nodulation and growth in the presence or absence of the two ineffective strains. In contrast, the second strain, which was effective when inoculated alone, gave poor nodulation and growth when the two ineffective strains were added at the same time. Robinson (1969a,b), however, found that clover plants tended to select for effective *Rhizobium* strains in agar, even when the effective strains were outnumbered by a factor of 10,000. Bell and Nutman (1971) were able to suppress effective native strains of *R. meliloti* for only 1 year by massive inoculation (50,000 rhizobia/seed) of alfalfa with ineffective rhizobia. In the second year, the indigenous, effective strains had overgrown the plots. Brockwell *et al.* (1972) reported similar results. In all probability, when more than one infective strain is present, factors in addition to effectiveness play a role in determining which strains predominate in nodulation, but effectiveness very likely plays an important role.

It is also possible that an inoculant strain gives excellent nitrogen fixation for a short time, but then it declines in abundance. Such was the case with *R. trifolii* TA1 inoculated onto subterranean clover in Australia, its inability to persist in certain soils leading to "second year clover mortality" (Chatel *et al.*, 1968). Although Brockwell *et al.* (1968) recommended early nodulation as one criterion for strain selection because early nodulation is correlated with high initial yields, it may be more important to determine yields for a long period, especially for those annuals which are planted seasonally but the growth of which is interrupted by winter, summer drought, or other conditions unfavorable to the plant (Lie, 1971). Some evidence has been gathered that yields of clover (Mulder *et al.*, 1966; Sherwood and Masterson, 1974) and soybean (Scudder, 1975) improve with time, yet no clear connection between yields and rhizobial effectiveness or survival has been made.

Certain "volunteer" strains of *R. trifolii* have proven to survive better in the field and give better yields than commercial inocula (Chatel *et al.*, 1968; Gibson *et al.*, 1976; Roughley *et al.*, 1976). Gibson *et al.* (1976) inoculated clover with five serologically distinct strains known to form effective symbioses with *Trifolium subterraneum*, and they sampled nodules present on the plants for four seasons. No naturally occurring strains were evident the first year. By the third season 10% and by the fourth season 9% of the isolates were not identifiable as any of the inoculant strains. Of the newly appearing rhizobia, over three-fourths were as effective or more effective than the bacteria used for inoculation. The authors suggested three sources for these new strains: (1) they were present at the beginning of the experiment in undetectable numbers and responded to a favorable environment in the rhizosphere; (2) genetic changes in the indigenous ineffective population; or (3) contamination from external sources. In addition, the inoculated strains may have changed their effectiveness and serological pat-

terns, although the results of Brockwell *et al.* (1977) tend to rule this out. Chatel *et al.* (1968) suggested that the ultimate source of the indigenous *R. trifolii* in Australia (legumes with fast-growing rhizobia not being native) was the first settlers from Europe, and that therefore these strains had 130 years to adapt and be selected in the soils. Regardless of their ultimate source, the volunteer strains show characteristics that fit the more complex criteria for successful inoculant strains (Brockwell *et al.*, 1968; Chatel *et al.*, 1968), including the ability to colonize the rhizosphere and infect legumes, participate in an effective symbiosis, and persist. The factors that allow strains to persist in these soils are at present almost entirely unknown.

Because of the considerable variation in tropical soils, weather patterns, and legume cultivars, an important concern is how to select and isolate such successful volunteer strains simply and rapidly. Can persistent strains be selected in laboratory experiments? In field experiments, should one sow uninoculated seeds and wait for a few vigorous plants to appear, or should the seeds be inoculated with an effective nonpersistent strain? Of 18 new isolates of *R. trifolii* found by Gibson *et al.* (1976) in the fourth season on uninoculated plots, all were as effective as or more effective than the commercial strains they used. Also, of 10 new isolates found in plots originally inoculated with a fairly persistent and effective strain, all were effective. Of 11 isolates recovered from plots originally inoculated with a poorly persisting strain, 5 were effective and 6 ineffective. Thus, the preceding evidence does not provide a clear strategy to use in trying to find high-performance *Rhizobium* strains. Nevertheless, such a procedure or set of procedures should be developed. Screening procedures have been made a high priority in some regions (Brockwell *et al.*, 1968; Bergersen, 1970; Lie, 1971; Graham and Hubbell, 1975), and possibly it will not be necessary to test every soil and wait until the third season before effective isolates appear.

4. Conclusions and Recommendations

After over three-quarters of a century of study, scientists are still only beginning to probe *Rhizobium* ecology. Many reports make no distinction between legume nodulation and rhizobial survival or confuse effects on the host plant or on the symbiosis with effects on the microsymbiont. Numerous stresses have been uncovered which potentially limit rhizobial survival, but there is little convincing evidence that these stresses are actually responsible for the microorganisms' failure to persist. Only a very few studies have looked at causal relationships. In most cases in which poor establishment of a legume results from poor rhizobial survival, scientific inquiry ended once a persistent strain was discovered. The great potential for determining the mechanism of persistence by comparing strains which survive with closely related strains which do not has not been pursued. For regional agricultural reasons, most research on *Rhizobium*

ecology has been carried out with *R. trifolii*, *R. meliloti*, and *R. japonicum*, whereas virtually nothing is known about the survival of the other species, expecially the tropical and cowpea-type rhizobia.

Yet, despite the lack of progress in answering the scientific questions, it is important to make recommendations for field practice, and thus certain tentative conclusions must be drawn from the limited information now available. The most serious natural environmental threats to rhizobial persistence appear to be: (1) pH, especially in the acid range; (2) drying, primarily in sandy soils; (3) high temperatures, particularly in moist or sandy soils; and (4) microbial toxins, especially in newly cleared land.

Obviously it is important to establish first whether a problem in obtaining high legume yields can be traced to poor rhizobial survival or to some other possibility. But to avoid expected problems, certain steps and precautions can be followed.

When in doubt about whether effective strains of *Rhizobium* are present, farmers should be encouraged to inoculate. In choosing an inoculum, priority should be given to a strain proven to be effective with the plant variety and known to persist in the soil type under consideration. If the soil pH is low (e.g., under pH 5.5-6.0 for *R. trifolii*, *R. meliloti*, *R. leguminosarum*, and *R. phaseoli* or below pH 5.0-5.5 for *R. lupini*, *R. japonicum*, and members of the cowpea group of rhizobia) and difficulty in establishing the legume is a good possibility, then the recommendation is to lime the soil or pellet the seed with lime. If soils are very alkaline, gypsum might be recommended. Inoculant strains for legumes in saline, alkaline, or acid soils should be isolated from effective nodules or plants growing on similar soils.

Dry sandy soils are a potential threat to survival of some species of *Rhizobium*, and the suggested remedies, aside from irrigation, are using a resistant strain of *Rhizobium* or, possibly and if practical, adding montmorillonite, illite, or ash to the soil. High temperatures pose a substantial threat, especially if soil is likely to be moist, or in light-textured soils that are dry. Means of alleviating high-temperature stresses on rhizobia are to adjust planting date, to maintain a plant cover, or to cover the exposed soil with plant remains. However, caution is in order if the field is newly cropped to a legume because leaving plant residues may be counterproductive. Changing the soil microflora is not as easy as removing one crop and replacing it with another, and in several instances, enough microorganisms which colonized a field produced toxins antagonistic to newly inoculated *R. trifolii*. The old plant residues possibly stimulated the growth of these antagonistic organisms. Thus, in this case, removal of plant residues may be desirable. Of primary importance is defining more precisely the reasons for poor survival of many rhizobia or of rhizobia in certain circumstances. At the same time, but particularly with this information, strains should be sought that are capable of enduring natural stresses on the root-nodule bacteria so that farmers and consumers can get greater benefit from the nitrogen-fixing activity of *Rhizobium* living in association with leguminous plants.

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