1983

Numbers and Activity of Nitrogen-Fixing Bacteria Associated with Spring Wheat

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NUMBERS AND ACTIVITY OF NITROGEN-FIXING BACTERIA ASSOCIATED WITH SPRING WHEAT

BY

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A thesis submitted
in partial fulfillment of the requirements for the
degree Master of Science
Major in Microbiology
South Dakota State University
1983

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BACTERIA ASSOCIATED WITH SPRING WHEAT

This thesis is approved as a creditable and independent investigation by a candidate for the degree, Master of Science, and is acceptable for meeting the thesis requirements for this degree. Acceptance of this thesis does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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Acknowledgements

I wish to dedicate this thesis to my family, whose continued trust and support are a part of everything I do.

My sincere thanks to Dr. Robert M. Pengra, my advisor, who made this work possible. I also wish to thank Miss Kay Lynn Slunecka and Mr. Wilbur Reitzel, who were my friends throughout.

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INTRODUCTION

Much of the food production in the world today depends on the presence of an adequate supply of nitrogen in the soil. Whether farming methods are simple or intense, nitrogen in usually the primary limiting factor in this production. In order to relieve this limitation, much of the world's agriculture has turned to the commercial manufacture and application of nitrogen fertilizers to increase crop output and to bring new lands into production.

The manufacture of nitrogen fertilizer is an energy intensive process requiring large amounts of petroleum. The increasing price of fossil fuels is then passed on in the form of higher food prices, making it increasingly difficult to meet the growing world-wide demand for food. On a global scale, the Haber-Bosch method of producing chemical fertilizer consumes more than 2 million barrels of oil daily.

As an alternative to this type of fertilization, the process of nitrogen fixation has come under close study. Significant contributions of nitrogen to crop production have been well documented in the cases of the legume-Rhizobium symbioses, and in the contribution of nitrogen to paddy rice by bluegreen bacteria (6). One aspect of nitrogen fixation that has not been as clearly defined, however, is that of chemoheterotrophic associative nitrogen-fixing bacteria.

There have been many encouraging, but also some contradictory, reports of plant growth increases caused by bacteria growing in the rhizosphere (5, 7,29,30,38). Any advancements made in this area could have significant effects on the production of non-legume crops such as corn and wheat. Also, since new discoveries involving nitrogen fixation by root-associated bacteria could potentially be used in nearly any type of farming, it represents an important area of study.

LITERATURE REVIEW

The fixation of molecular nitrogen by bacteria in vitro was reported as early as the late 1800's by Winogradsky (44). In this country, Skinner (39) reported in 1928 that Bacterium aerogenes (Escherich) Chester grew sufficiently to produce turbidity in Koser's medium, even though the uric acid was omitted, making it essentially nitrogen-free. The bacterium was a gas-forming slime producer. He also reported nitrogen fixation by Bacterium radiobacter and by Bacterium radicicola (39).

A nitrogen balance study in the Broadbalk continuous wheat experiment carried out between 1843 and 1967 in England, showed an average annual gain of 34 kg/N ha, removed as straw and grain (16). However, values that have been extrapolated from acetylene reduction assay of soil cores seem much lower, 2 to 3 kg/N ha (11). It was shown that the nitrogenase activity of soil cores containing wheat was significantly higher that bare soil (29). Other long-term experiments by researchers at Rothamstead Experiment Station, England, suggested that considerable amounts of nitrogen gain occured from nonsymbiotic nitrogen fixation and that the nitrogenase activity was highly and positively correlated with soil moisture levels (11). Pederson, et al, concluded from these studies that bacterial or algal nitrogenase activity is associated with many plant roots, especially in tropical and subtropical regions (34).

In the Broadbalk experiment a large part of observed nitrogen fixation was attributed to bluegreen bacteria, but significant amounts were also attributed to anaerobic or facultatively anaerobic bacteria. Nelson, et al, isolated nitrogen-fixing strains of Enterobacter cloacae, Bacillus

macerans, and B. polymyxa from wheat roots in Oregon (28). Their attempts to correlate root-piece activity with enrichment have been unsuccessful.

Results obtained by Wright and Weaver (45) illustrated an almost ubiquitous association between diazotrophic bacteria and grass roots in subtropical regions. Populations of bacteria on the roots seem potentially high enough for significant rates of nitrogen fixation to occur in a relatively short time when the proper conditions for proliferation occur. Two species of bacteria, Klebsiella pneumoniae and Enterobacter cloacae, were the principle diazotrophs isolated from the roots. Grass species, soil pH, or soil texture did not seem to influence the populations of the bacteria.

Schank, et al (38), found that when Digitaria sp. growing in non-sterile soil were inoculated with Azospirillum brasilense, yield increases with the live bacterial inoculants were 8.5%. The higher yielding plants had significantly lower percent nitrogen, but when total nitrogen of the tops was calculated, the inoculated had a higher total N than did the uninoculated ($P=0.05$). Data from sandy and clay soils show significant ($P=0.02$) green weight and dry matter increases from inoculated plants. Inoculation increased the dry matter forage yields 23% in sandy soil and 8.5% in clay soil. Their observation, based on plant appearance and root mass, in addition to the previous data, were that inoculation caused plants to grow larger and faster, and to mature earlier. This resulted in a lower %N than that of the uninoculated plants, but a higher dry matter yield. It was noted that these increases were all seen, although reduction of acetylene could not be

detected.

Nur, et al (30), when inoculating Zea mays and Setaria italica with Azospirillum spp. in nonsterile soil, found that the inoculated plants showed increases in plant dry weight and total nitrogen content when compared to controls. The %N of the shoots was higher in the uninoculated plants, but differences were not significant. In the plants inoculated with a strain designated "Cd" there was a 128% increase in total nitrogen. No azospirillum cells were found in the roots of the controls. Overall, increases in total nitrogen content of inoculated plants were between 50-200%. Acetylene reduction was detectable within 6 hours of inoculation.

When Bowen and Rovira (7) studied the development of roots in sand and in agar, they reported that in some cases, the presence of microorganisms greatly stimulated growth. However, other areas of their study also showed marked decreases in plant growth with some duplicate test plants responding differently. Their examination of six-week old subterranean clover grown in sand inoculated with soil suspensions revealed marked differences between the root hairs of plants grown in sterile and nonsterile sand. In nonsterile treatments, root hairs were sparse and less well developed. Their results showed that the rhizosphere population can significantly effect the development of roots and root hairs. They concluded that the activity of microorganisms causing root and root hair supression in the field could materially influence crop yield (7).

Mixed inoculations were used by Bennett and Lynch (5) to study bacterial growth in the rhizosphere of gnotobiotic cereal plants.

It was found that when cells of mycoplana, psuedomonas, and curtobacterium were co-inoculated onto wheat, large differences were observed in the growth of each species as compared to when they were inoculated monaxenically. The numbers of pseudomonads during the early stages of plant growth were increased by the mixture, though the final population was similar to when it was used as sole inoculant. In contrast, the effect on mycoplana was one of supression, with the final population only 10% of that when it was the sole inoculant species. The most marked effect was on the cells of curtobacterium, which increased slightly in numbers during the first 24 hours after inoculation, but then disappeared completely. Evidently it was a poor competitor under experimental conditions.

With wheat, the bacteria were found to aggregate along the length of the root apparently in the intercellular spaces, or in discrete microcolonies, some of which appeared to be on the surface of root cells and not in the intercellular spaces (5).

Starkey (40) in 1929, studied plant-bacteria relationships regarding the various growth stages of the various host plants. Different plants, showing a variety of growth characterisics, affect soil populations differently. He found that the influences of many plants are not great in the early stages of growth and that the maximum effects generally appear only after the plants have attained considerable size, have reached the height of vegetative growth, bloomed, or have started vegetative degeneration. Subsequent to the death of the plants there was generally a pronounced decline in the number of bacteria. He concluded

that this periodic influence of the plants may be logically correlated with the periods of youthful root development, of stabilized root activity, and finally of root degeneration and death. It was also noted that there appeared to be a tendency for the bacteria in the unplanted soils to decrease throughout the period of the study. This he expected, since in fallow soil the readily decomposable organic matter is continuously depleted. It was concluded that the development of higher plants has in practically all cases increased the abundance of bacteria which are detected by plate methods (40).

Starkey (41), in a review of interrelations of microorganisms and plant roots, concluded that the non-sporulating, Gram-negative rods are the most prominent group of bacteria in the rhizosphere, as shown by direct staining and by agar plate recovery methods. He went on to report that microbial populations in the rhizosphere are quantitatively and qualitatively different from that of the soil. The aerobic cellulose decomposing bacteria, anaerobic gas-producing bacteria, and anaerobes in general were more abundant in the rhizosphere of wheat and mangels, whereas the nitrifying and anaerobic cellulose decomposing bacteria were less numerous. Azotobacter showed no apparent rhizosphere effect. He noted that these conditions are continuously changing because the rhizosphere population is determined to a large extent by the root metabolism, which is affected by soil and climatic conditions, and by the stage of plant growth.

The exudation of organic compounds from intact roots had been reported for many plant species (2,18,22) though many of these are water-soluble exudates and provide no information on the site of root exudation. There

are studies that indicate greater exudation of ninhydrin-positive compounds occurs at root tips, in comparison with older parts of the root (32).

Barber and Lynch (3) grew barley plants in solution, either axenically or with mixed populations. They found close agreement between the calculated and the observed biomass produced in the first four days, but thereafter a much greater population of organisms was present than could be supported by the quantities of carbohydrate apparently released by the plants which grew under axenic conditions. Even ignoring the energy that would have to be used in maintaining the population, the yields of bacteria were much greater than could have been derived from the quantities of carbohydrate exuded under aseptic conditions, particularly at the later harvests.

This suggests that microbes on and around the roots enhanced the release of substrates possibly both directly by the production of substances which stimulate the process, or indirectly by utilizing the exudates and preventing their buildup in solution, thus increasing outward diffusion. They finished by concluding that apart from the zones immediately around recently incorporated plant and animal residues, an appreciable and continuous microbial activity in soils can only be expected in the rhizosphere.

Earlier (40) it had been observed that young roots exert comparatively slight effects on soil organisms. Further, periods close to fruiting or advanced growth were where the influences were most marked. This effect was attributed to the quantity and quality of organic excretions by roots.

In examining the release of radiolabeled exudates into sterile and nonsterile soil by barley and wheat, Barber and Martin (4) found that growth of the shoots was unaffected by soil treatment, but that root growth was considerably greater in the nonsterile soil. Depending on plant species and day length, the sterile roots released material equal to 7-13% of the total dry matter production of the plants during three weeks. In unsterilized soil, the losses increased to 18-25%. A higher loss of material by barley than wheat was also apparent, and was probably responsible for the higher numbers of bacteria present. Moisture also appeared to have considerable influence on the amount of soluble material exuded by the roots.

In a previously cited study of root-microbial interrelationships (41), it was seen that one of the most consistent results was the presence of a higher percentage of amino acid requiring bacteria in the groups isolated from the rhizosphere than in the groups obtained from the soil. Among the preformed organic materials frequently required was the sulfur-containing amino acid methionine. Since vitamins and amino acids are susceptible to decomposition, they are likely to be more available in those regions of the soil where there is extensive root or microbial development which would supply a continuing source of these compounds.

Numerous rhizosphere studies (29,40,41) indicate that the abundance of bacteria is affected by the kind of plant and its stage of development and vigor. The greatest effect occurs during periods of active plant development, and the effect disappears promptly upon death of the plant.

Plant vigor, however, is also affected by illumination, humidity,

temperature, and disease. These effects will then be reflected in the microbiological status of the rhizosphere. To some degree, the rhizosphere population is also directly affected by soil conditions.

Coker and Schubert (10), when studying CO_2 fixation in soybeans found that the availability of photosynthetically reduced carbon (photosynthate) is the major factor limiting symbiotic nitrogen fixation in agronomically important legumes. The reduction of nitrogen and the assimilation of NH_3 require large amounts of photosynthate to support the increased respiratory burden of nodulated roots and to provide the carbon skeletons necessary for synthesis of organic forms of nitrogen which are exported from the nodule to the leaves and pods. In many cases, these energy costs may represent 15 to 35% of the total photosynthetic capacity of the host. For this reason, any factors which affect the rate of photosynthetic carbon reduction have a direct effect on nitrogen fixation.

One legume study, conducted during a 9-day period of active photosynthesis and nitrogen fixation showed this: 32% of the total photosynthate was translocated to the nodules, where 5% was utilized in nodule growth, 12% was consumed in respiration, and 15% was returned to the shoots via the xylem as amino compounds generated from nitrogen fixation (6).

Neyra and Dobereiner (29) reported that the seasonal pattern of nitrogenase activity associated with plant ontogeny and the genotypic differences observed with several plants show that the physiology of the host can control the level of nitrogenase activity of the bacteria associated with the roots. An example of this activity would be

the highly specific association of a nitrogen-fixing Bacillus sp. with a genetically defined line of wheat (21). This indicates the potential of plant breeding as a tool for acheiving better plant-bacteria associations with greater nitrogen fixing ability.

It was also reported that (29) in general, maximum nitrogenase activities are found during reproductive growth of plants. In field-grown maize, two peaks of nitrogenase activity have been observed. The first peak is associated with silk emergence, and the second peak appeared at the onset of grain-filling. Conversely, very little nitrogenase activity is observed before taselling of the plants, and after mid grain-filling. In field-grown sorghum, maximum activities were reported at flowering. Maximum nitrogenase activity in rice was observed at the heading stage, and then declined rapidly. It was proposed that the competition for available photosynthate by the grain could be the cause for the observed decline of nitrogenase activity during the seed-filling stage.

Peat, et al (33), found that in soybeans, removal of reproductive structures caused a marked reduction in nitrogenase activity, which was reflected in the reduced nitrogen content of the treated plants when compared to those that did not have reproductive structures removed. Their work was based on a pattern stated by Brun (9) that the seasonal nitrogen fixation of soybeans has three phases: 1) during the vegetative period, when the fixation rate is low, 2) when the fixation rate increases rapidly and reaches a peak sometime after flowering, and 3) when the nitrogen fixation rate declines rapidly during the early seed-filling period. Peat, et al, suggested that although the

presence of reproductive structures may promote nitrogen fixation during the early part of the reproductive period, the rapidly growing seeds produced from these structures may later compete successfully with the roots and nodules for photosynthate (33).

Livingstone and Patriquin (22) and Patriquin and McClung (31) found that an association between roots and bacteria, similar to those in tropical grasses, occurs in the temperate salt marsh grass, Spartina alterniflora Loisel. The acetylene reduction by this system was at maximum during seed-filling. There was a high degree of correlation between acetylene reduction at 20^{o} C and the total ethanol soluble carbohydrates of below ground tissue measured two weeks previous to the reduction test ($P=0.05$), but not between those measured on the same day. This indicated that the seasonal variation in acetylene reduction is related to seasonal changes in carbohydrate concentration of below ground tissue. The fact that there was a two week lag between underground carbohydrate production and peak acetylene production was attributed to the finding that most of the nitrogen-fixing bacteria found in association with S. alterniflora required malate, not sugars to fix nitrogen.

In a study conducted by Martin (24) using wheat, ryegrass, and clover grown in nonsterile soil, it was found that the control treatment with no plants gave high bacterial counts at the first wash, shortly after wetting the air-dried soil, but the numbers decreased significantly ($P=0.001$) with succeeding washes until a steady value was reached at the fourth week. Growth of each of the three plants reversed this trend. The numbers of bacteria in the water leached from the pots increased to

give a double peak, the first peak coinciding with the beginning of flowering. There was a significant decrease (P=0.001) in bacterial counts of the solutions leached from the wheat pots from the seventh to the eleventh week. The wheat was almost completely ripe by week eleven, after which counts were not significantly different from controls. The rhizosphere effect for wheat was greatest at the time of grain formation and was weak or absent after ripening.

When Setaria italica was grown in sterile sandy loam and inoculated with Azospirillum brasilense Cd (17) acetylene reduction increased with plant age. Peak reduction activities (2350 nmol/hr/plant) were seen at flowering stage, after which the activities gradually decreased. It was noted that testing acetylene reduction during either very early or very late stages of plant growth may lead to an underestimation of nitrogen fixation potential. For example, acetylene reduction was very high at the booting stage, while no activity was detected at the seedling stage.

Pederson, et al (34), chose wheat for their acetylene reduction studies because it is a well-studied major food source. It uses the C-3 pathway for photosynthetic CO_2 fixation, and is often grown with little or no fertilizer. They found that rhizosphere samples of a commercial winter wheat field of Scout-66, located near Chappell, NE, did show appreciable acetylene reducing activity. The field was sampled four times, from early heading to late dough stage, and a range of 1.0 to 9.1 nmol of C_2H_2/hr/g dry weight of roots was obtained, based on total root systems. When roots were cut into segments, a maximum of 290 nmol of C_2H_2/hr/g dry weight of roots for 2 cm segments was obtained

from directly below the crown. This area represented 85% of the total acetylene reducing activity. Of the organisms isolated from active roots, almost all were Klebsiella pneumoniae, with a few Enterbacter cloacae . Activities were seen to increase up to the soft dough stage, then returned to nearly undetectable in the next two weeks. Scout-66 was tested in at least 10 other locations, and did not exhibit a high nitrogenase activity.

Neal and Larson (27) and Larson and Neal (21) found one recurrent morphotype of nitrogen fixer in their study of acetylene reduction in the wheat rhizosphere. This was a Gram-positive rod with a sub-terminally located spore. The strain was similar to those found in the rhizosphere of other plant species with respect to the inhibition of nitrogenase activity by either O_2 or by inorganic nitrogen additions. They noted that while this strain was consistently isolated from the rhizosphere of one wheat genetic subsitution line, none were recovered from the recipient parent, Cadet, or from the chromosone donor, Rescue. In monaxenic studies, the same bacterial strain was an active nitrogen fixer. It was clustered about the roots, especially in the area of newly forming rootlets. The cells were covered with a slime, and were found to invade intercortical cellular spaces.

When Hess and Kiefer (14) inoculated wheat and sorghum in vitro, they saw that growth and development of plants was usually impaired by bacterial presence, especially by Rhizobium sp. Roots were less branched and stumpy, and leaf growth was retarded when compared to axenic controls. In association with wheat, Azospirillum lipoferum reduced acetylene within a few hours of acetylene addition, whereas this activity with

sorghum was much slower. Bacteria or plants alone gave little and no reduction, respectively. Within a few days of inoculation, the bacteria developed a thick slime on the agar surface, and glove-like veils around the roots. It was concluded that although the ability of wheat to induce nitrogenase activity was not as high as sorghum, it was still by no means negligible.

Kundu and Gaur (20) found that mixed inoculants worked better for wheat than did monaxenic treatments. They tested wheat in sterile and nonsterile soil, with and without 2% added barnyard manure. The highest yields were found in sterilized soil and in sterile soil with added organic matter. They also found that the presence of Pseudomonas striata or Bacillus polymyxa stimulated the populations of Azotobacter chroococcum in the root zone, although this effect was less marked in nonsterile soil. The better response in sterilized soil may be due to lack of competition and better availability of nutrients for the introduced organisms.

In a study of plant growth substances produced by microorganisms in the rhizosphere, Brown (8) obtained these results. Isolates from the soil were found to produce known plant growth promoting substances. The genera responsible for producing these substances showed a significant increase in population in the wheat rhizosphere between days 42 and 82. Up to 67% of the rhizosphere population, at the 42 day stage, was found to produce these substances. Measurements were also taken to detect plant growth inhibiting substances. Pseudomonas spp. and Achromobacter spp. were found to produce plant growth inhibitors. The latter genera were stimulated more at the early stages of plant growth than at the

late stages. Paper partition chromatography was used to isolate and identify the various substances, with test samples taken from liquid cultures of the soil isolates.

The nitrogenase reaction is divided into two parts, 1) electron activation (the rate limiting step), and 2) substrate reduction. Substrate reduction may use endogenous electron acceptors, H+, or exogenous electron acceptors, HCN, N_2, or C_2H_2 (13). In the absence of substrate, protons are reduced to H_2. The activity of nitrogenase on HCN has led to speculation that the enzyme may have developed for cyanide detoxification (25). The use of acetylene in place of nitrogen forms the basis of the acetylene reduction assay. The limiting reaction of nitrogenase is hydrolysis of ATP to ADP and Pi coupled with electron transfer to form e*, a postulated activated electron or reduced species. Electron transfer measured as a function of ATP consumption or total reduction product formation is independent of exogenous electron acceptors, H_2, or CO_2. Activation energy for ATP consumption is also independent of exogenous substrate (13).

Nitrogenase reduces acetylene to ethylene but not to ethane. The ethylene is released by the protein and does not become a competitive inhibitor, as does N_2 in the acetylene reduction assay. Usually, Argon or Helium is mixed with the acetylene to create the test atmosphere. The inert nature of Argon and Helium prevents them from inhibiting acetylene reduction. Hardy, et al (13) reported that nitrogenase activity is stereospecific, producing only cis-1,2-dideuteroethylene. This disagrees, however, with a report by Tanaka, et al (43), that various deuterated ethylenes are seen.

The acetylene reduction test itself provides strong evidence for nitrogenase activity, and is of considerable value with both ecological and enzymological studies. With a good gas-liquid chromatograph, its sensitivity is about 10^3 times that of Kjeldahl analysis, measurements of NH_3 production, or isotopic tests (36). Reasonable correlations have been obtained between environmental acetylene reduction and direct methods for assaying nitrogen fixation. In the majority of cases, the ratio of mol C_2H_4:mol N was between 3.0 and 4.5, with one report giving a wheat rhizosphere value of 5.5 (42).

Hydrogen-flame ionization, suggested as the gas chromatographic detection system in the initial proposal of the assay, has remained the most widely used method. It provides adequate sensitivity, even necessitating corrections for C_2H_4 present as an impurity in commercial acetylene. Alumina was used as the original column packing material, but it may be replaced with other materials of shorter retention time, lower operating temperatures, greater reproducibility and increased sensitivity. A variety of column packing materials are now being used (13).

Important factors in the acetylene reduction assay are 1) the specific electron acceptor, N_2 or C_2H_2, does not influence the rate of electron transfer, 2) the rate of substrate reduction is proportional to the rate of electron transfer and inversely proportional to the electron requirements per molecule, 3) when ATP reductant is limiting, as may occur at low carbohydrate levels, electron transfer by nitrogenase decreases and this produces a corresponding decrease in acetylene or

nitrogen reduction, and 4) nitrogenase activity, and not necessarily enzyme concentration is measured (13).

One of the bacterial strains used in this thesis study was Klebsiella terrigena L89. Since the L group of Klebsiella was only recently submitted for speciation to the International Journal of Systematic Bacteriology, literature on K. terrigena is limited (15). For this reason, a short review of its delineation will be given.

In 1977, Gavini, et al (12) showed, by numerical analysis of the phenotypic properties of 122 strains belonging or related to the genus Klebsiella, the existence of 2 groups. One of these was group L, which consisted of 37 strains of nonclinical origin (soil and water). Group L was found to be related to K. pneumoniae, although the 37 strains possesed tetrathionate reductase and utilized m-hydroxybenzoate. In a more recent taxonomy study of 180 strains isolated from clinical and nonclinical origin, Naemura, et al (26), confirmed the existence of group L.

Eighteen of the twenty selected L strains had DNA-DNA reassociation values of at least 87% with the phenotypic centrostrain (15). Thirty-two of the thirty-seven strains displayed very similar protein electrophoretigrams. These results indicate that the majority of the strains form a genetically homogenous group. Also, the DNA relatedness with other species was lower than 48%, with the exception of Enterobacter aerogenes, which was 50 to 63% (15).

The type strain is Klebsiella terrigena L84. Pengra and Dott have found (unpublished data) that K. terrigena strains L66, L73, L80, L84, and L89 actively reduce acetylene while achieving a high turbidity in

a nitrogen-free medium.

This study was conducted with the following objectives:

1) to determine if the roots of spring wheat display a "rhizosphere effect" on nitrogen-fixing bacteria,
2) to determine if the stage of growth of the plant affected the activity of these bacteria, and
3) to determine the effect of inoculation of known nitrogen-fixing bacterial species onto the wheat plant. That is, do the applied bacteria survive in high numbers in the rhizosphere and do they promote nitrogen increases in the plant.

MATERIALS AND METHODS

Media The highly buffered medium of Pengra and Wilson (35) was used for the most probable number (mpn) counts of facultatively anaerobic nitrogen fixing bacteria and for the preparation of bacterial inoculants used in the greenhouse study. It contains, per liter of deionized water, Na_2HPO_4, 12.5 g; KH_2PO_4, 1.5 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $CaCl_2$, 0.01 g; $FeCl_3$, 0.003 g; $Na_2MoO_4 \cdot 2H_2O$, 0.25 mg; sucrose, 20 g. This medium is free of nitrogen.

Culture Conditions For mpn counts, 7 ml aliquots of the medium were dispensed into 16 x 150 mm pyrex tubes. Racks of tubes were covered with aluminum foil and autoclaved for 20 min at 121° C. Following sterilization, serum stoppers handled with ethanol-sanitized forceps were placed in the tubes. The atmosphere in the tubes was then displaced with nitrogen gas. This was accomplished by evacuating the tubes to 0.1 atm, then refilling with tank nitrogen. The evacuation and refill was then twice repeated. This was done using a 12-port manifold that was connected to both the vacuum pump and nitrogen tank. The barrels of 1.0 ml tuberculin syringes were filled with cotton and connected with rubber tubing to the manifold. Serum stoppers were swabbed with 70% ethanol prior to penetration with the syringe needles.

Fifteen tubes of medium were used for each mpn count; five tubes each at three ten-fold dilutions. For those tubes requiring 0.1 ml inoculum, a disposable 1.0 ml syringe was used. For those requiring a 1.0 ml inoculum, a 10 ml Becton-Dickinson glass syringe was used. The glass syringe was rinsed with 70% ethanol between samples. In this way, one-half of a full syringe could be used to inoculate the five

tubes at a particular dilution level. Serum stoppers were swabbed with 70% ethanol prior to inoculation. Tubes were incubated statically in the dark at 23° C for five days.

Measurement of Growth Growth of nitrogen-fixing microorganisms in the tubes was verified by evaluating nitrogenase activity using the acetylene reduction assay (13). Tubes that showed visible turbidity were injected with 1.5 ml of tank acetylene. This created an acetylene concentration in the headspace of approximately 10%. Incubation was continued for 12-18 hours, after which gas chromatography was used to detect ethylene production. The chromatograph used was a Varian Aerograph series 1520, equipped with a flame-ionization detector and a 180 cm x 1.2 mm alumina-packed column. Nitrogen was the carrier gas. Fifty ul samples were injected into the chromatograph. Tubes demonstrating ethylene production were counted as positive. Microorganisms were enumerated as described by Postgate (37) and reported as cells per gram of soil.

Field Studies Field studies were conducted in cooperation with the Plant Science Department, South Dakota State University. Wheat test plots were part of the Lake County experiment site. The plots were selected because they had not been heavily fertilized for several years prior to this study. The field, owned by Mr. Ray Dick, was located one mile west and three miles south of Madison, S.D. Hard red spring wheat, Triticum aestivum L., Butte variety, was drilled in plots measuring 1.2 m x 7.7 m. Seed was supplied by the Plant Science Department, South Dakota State University. Test plots were either

fertilized with 102 kg/N/ha or unfertilized.

Soil samples were taken (1) at planting time for soil analyses which were conducted by the Soil Testing Laboratory at South Dakota State University. The unfertilized plots had an organic matter content of 3.4%, 15 kg/P/ha, 622 kg/K/ha, a 0.4 ratio of soluble salts at a 1:2 dilution, 22 kg/N/ha, 39.4 ppm Fe, and a pH of 6.2 at a 1:2 dilution. The fertilized plot had an organic matter content of 3.4%, 24 kg/P/ha, 633 kg/K/ha, a 0.5 ratio of soluble salts at a 1:2 dilution, 53 kg/N/ha, 47.6 ppm Fe, and a pH of 6.1 at a 1:2 dilution. Nitrogen values are NO_3^-/NH_3 nitrogen, taken at a depth of 0-15 cm. Soil texture was loam.

Most probable number counts were conducted weekly starting at planting time. Three samples per week were enumerated from rhizosphere, fertilized; nonrhizosphere, fertilized; rhizosphere, unfertilized; and nonrhizosphere, unfertilized. A 2.0 cm x 10.0 cm corer was used to obtain these samples. Rhizosphere samples were obtained by lowering the corer directly over the plants. Nonrhizosphere samples were taken from bare soil between rows. Samples were placed in labeled plastic jars, capped, and returned to the lab for processing on the same afternoon. Samples were kept at 25° C. In the lab, samples were lightly chopped, mixed, and 11 g was placed in 99 ml dilution blanks. After shaking, 1.0 ml of this dilution was transferred to another 99 ml dilution blank, acheiving a 10^{-3} dilution. For each series of 15 tubes, 5 were inoculated with 0.1 ml per tube from the 10^{-1} dilution, 5 were inoculated with 1.0 ml of the 10^{-3} dilution, and 5 were inoculated with 0.1 ml of the 10^{-3} dilution. This resulted in final in-tube dilutions of 10^{-2},

10^{-3}, and 10^{-4}. Controls were inoculated with sterile diluent.

After plant emergence, small soil cores were removed and tested for nitrogenase activity using the acetylene reduction assay. This activity was assayed twice weekly and at the same time of day (11:00 am - 1:00 pm). The soil corer had an inside diameter of 1.4 cm and the sample was withdrawn from 2.0 to 4.5 cm depths. Samples were placed individually in 5 ml micro Fernbach flasks, then fitted with serum stoppers. Three samples each were taken from rhizosphere, fertilized; nonrhizosphere, fertilized; rhizosphere, unfertilized; and nonrhizosphere, unfertilized. The stoppers were then pierced with two syringes, one of which was connected to a hand vacuum pump, the other to a small reservoir tank containing a mixture of 85% Argon and 15% acetylene. A vacuum was drawn within the flasks to reduce the ambient air pressure to 0.15 atm. The reservoir line was then opened to replace flask atmosphere with the Argon/acetylene mixture. Vacuum and refill procedure was then twice repeated. Flasks were incubated at 25° C for three hours and during transportation to the lab. After incubation, 50 ul samples were injected into a portable Taguchi Gas Sensor gas chromatograph (23). Air was used as the carrier gas. This unit had a 44 mm column packed with 22 mm of Porapak N and 22 mm of Porapak R, followed by a thermoconductivity sensor. Measurement of peak sizes was performed by connecting the unit to a Sargent Recorder model SR. Samples with an air atmosphere were used as controls.

The experiment plots were harvested by the Plant Science Department on August 5, 1981.

Greenhouse Project A greenhouse study was performed by planting the same wheat variety into one quart, nondraining crocks. Each crock contained a mixture of 50% loam soil and 50% sand. This mixture assured a low concentration of available nitrogen. Analyses of the soil mixture gave values of 0.7% organic matter, a pH of 8.2 at a 1:2 dilution, 17 kg/N/ha, 41 kg/P/ha, 158 kg/K/ha, a soluble salts ratio of 0.6 at a 1:2 dilution, and 2.9 ppm Fe. After filling the crocks with this mixture, half of them were wrapped individually and autoclaved for 4 hrs at 121^{o} C.

Several seeds were placed in each pot and watered with 100 ml of tap water. At two weeks, the wheat was thinned to three plants per pot.

Inoculations were performed two days after emergence (eight days after planting). Inocula used were Klebsiella pneumoniae M5a1, Klebsiella terrigena L89, and wheat isolate "A" (a Gram-positive facultatively anaerobic rod that was isolated from the wheat rhizosphere during the field trials. It formed subterminal spores and actively reduced acetylene while acheiving high turbidity in a nitrogen free medium). These species were grown in the same medium that was used in the mpn procedure. Cultures were prepared in the following manner: the medium was dispensed in 100 ml aliquots into 250 ml Erlenmeyer side-arm flasks. Flasks were autoclaved and fitted with sterile rubber stoppers. The rubber stoppers had previously been fitted with glass tubing which had rubber tubing attached to the exposed end. The rubber tubing was attached to a vacuum pump-nitrogen

tank apparatus. Flask atmosphere was changed to nitrogen gas in the same manner as described for the mpn tubes. A cotton-filled sterilized tube was used to attach flasks to the gas displacement apparatus.

Inocula for these flasks were obtained from 48 hr cultures of the various organisms, cultivated in brain heart infusion broth (Difco) supplemented with 0.5% yeast extract. Tubes had been incubated at 23° C and were vigorously shaken with a vortex prior to use. Inoculum size was 2 ml.

Sterile tuberculin syringes were used to facilitate inoculation through the rubber tubing.

Flasks were then incubated with shaking (200 rpm), in the dark at 23° C. Growth was measured spectrophotometrically (Bausch and Lomb model 340) at 640 nm. Flasks were removed when the cultures reached late exponential phase. This occured at an optical density of 0.54 for the two Klebsiella species, and at 0.46 for isolate "A". Additional cultures were prepared as described for use as controls.

Each pot was inoculated monoxenically, with five pots per treatment series, as follows:

Sterile Soil	Nonsterile Soil
no inoculum	no inoculum
sterile M5a1	sterile "A"
live "A"	sterile M5a1
live M5a1	sterile L89
live L89	live "A"
	live M5a1
	live L89

After inoculation of the wheat plants, pots were labeled and

distributed randomly. Pots were randomized twice again during the experiment to reduce any variations which may have existed in light intensity or watering technique. Daylight was extended to 14 hrs per day with the use of overhead fluorescent lights. Average daytime temperature in the greenhouse was 27° C. Pots were watered once weekly; 50 ml per week for the first six weeks, and 100 ml per week for the final two weeks.

One day prior to harvest, a small soil sampler was used to extract a 2.5 cm x 1.4 cm core sample of rhizosphere soil from each of the treatment and control pots. Cores from like treatments were combined and used for an mpn count.

At harvest time a general description of plant appearance was recorded along with average plant height. Soil was shaken loose from around the roots and whole plant wet weights were recorded. Due to root intertwining, plants were not individually separated for these measurements. Each group of three plants from a pot was treated as a single sample. After weighing, roots were separated from shoots and wrapped in labeled aluminum foil. Foil packets were dried for 96 hrs at 70° C, and dry weights of roots and shoots were recorded.

Dried shoot samples were ground and used to determine nitrogen content via the semi-micro Kjeldahl method. A miniature Wiley mill equipped with a 60-mesh screen was used to prepare these samples.

Duplicate nitrogen determinations were made of each shoot sample. Approximately 0.020 g of ground shoot sample was added to 100 ml Kjeldahl flasks. To each flask was added 3 ml of H_2O, 5 ml of Kjeldahl

sulfuric acid digest mix, three glass beads, and one drop of castor oil to reduce foaming. Similar duplicates were prepared to be used as blanks, only with no sample added. After digestion over high heat, samples were quantitatively transferred into 50 ml volumetric flasks and brought to volume with deionized water.

After the dilutions were cooled and mixed, 1.0 ml from each flask was added to individual 13 mm spectrophotometer tubes. To each tube was then added 3.0 ml of Nessler's alkali and 2.0 ml of Nessler's reagent. Tubes were mixed and allowed to stand for 15 min. Absorbance was recorded at 490 nm. Absorbance values were compared to a standard curve obtained from tubes containing samples with concentrations of 0, 5, 10, 20, 30, and 40 ug ammonia-N/tube (35).

Nitrogen values were adjusted to reflect total nitrogen content of dry plant material per pot.

RESULTS AND DISCUSSION

In the field studies, no acetylene reduction was detected in wheat rhizosphere soil or in adjacent soil throughout the growing season. Several factors could account for the failure of these samples to reduce acetylene. The combination of small sample size and short incubation period may have limited acetylene reduction to undetectable levels. Soil moisture, although adequate for plant growth, may have been inadequate to provide necessary conditions for detectable microbial activity. This may have limited the development of strong plant-bacterial associations by not acheiving the proper levels of moisture and of oxygen exclusion necessary for nitrogen fixation. Figure 1 shows that the root crown area of the wheat rhizosphere had adequate protection from high daytime air temperatures. However, nearly all of the temperatures recorded are below those usually associated with active nitrogen fixation, 20-35° C. Finally, the mpn counts in Figures 2 and 3 show that populations of nitrogen fixing bacteria usually remained well below 10^3 per g of soil, and sometimes below 10^2 per g. Koch and Oya (19), when studying Hawaiian soils, found that total counts of 2 x 10^3 per g of soil were necessary before acetylene reduction was detected.

Rainfall, shown in Figure 4, was a rough indicator of soil moisture. On planting day, soil moisture was very high and remained high for the next two weeks. Around day 20, warmer air temperatures and very high winds dried the soil quickly. These conditions, combined with the absence of rainfall for 10 days, brought topsoil to near dust condition by day 25. Steady rains did not begin until day 35. From this time on,

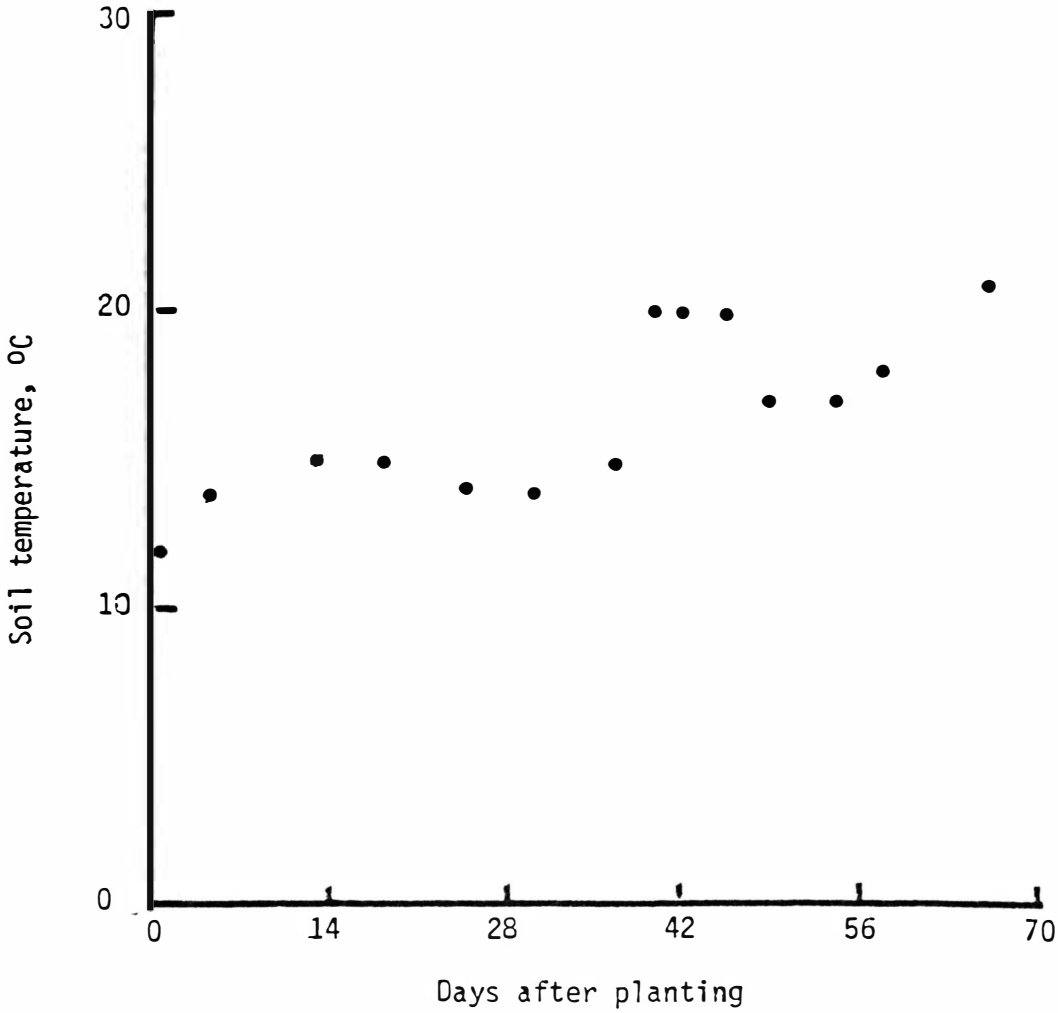

Figure 1. Soil temperature in degrees Celsius versus days from planting. Measurements were taken at a depth of 2.5 cm, between 11:30 am and 12:30 pm.

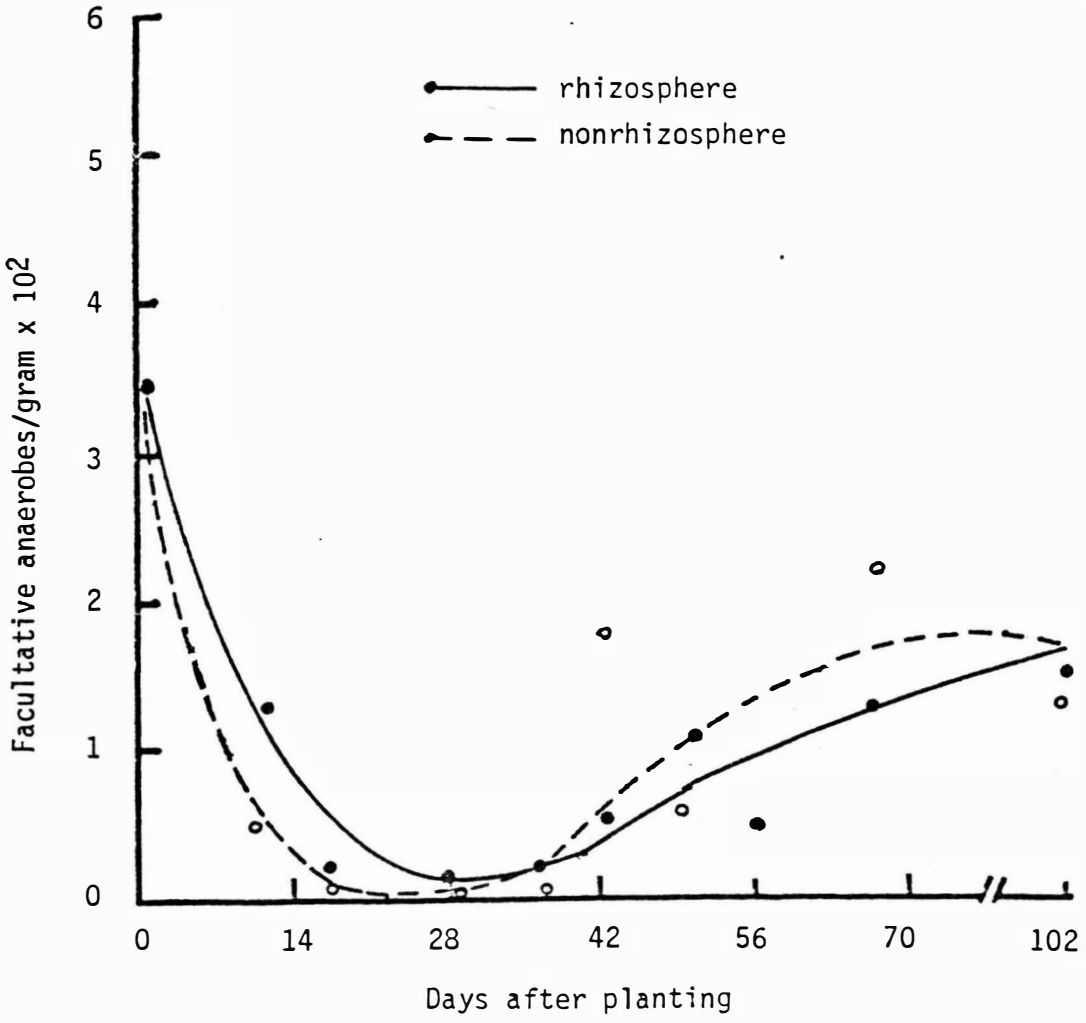

Figure 2. Facultatively anaerobic nitrogen fixing bacteria per gram of soil versus days from planting. Samples taken from fertilized wheat plots.

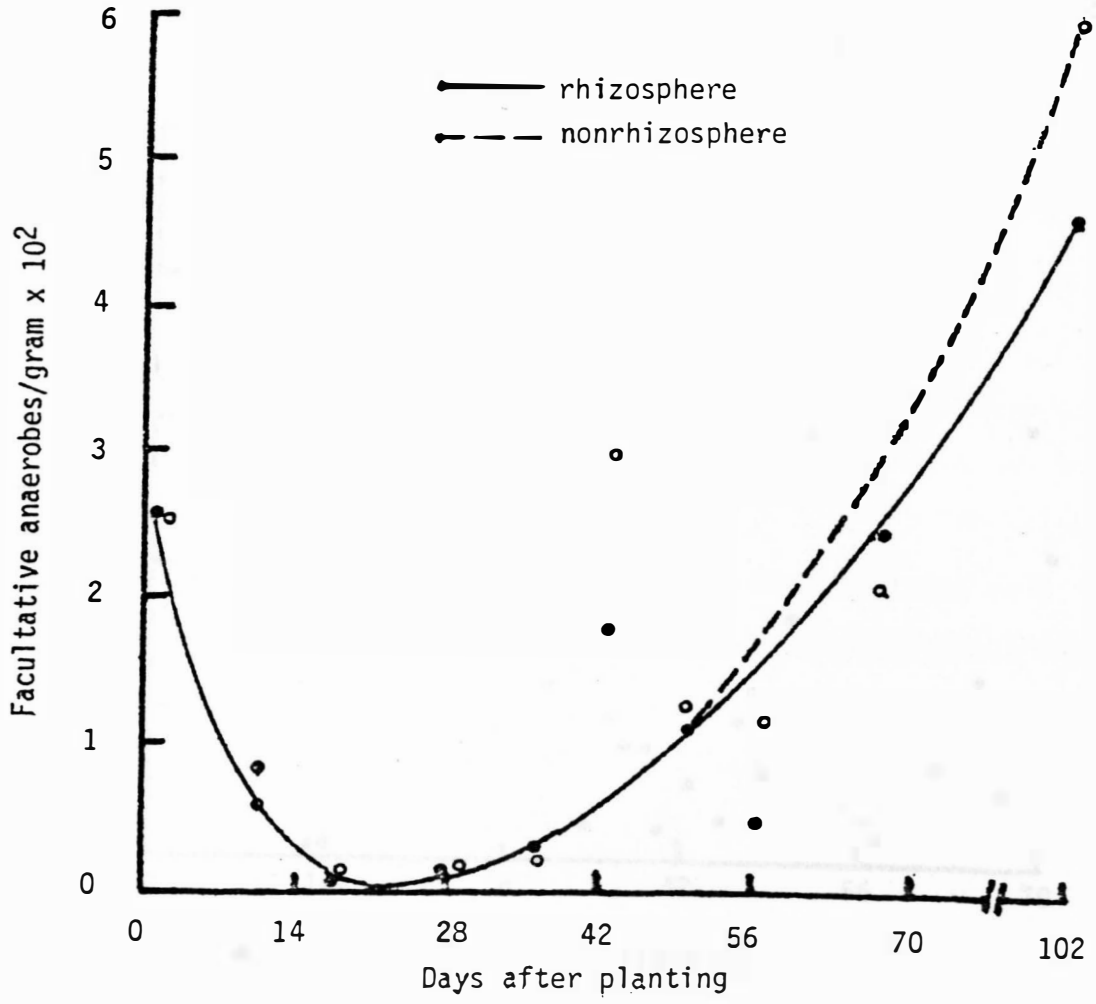

Figure 3. Facultatively anaerobic nitrogen fixing bacteria per gram of soil versus days from planting. Samples taken from unfertilized wheat plots.

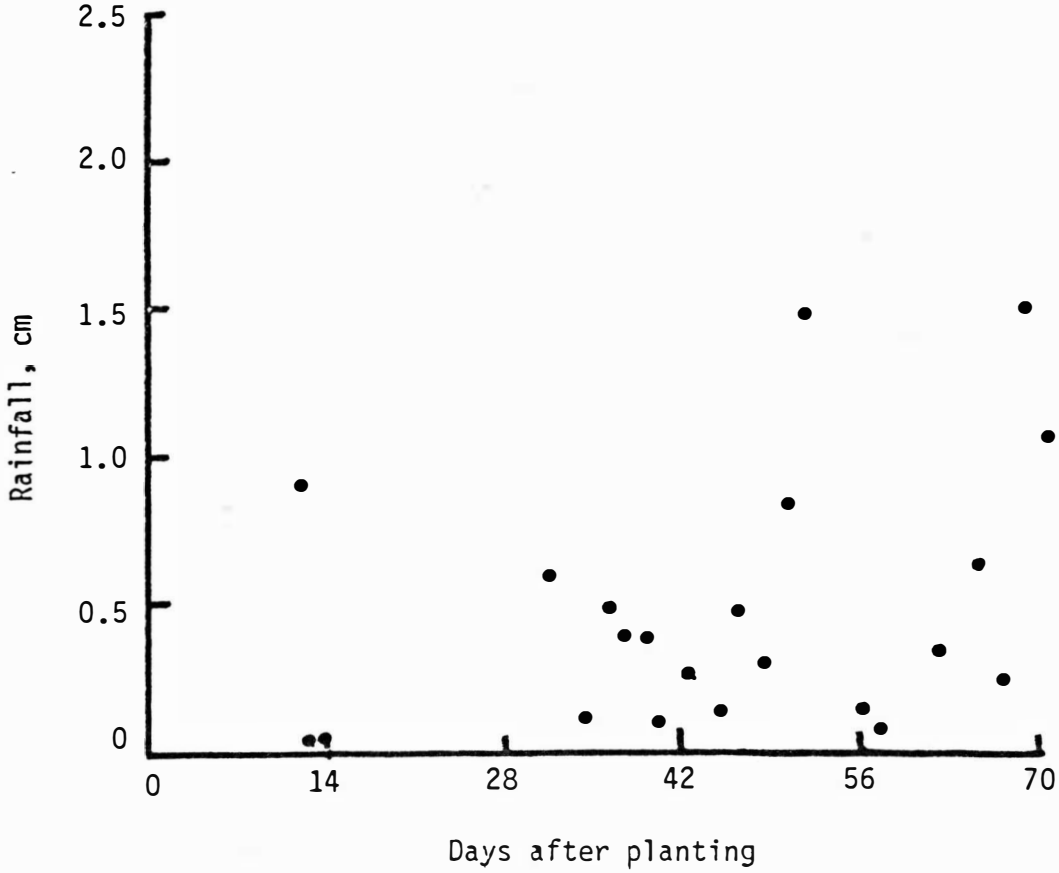

Figure 4. Rainfall in cm versus days from planting. All measurements above trace level are shown. Measurements made by National Weather Service Cooperative Network. Madison Experiment Farm, Madison, S. D.

rainfall was adequate but not saturating.

Figures 2 and 3 show the values obtained for mpn counts of facultatively anaerobic nitrogen fixing bacteria. Each figure shows values from rhizosphere and nonrhizosphere soil. Figure 2 is from the fertilized wheat, and Figure 3 from unfertilized. Fertilized plots showed a slight rhizosphere effect during the first four weeks. Unfertilized wheat showed an all but undetectable rhizosphere effect during the same period. Neither plot showed any distinctive rhizosphere effects for the next 2-3 weeks. For the remainder of the growing season, there appeared to be no stimulation of nitrogen fixing bacteria in the rhizosphere. Most probable number counts from day 50 onward showed higher numbers in nonrhizosphere soil, for both fertilized and unfertilized wheat.

When mpn counts are compared to the visual and physical descriptions of soil moisture recorded during the season, a pattern emerges. Most probable number counts were high early in the season, when the spring thaw and rains provided for relatively high soil moisture. During periods of high winds and low rainfall (e.g. days 14-35), soil moisture was low and nitrogen fixing bacteria were nearly undetectable. From day 40 onward, rainfall was adequate and both soil moisture and mpn counts increased.

When comparing Figures 2 and 3, one of the more obvious results is that as the season progresses, the mpn counts for the unfertilized plots are higher than the mpn counts for the fertilized plots. It appears that the addition of available nitrogen to soil may decrease any

competitive advantage held by those bacteria that fix nitrogen.

Tables 1, 2, and 3 summarize the data obtained from the greenhouse studies where wheat was inoculated with cultures of known nitrogen fixing bacteria. Plate counts derived from the Klebsiella pneumonaie, Klebsiella terrigena, and isolate "A" inocula as they reached late exponential phase were 4.5×10^8, 1.1×10^9, and 1.3×10^8 per ml. Kjeldahl analysis showed that each flask represented an addition of 5 mg nitrogen to the pots. Table 1 lists the physical descriptions and measurements made of the wheat plants at harvest time. Visual description of the plants showed that the uninoculated plants of both groups (sterile and nonsterile soil) had a healthier appearance than did the inoculated plants. Plant height was significantly greater ($P=0.01$) for those grown in nonsterile soil than for those grown in sterile soil. Wet weight measurements showed no significant differences between inoculated and uninoculated plants. However, the sterile soil plants were heavier as a group ($P=0.001$) than those grown in nonsterile soil.

The sterile soil pots did not appear to be contaminated with nitrogen fixing bacteria from watering or from dust settling. This was indicated by the mpn values listed in Table 1. Klebsiella terrigena L89 and isolate "A" showed moderate survivability in sterile soil, but they did not survive in high numbers in nonsterile soil. Values from the nonsterile soil pots showed that none of the nitrogen fixing organisms were present in high numbers at the end of the study.

Average dry weights of roots and shoots per pot are shown in Table 2. Inoculation did not significantly change the dry weight of

Table 1

Description of plants, average plant height, average wet weight, and mpn of root associated soil. Measurements taken at harvest.

Treatment	Description of Plants	Average Height*	Average Wet Wt.**	MPN
Grown in Sterile Soil				
no inoculum	strong, good grain & health	25.8	2.10	0
Klebsiella pneumoniae (autoclaved)	strong, good grain & health	21.8	1.94	0
isolate "A"	average grain and health	19.4	2.03	450
Klebsiella pneumoniae	avg. to good grain, avg. health	18.6	1.85	60
Klebsiella terrigena	strong, good grain and health	21.6	2.16	600
Grown in Nonsterile Soil				
no inoculum	average grain, good health	30.0	1.40	0
isolate "A" (autoclaved)	weak, avg. grain and health	27.0	1.49	45
K. pneumoniae (autoclaved)	good grain and health	22.6	1.68	50
K. terrigena (autoclaved)	average grain and health	26.8	1.60	175
isolate "A"	weak, avg. grain and health	23.0	1.42	50
K. pneumoniae	weak, avg. grain and health	25.2	1.34	25
K. terrigena	average grain and health	26.4	1.33	35

* Average height measured in centimeters
** Average wet weight measured in grams. Values represent total plant matter produced per pot.
All values are averages of five pots per treatment series.

Table 2

Average dry weight of roots and shoots
Butte variety spring wheat

Treatment	Average Dry Weight, Roots (g)		Average Dry Weight, Shoots (g)		Total
Grown in Sterile Soil					
no inoculum	0.4423	+	0.4807	=	0.9230
Klebsiella pneumoniae (autoclaved)	0.3472		0.5100		0.8572
isolate "A"	0.4114		0.4449		0.8563
Klebsiella pneumoniae	0.3197		0.4501		0.7698
Klebsiella terrigena	0.3971		0.5326		0.9297
Grown in Nonsterile Soil					
no inoculum	0.1610	+	0.4732	=	0.6343
isolate "A" (autoclaved)	0.1385		0.4265		0.5650
Klebsiella pneumoniae (autoclaved)	0.1563		0.4981		0.6545
Klebsiella terrigena (autoclaved)	0.1923		0.4144		0.6067
isolate "A"	0.1433		0.3869		0.5302
Klebsiella pneumoniae	0.1744		0.3742		0.5486
Klebsiella terrigena	0.1389		0.3920		0.5309

Values represent total dry matter production per pot. All values are averages of five pots per treatment.

Table 3

Percent nitrogen of wheat, dry matter, and total nitrogen of ground wheat samples

Treatment	% Nitrogen*		Total Dry Weight (g)		Total Nitrogen Per Pot Average (g)
Grown in Sterile Soil					
no inoculum	4.7	x	0.9230	=	0.044
Klebsiella pneumoniae (autoclaved)	5.1		0.8572		0.044
isolate "A"	4.9		0.8563		0.042
Klebsiella pneumoniae	5.3		0.7698		0.041
Klebsiella terrigena	5.0		0.9297		0.047
Grown in Nonsterile Soil					
no inoculum	3.6	x	0.6343	=	0.023
isolate "A" (autoclaved)	3.2		0.5650		0.018
Klebsiella pneumoniae (autoclaved)	4.2		0.6545		0.028
Klebsiella terrigena (autoclaved)	2.9		0.6067		0.018
isolate "A"	3.7		0.5302		0.020
Klebsiella pneumoniae	3.9		0.5486		0.021
Klebsiella terrigena	3.3		0.5209		0.018

*Percent nitrogen values obtained by semi-micro Kjeldahl analyses of dried shoot samples. Samples dried at 70° C for 96 hours, then milled and sifted through 60 mesh screen. Each of five pots in a treatment series was tested in duplicate for percent nitrogen.

roots or shoots. However, soil sterilization appeared to cause increases in plant dry matter (P=0.05).

Results of Kjeldahl analysis of wheat shoots are presented in Table 3. In sterile soil inoculation did not seem to increase the percent nitrogen of the plants. The same was true for inoculations carried out in nonsterile soil.

Throughout the study, values for total nitrogen did not seem to be influenced by inoculation. However, it is clear that plants grown in sterile soil contained more total nitrogen (P=0.01) than did plants grown in nonsterile soil.

SUMMARY AND CONCLUSIONS

Most probable number counts taken during the field tests showed that strong plant-bacterial associations failed to develop. The plant roots seemed to have a slight stimulating effect on the numbers of facultatively anaerobic nitrogen fixing bacteria in the fertilized wheat for the first 4 weeks of the growing season. During the second 4 weeks this rhizosphere effect was not seen. The final 7 weeks of growth actually showed slightly lower numbers of nitrogen-fixers in the rhizosphere. As the season continued, counts from the unfertilized plots were progressively higher than those from the fertilized plots. This suggested that the addition of available nitrogen to soil may reduce any competitive advantage held by nitrogen fixing bacteria. Environmental factors seemed to be more important than root presence in encouraging these bacteria. Soil moisture and available nitrogen had greater effects on population density than did root presence.

In the greenhouse study, inoculation with known nitrogen fixing bacteria on Butte variety spring wheat failed to increase dry weight or percent nitrogen of the plants. This may be related to the fact that pre-harvest mpn counts showed moderate to poor survivability of the applied bacteria. The failure to establish effective rhizosphere populations would limit plant gains from nitrogen fixation.

One unexpected result found was that plants growing in previously sterilized soil acheived significantly higher dry weight and percent nitrogen than those growing in nonsterile soil. Possible explanations may be 1) the sterilization process may cause some soil components to be converted to forms more readily available to the plants, and/or 2)

sterilization eliminated certain bacteria or fungi that may have been inhibitory or phytopathogenic.

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