# T H E S I S

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# SOME FACTORS INFLUENCING THE ACTIVITY OF AZOTOBACTER IN SOILS

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Submitted by

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In partial fulfillment of the requirements

for the Degree of Master of Science


Colorado State College

of

Agriculture and Mechanic Arts

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# SOME FACTORS INFLUENCING THE ACTIVITY OF AZOTOBACTER IN SOILS

by Lynn L. Gee

## INTRODUCTION

The problem of non-symbiotic nitrogen-fixation has received a vast amount of study in an attempt to determine the role this unique microbiological process plays in the maintenance of soil fertility. It is known that there are living free in the soil various types of microorganisms that have the power to utilize the vast supply of atmospheric nitrogen in their protein synthesis. Through this mechanism there is added to the soil, from a practically inexhaustible source, that highly important plant nutrient, nitrogen. It is only natural, then, that this problem should receive a large amount of study in an attempt to more thoroughly understand the mechanism so that it might be utilized more profitably in practical agriculture.

Lohnis (45),[1] in pointing out the economic value of bacterial nitrogen, states that experiments show that leguminous bacteria may fix 50 to 100 or more pounds of nitrogen per acre per year, while those living free in the soil may store in their bodies 10 to 40 pounds per

1. Reference by number (in parenthesis) is to "Literature Cited."

acre per year. But the lower efficiency of these non-symbiotic organisms is more than counter-balanced by their general distribution. There are in round numbers about 375 million acres in the United States planted annually to harvested crops; leguminous plants are grown on about 10 percent of the total area. If it is assumed that 60 pounds of nitrogen per acre per year are added under legumes and only 10 pounds of nitrogen per acre per year are added under non-legumes, the annual total effect would be 1 million tons of nitrogen fixed annually by the root-nodule bacteria and 1 1/2 million tons of nitrogen fixed by the non-symbiotic soil bacteria, making a total of 2 1/2 million tons of nitrogen which are added to the soil annually through the agency of bacteria. When it is considered that not much more than 200,000 tons of combined nitrogen are put into the soil by commercial fertilizers, the importance of bacterial nitrogen fixation at once becomes evident. It would seem, then, that any step forward in the study of bacterial nitrogen-fixation is an approach to the control of this important mechanism and its more economic utilization in the maintenance of soil fertility.

It is generally believed that the Azotobacter are the most important organisms concerned in the non-symbiotic fixation of nitrogen under agronomic conditions, hence the present study is confined to factors influencing

the activity of this species of organism in the soil. The factors which are to be studied in this paper are:

1. Energy sources, such as straw, mannitol and sodium benzoate.
2. Inorganic nutrients, such as phosphorus, potassium, iron, and molybdenum.
3. The presence of combined nitrogen and the carbon-nitrogen ratio.

## Review of Literature

The first indication that this process of fixation of atmospheric nitrogen by free living organisms was going on in the soil was probably made by Boussingault (8) in the middle of the ninteenth century when he noted that soil kept moist, by the addition of nitrogen-free water, for a period of three months showed an increase in total nitrogen and a decrease in total carbon. Then, later, in 1883, Berthelot undertook the study and was the first to show that gains in combined nitrogen in bare unsterilized soil was due to microscopic organisms.

This introductory work attracted the attention of S. Winogradsky, who in the year 1895 (65) discovered in the soil an organism which was capable of living in nitrogen-free medium and of assimilating the free nitrogen of the air. To this organism he gave the name Clostridium pastorianum.

The next important step in the problem of the non-symbiotic fixation of nitrogen was made by Beijerinck (4) in 1901 when he described a group of large aerobic bacilli which were capable of utilizing atmospheric nitrogen in their metabolic process. To this group he gave the generic name Azotobacter, of which he isolated two species: Azotobacter chroococcum, isolated from garden soil, and Azotobacter agilis which was isolated from canal water.

Other species of Azotobacter have been isolated by various investigators and may be listed as follows: Azotobacter vinelandii - Lipman 1903 (38); Azotobacter beijerinckii - Lipman 1904 (39); and Azotobacter vitreum - Lohnis and Westerman 1908 (43). Lohnis and Smith (44) recognized only two species of Azotobacter isolated so far: Azotobacter chroococcum and Azotobacter agile -Beijerinck (Synonym Azotobacter vinelandii - J. G. Lipman). Azotobacter beijerinckii - Lipman - was looked upon as a variety of Azotobacter agile.

The first attempt to utilize the process of non-symbiotic nitrogen-fixation artificially in agriculture came as a result of Caron's studies with Bacillus ellenbachensis. He found that soils inoculated with bouillon cultures of this organism gave higher yields than soils receiving the sterile bouillon only. This

discovery led to the use of his cultures commercially in an attempt to use this method of nitrogen-fixation to supply the soil with this much needed nutrient. One of the most publicized and most studied of these cultures was the "alinit", which was found to have a slight nitrogen-fixing power, but needless to say this attempt to supply the soil with nitrogen soon met with failure, but the subject had received a vast amount of publicity and scientists as well as the farmer were interested to know the actual role this process was playing in maintaining the fertility of the soil, and to determine whether or not the process could be modified in such a way as to furnish this much needed nutrient to the soil in sufficient quantities to supply that needed to support plant growth.

A great amount of work has been done to determine what factors influence this form of nitrogen-fixation and in determining its capabilities and limitations. The majority of this work has been carried out under conditions which depart widely from those in nature and though this is an approach to the subject it must be remembered that all organic beings react to their environment and what is found to be true under one set of conditions may be little indication as to what may happen in another environment, hence certain changes which have been carefully studied in the laboratory may fail completely to have a counter-

part in their natural habitat. It must not be assumed, however, that this vast amount of work carried out under artificial conditions is valueless because it is only through this approach that it is possible to determine the capabilities and limitations of these organisms. This round-about approach also allows us to build up certain technics which may serve as a short cut in determining their true role in the soil.

It must not be considered that Azotobacter is the only organism that has the power to fix atmospheric nitrogen. Emerson (18) has shown that a soil which he examined contained 2,400,000 organisms capable of developing on nitrogen-free medium, this number of organisms constituting at least four distinct groups. The reason Azotobacter is most commonly referred to as the free-living nitrogen-fixer is because it is the most important nitrogen-fixer that has yet been described, hence the term "nitrogen-fixer", unless otherwise designated, will refer to the Azotobacter group.

One of the reasons the Azotobacter has assumed such importance in the field of nitrogen-fixation and one of the reasons it is assumed to play such an important role in maintaining the nitrogen supply in the soil is its extensive distribution in the soil. Lipman and

Burgess (41) have shown that in 46 soils gathered from all parts of the world about one-third showed the presence of Azotobacter and though all agricultural soils studied were capable of fixing nitrogen, those in which Azotobacter makes up part of the soil flora are capable of fixing more nitrogen than those in which Azotobacter is lacking.

Source of Energy

Lohnis and Pillai (42), working with this, have shown the following as acting as a source of carbon: Mannitol, xylose, lactose, laevulose, inulin, galactose, maltose, arabinose, dextrin, sucrose, dextrose, starch, sodium tartrate, glycerine, sodium succinate, calcium lactate, sodium citrate, sodium propionate, potassium oxalate, calcium butyrate, and humus. The order in which they are listed is the order of effectiveness recognized by most of the workers. Diehm (16) has shown that mixed cultures of soil microorganisms are capable of utilizing mannan, galactan, and xylan as sources of energy for nitrogen-fixation and that these hemicelluloses may not be utilized significantly by pure cultures of Azotobacter. Fuller and Rettger (20) in determining the influence of combined nitrogen on the growth and fixation of nitrogen have shown that Azotobacter are capable of utilizing such nitrogenous substances as glutamic acid, aspartic acid, cysteine hydrochloride, and glycocoll. They conclude by saying, "The simpler organic substances including the

lower amino acids, and the inorganic compounds seem to be utilized by Azotobacter with the result that growth is increased and nitrogen fixation either relatively or actually depressed."

The probable role of cellulose in the supply of carbon for nitrogen-fixation has been of interest to the investigator for it was early recognized by Beijerinck that certain decompositions of cellulose can serve as a source of energy for Azotobacter. However, according to Tourila (60) Azotobacter cannot use cellulose as a source of energy when in pure culture, but derives energy from this source only when mixed with other cultures. It is unknown just how far cellulose decomposition must proceed before the decomposition products are available to the Azotobacter, but cellobiose cannot be used by Azotobacter as a source of energy (37). It is pointed out by Sanborn and Hamilton (55) that a metabiotic relationship may exist between the cellulose decomposers and the Azotobacter, the levorotatory gum produced by the latter aiding the action of the cellulose destroyers. This relationship has also been noted by others (13), (48), (46), (47). The Azotobacter organisms are apt to derive more utilizable carbonaceous material if the cellulose is decomposed by crude cultures (13).

Doryland (17), studying the possibility of obtaining nitrogenous fertilizers by utilizing waste

materials for the fixation of nitrogen by nitrogen fixing bacteria, has shown that molasses, orange juice, grapefruit juice, wheat, oat, barley, rye, or flax straw are materials that may serve as a source of energy for the nitrogen fixers. They also state that waste paper, wood wastes, grass residues, and beet and cane sugar residues will probably serve in the same way.

Hutchinson (36) noted that additional sugars to soil was followed by an increase in the soil nitrogen as was the addition to the soil of plant residues.

McBeth (49) has shown that green manures furnish a valuable source of energy for the non-symbiotic fixation of nitrogen and when added to the soil tend to increase the total amount of nitrogen therein. Desai (15) also points out that fixation of nitrogen does take place when green manures, farmyard manures, straw or any other organic material is added to the soil under favorable conditions of temperature and moisture. He also shows that non-leguminous plants are much better than legumes for energy source in the non-symbiotic fixation of nitrogen. This was earlier pointed out by Fulmer (21) who believed the effect to be due to the nature of the carbohydrate content of the non-legume.

Greaves (29), who conducted a similar experiment on Western dry farm soil, however, found that the leguminous plants when added to the soil caused much more

nitrogen to be fixed than did the addition of non-legumes, the latter in some cases actually causing a loss in nitrogen.

More recently Salter (54), studying the relation of carbon-nitrogen ratio to the accumulation of organic matter in the soils, points out that regardless of the source of carbon the quantities present at the end of the year fall within a narrow range and that the more carbonaceous materials show the greatest increase in nitrogen, but that the nitrogen thus fixed may not become available to the plants until the carbon-nitrogen ratio is reduced. The addition of highly carbonaceous material causes a pronounced depression in the nitrate nitrogen of the soil, hence any addition must be done at a time when the plants and microorganisms will not be competing for the soil nitrogen. The matter of the amount of carbonaceous material to be added for most efficient fixation of nitrogen is also of importance because, as is pointed out by Murray (50) in working with straw as a source of energy for the nitrogen fixers, the amount of fixation is not dependent upon the amount of straw applied. To get the maximum benefit from addition of straw, then, the amount to apply would have to be determined for a given condition.

Greaves has done considerable work with nitrogen-fixation in the soil and states (28) that the yearly

application of 5 tons of manure to fallow land per acre over a period of 11 years very materially increased the nitrogen-fixing power of the soil. This application increased the nitrogen content by 1370 pounds. This is 486 pounds greater than the nitrogen applied in the manure, hence it may be assumed the manure furnished a source of energy for nitrogen fixers and they were responsible for the additional nitrogen. This figure closely approximates that of Bear (3) who estimates that under conditions favoring the Azotobacter they may add as much as 40 pounds of nitrogen per acre per year. This figure is accepted by most investigators as the best estimate of the amount of nitrogen added to the soil by the nitrogen-fixing organisms of the non-symbiotic type.

## Inorganic Nutrients

The role played by inorganic nutrients in the fixation of nitrogen by non-symbiotic fixers has been studied closely by a large number of investigators. Phosphorus is probably one of the most important of the inorganic nutrients for these organisms, (51), (62), (14), (1) being used by the organism in the building up of phospho-proteins and nucleo-proteins in which their bodies are very rich. So important is phosphorus in the nutrition of these organisms that their growth on soil plaques has been used for determination of available phosphorus in the soil (53), (68).

The presence of nitrogen plays an important part in the fixation of atmospheric nitrogen. According to Brown and Hart (10) small quantities of nitrogen increased the nitrogen fixing power of Azotobacter chroococcum, vinelandii, and beijerinckii, in cultural solutions. The inorganic forms proved to be more stimulating than the organic forms. Burk and Lineweaver (11) have shown that the amount of readily available fixed nitrogen in culture medium required to inhibit nitrogen-fixation by Azotobacter is 0.5 mg. per 100 cc. In soil the toxicity of various salts was determined by Greaves, Carter and Lund (27) by the tumbler method. It was found that concentrations of $NaNO_3$ at 460 p.p.m., $Ca(NO_3)_2$ at 400 p.p.m. and manganese nitrate at 550 p.p.m. were not detrimental to nitrogen-fixation. Hence, it is concluded that the common soil alkalies would have to be present in quantities large enough to greatly retard plant growth before nitrogen-fixation would be retarded. It was earlier shown by Lipman (41) that in solution culture-50 p.p.m. $KNO_3$, or 100 p.p.m. of peptone depressed nitrogen-fixation by Az. vinelandii.

Hills (32), (33), also shows that small quantities of nitrates stimulate Azotobacter growth and that more atmospheric nitrogen is assimilated in the presence of small amounts of nitrate than in its absence.

This stimulatory effect continues to increase the numbers of Azotobacter up to a certain limit, after which an increase in nitrate concentration causes a retardation in growth and a concentration limit is reached at which Azotobacter growth ceases entirely.

Fuller and Rettger (20), studying the effects of a large number of nitrogenous compounds of the fixation of nitrogen by Azotobacter, noted that most of the non-toxic compounds did not influence the nitrogen fixation to any marked extent. Certain compounds, such as aspartic acid, cysteine hydrochloride, glycocoll, creatine, creatinine, and urea, almost inhibited the fixation of nitrogen.

Greaves (26) found that soils comparatively rich in combined nitrogen fixed more nitrogen than those poorer in organic nitrogen.

Brown and Allison (9), studying the effect of manure and various plant residues, found that the carbon-nitrogen ratios of the materials used were of little value in indicating their effects on nitrogen-fixation.

Thompson (59) has shown that the nitrates of sodium and potassium, urea, and the amide nitrogen of asparagin were readily attacked by Azotobacter with the production of ammonia, which was then utilized by the organisms. These compounds inhibited nitrogen-fixation almost entirely, and in some cases there was a loss of ammonia. Many of the complex nitrogenous compounds tested

were not utilized except to a small extent by Azotobacter vinelandii, and nitrogen-fixation was usually not affected or was stimulated.

Thompson (58) in another paper shows that the initial addition of straw greatly reduces the nitrate content of the soil, but finally this content is practically the same as that of the untreated soil. This depressing effect may be overcome somewhat by the addition of sodium nitrate. Soils lowest in nitrate nitrogen generally made greatest gains in total nitrogen and, in general, these soils contained the smallest numbers of Azotobacter. These organisms seem to utilize fixed nitrogen in preference to free nitrogen, but will use the latter when there is a deficiency of the former.

Horner and Burk (34) found that the concentrations of Mg, Ca, and Fe required for the maximum growth of Azotobacter in free nitrogen were 0.05 - 0.1 millimolal, 0.1 - 0.2 millimolal, and 0.0004 - 0.001 millimolal, respectively. The requirement for P appeared to be 0.1 millimolal, and for S, K, Mo, and V equal to or less than that for Fe, Bortels (5) earlier found that the growth of Azotobacter chroococcum in a nitrogen-free nutrient solution was stimulated by the addition of 0.0005 percent of $Na_2MoO_4.7H_2O$. He also noted (6), (7) that both molybdenum and vanadium increase nitrogen fixation as much as one hundred fold. Burk,and Horner (12) state the

acceleration of growth of Azotobacter may be used as an extremely delicate test for molybdenum and vanadium. Baier (2) indicates that the stimulating effect of sewage and humic acid on the growth of Azotobacter may be due to the addition of traces of Fe, Mo, Al, Zn, Cu, and S. Greaves (30) has found that in addition to Mn and $Fe^{++}$, iodine also enhances Azotobacter growth in liquid culture.

Reaction, Temperature and Aeration

Winogradsky (65) in his original work on the fixation of nitrogen added to his nutrient medium an excess of calcium carbonate to neutralize the acids formed. It was Stoklasa (56), however, who produced the necessary evidence for a correct understanding of the function of calcium carbonate in nitrogen-fixation experiments by demonstrating quantitatively the formation of organic acids in cultures of Azotobacter.

Gainey (24), (22), (23) has shown that in soils a pH below 6 will not support Azotobacter. He concludes that the presence or absence of Azotobacter in natural soils is very closely associated with, if not dependent upon, the absolute reaction of the soil solution. In solution culture Gainey (25) found the limiting pH for the Azotobacter cultures employed to be between 5.9 and 6.1 and that the optimum pH for nitrogen fixation is apparently somewhat higher than the optimum pH for growth.

Yamagata and Wilson (66) found that for the

greatest increase in the number of Azotobacter cells for the three types of Azotobacter the following pH values were best: Azotobacter beijerinckii - 6.6, Azotobacter chroococcum - 6.8, and Azotobacter vinelandii - 7.0. Yamagata and Itano (67), by more refined methods, determined the following pH values for growth of Azotobacter:

Azotobacter chroococcum: Optimum = 7.45 - 7.60.
Limiting = 5.80

Azotobacter beijerinckii: Optimum = 6.65 - 6.75.
Limiting = 5.80

Azotobacter vinelandii: Optimum = 7.50 - 7.70.
Limiting = 5.90.

It has been determined by Panganiban (52) that nitrogen-fixation proceeds between 15°C. and 40°C. with an optimum temperature between 25°C. and 30°C. Greene (31) found the optimum temperature for nitrogen-fixation to be 32.5°C. and the maximum temperature at which nitrogen-fixation could take place to be 40°C.

Desai (15) believes aeration to be a main factor in nitrogen-fixation by organisms. The importance of aeration in stimulating the growth of Azotobacter has also been shown by Hunter (35), working with solution cultures, and Turk (61) who states that the nitrogen-fixing capacity of soils is favored by well-aerated conditions.

## EXPERIMENTAL

The experiments described below were conducted to determine the effect of various environmental conditions on the growth and fixation of nitrogen by Azotobacter. The Azotobacter vinelandii used was secured from the New Jersey Agricultural Experiment Station. All other Azotobacter strains used were crude cultures isolated from soil taken from the Colorado Agricultural Experiment Station farm located just east of Fort Collins. The cellulose decomposing organism used has been designated as cellulose decomposer number 12.

All sterilization of soil was accomplished by autoclaving at 15 pounds pressure for two hours on each of three successive days. This procedure was adopted since the customary two to three hours at 15 pounds pressure leaves some question as to the sterility of the soil.

Nitrogen analyses of the samples were made before and after incubation. The ammonia nitrogen and nitrate nitrogen were determined on the same KCl extract. The nitrate nitrogen was determined by the Devarda's alloy reduction method. Total nitrogen was determined by the Kjeldahl method modified to include nitrates by the salicylic acid method. Copper sulfate was used as the catalyst. Acid and alkali .07142 N were used in all nitrogen

determinations.

The incubation was carried out in the 28° C. constant temperature room.

The carbon dioxide evolution by decomposing organic matter was determined by its neutralization of standard alkali (0.1666 N sodium hydroxide). The flasks were aerated for 5 hours daily, and the carbon dioxide determined daily.

The medium in making Azotobacter counts was Wenzl's medium (64) to which sodium molybdate was added. The composition of the medium was as follows:

| | |
|---|---|
| Agar | 10.00 gm. |
| Mannitol | 20.00 gm. |
| $MgSO_4.7H_2O$ | 0.30 gm. |
| $K_2HPO_4$ | 0.75 gm. |
| NaCl | 0.20 gm. |
| $FeSO_4.7H_2O$ | 0.02 gm. |
| $Al_2(SO_4)_3$ | 0.02 gm. |
| $Na_2MoO_4.2H_2O$ | 0.00025 gm. |
| $CaCO_3$ | 5.00 gm. |
| Water | 1000. cc. |

The numbers of Azotobacter in the soil at the beginning and at the end of the incubation period were determined by plating out on the above medium. The agar was allowed to solidify in the petri dishes and was then inoculated by pouring 1 cc. of the final dilution on the

surface of the agar and tipping the plate in order to distribute the inoculum over the entire surface of the plate. The lids were removed from the plates, which were allowed to remain uncovered in the 28° constant temperature room until the inoculum had evaporated. Care was taken to see that the agar did not dry. This method of plate inoculation was adopted because it gave higher counts than did the more usual procedure of pouring the cooled agar into plates to which the inoculum had previously been added. Check plates poured in conjunction with the dilution plates failed consistently to show the development of any bacterial colonies.

Though many minute colonies appear on the dilution plates, the Azotobacter colonies are readily distinguishable by their luxuriant growth. In the experience of the author there has been but one organism appearing on soil dilution plates that could be confused with Azotobacter. This organism could, however, be distinguished by its capitate colony formation and absolute lack of pigmentation regardless of age, while the Azotobacter colonies tend to spread over the surface and take on a milky color.

Where possible all results were analyzed statistically by one or more of the following methods: (A) the analysis of variance (19), (B) "Students" method

of paried comparisons (57), and (C) coefficient of correlations (63). The general procedure followed in these analyses will be given with the first application of each, thereafter only the constants sought will be recorded.

Fig. 1. Carbon Dioxide Apparatus

Series 1.

One hundred grams of Agronomy Farm soil was placed in 500 cc. Erlenmeyer flasks. The flasks were treated as follows:

Flasks Nos. 1-4 - 100 gms. soil containing 2 percent mannitol. Sterilized and inoculated with Az. vinelandii.

Flasks Nos. 5-8 - 100 gms. soil containing

2 percent mannitol. Unsterilized.

Flasks Nos. 9-12 - 100 gms. soil containing 1.365 percent sodium benzoate. Sterilized and inoculated with Azotobacter vinelandii.

Flasks Nos. 13-16 - 100 gms. soil containing 1.365 percent sodium benzoate. Unsterilized.

Flasks Nos. 17-20 - 100 gms. soil. Untreated. Sterilized and inoculated with Azotobacter vinelandii.

Flasks Nos. 21-24 - 100 gms. soil containing 2 percent wheat straw. Unsterilized.

Flasks Nos. 25-28 - 100 gms. soil containing 5 percent wheat straw. Unsterilized.

Flasks Nos. 29-32 - 100 gms. soil containing 10 percent wheat straw. Unsterilized.

Flasks Nos. 33-36 - 100 gms. soil. Untreated. Unsterilized.

By adding 2 percent mannitol and 1.365 percent sodium benzoate the amount of carbon added to the soil by the two treatments is the same. The carbon content of the straw, as determined by the wet combustion method, was 40.27 percent.

The flasks were fitted to the carbon dioxide apparatus in the 28° C. constant temperature room and the carbon dioxide evolved was determined daily for 20 days. Thus it was possible to follow the general trend of decomposition of the organic material added to the soil.

The average daily evolution of carbon as carbon dioxide is given in Table I and shown graphically in Figs. 2 and 3.

From these figures it will be seen that the straw treated soils show very much the same general trend in carbon dioxide evolution, and that the mannitol and sodium benzoate have similar decomposition curves. An analysis of variance on the three straw treatments, the unsterilized mannitol treatment, the unsterilized sodium benzoate treatment, and the unsterilized check using average daily evolution of carbon as the index figure (Table II), shows that the straw treatments differ significantly from each other and that the mannitol and sodium benzoate treatments differ significantly from the straw treatments occupying a position between the 5 percent and the 10 percent straw treatment. All five treatments are much higher than the check. There is, however, no significant variation in the average daily evolution between the mannitol and the sodium benzoate treatments. This would indicate that the decomposition of the two had proceeded to approximately the same point by the end of the 20 days incubation period.

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## TABLE I

### Averages* of Carbon Dioxide Evolved (in mg.)

| Soil Treatment | Time in days: 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1. 2% mannitol-sterilized-Inoculated with Az. Vinelandii | 0.76 | 0.45 | 0.41 | 0.25 | 0.43 | 0.95 | 0.54 | 0.49 | 0.55 | 0.56 | 0.43 | 0.41 | 0.38 | 0.30 | 0.31 | 0.36 | 0.96 | 0.41 | 0.39 | 0.36 |
| 2. 2% mannitol-unsterilized uninoculated | 7.80 | 21.38 | 34.13 | 36.20 | 32.63 | 30.61 | 26.45 | 22.48 | 25.65 | 18.85 | 19.51 | 14.31 | 14.20 | 13.26 | 12.20 | 11.42 | 10.61 | 10.91 | 10.58 | 9.79 |
| 3. 1.356% sodium benzoate sterilized + Az. vinelandii | 0.68 | 0.36 | 0.43 | 0.28 | 0.46 | 0.70 | 0.48 | 0.46 | 0.31 | 0.51 | 0.33 | 0.33 | 0.32 | 0 30 | 0.37 | 0.37 | 0.70 | 0.35 | 0.30 | 0.31 |
| 4. 1.356% sodium benzoate unsterilized - uninoculated | 5.59 | 15.16 | 21.61 | 28.54 | 26.65 | 23.85 | 23.11 | 24.00 | 28.78 | 21.86 | 21.97 | 17.31 | 15.25 | 16.59 | 16.00 | 15.62 | 14.06 | 14.46 | 13.91 | 13 22 |
| 5. Soil sterilized-Inoculated with Az. vinelandii | 0.65 | 0.46 | 0.71 | 0.72 | 0.92 | 1.01 | 1.04 | 0.66 | 0.76 | 0.70 | 0.52 | 0.72 | 0.75 | 0.60 | 0.80 | 0.51 | 0.96 | 0.60 | 0.70 | 0.69 |
| 6. 2% straw-unsterilized | 25.85 | 24.86 | 18.08 | 15.94 | 8.29 | 9.01 | 7.55 | 7.51 | 6.06 | 5.72 | 5.41 | 5.47 | 5.51 | 5.49 | 5.62 | 5.45 | 5.19 | 5.96 | 5.51 | 5.49 |
| 7. 5% straw-unsterilized | 33.46 | 32.94 | 27.75 | 27.82 | 19.31 | 20.04 | 14.99 | 15.28 | 11.74 | 11.88 | 10.70 | 10.50 | 10.59 | 10.41 | 9.88 | 9.85 | 9.66 | 10.08 | 7.45 | 7.31 |
| 8. 10% straw-unsterilized | 45.31 | 44.52 | 39.80 | 40.28 | 34.55 | 33.36 | 27.52 | 27.19 | 25.86 | 21.94 | 21.09 | 15.30 | 17.62 | 14.86 | 16.38 | 13.58 | 13.62 | 14.15 | 13.76 | 13.32 |
| 9. Soil-untreated unsterilized | 1.61 | 2.31 | 1.59 | 1.76 | 1.41 | 1.55 | 1.55 | 1.40 | 1.36 | 1.55 | 1.45 | 1.28 | 1.31 | 1.35 | 1.17 | 1.24 | 1.29 | 1.36 | 1.40 | 1.39 |

*Each figure given is the average of the four replicates.

Fig. 2. Average daily evolution of carbon as carbon dioxide

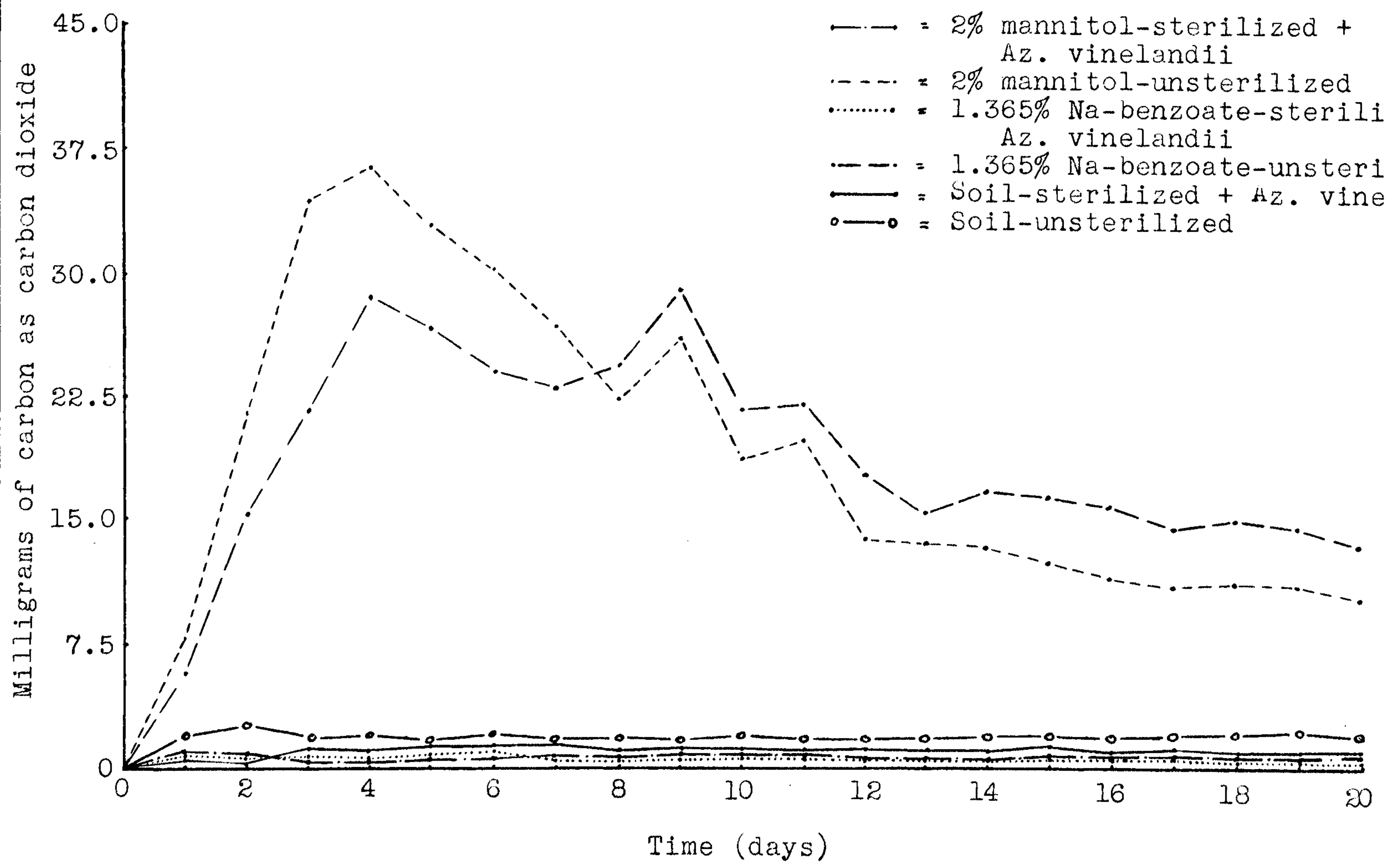

Fig.3 Average daily evolution of carbon as carbon dioxide

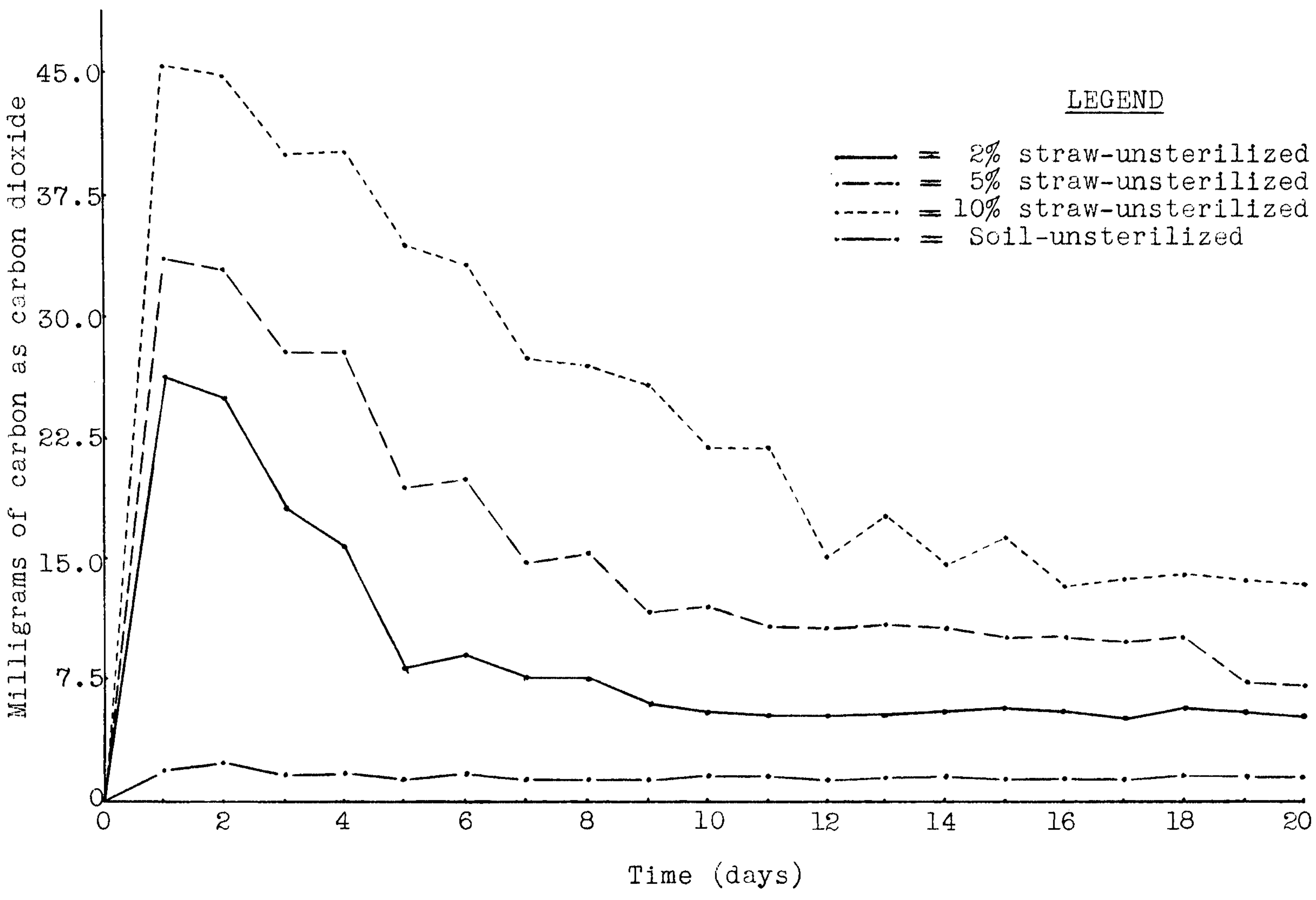

TABLE II

Analysis of Variance of Average Daily Carbon Dioxide Evolution in Relation to Treatment

| Variance due to | Sums of squares | Degrees of Freedom | Variance | F | $\sigma_{\Delta}$ |
|---|---|---|---|---|---|
| Treatment | 1,358.2362 | 5 | 271.6500 | 788.305 | |
| Replication | 0.1833 | 3 | 0.0611 | 0.177 | |
| Error | 5.1689 | 15 | 0.3446 | | 0.587 |
| Total | 1,363.5884 | 23 | | | |

$\sigma_{\bar{x}}$ = 0.2935 $\sigma_{D}$ = 0.4150 Level of significance (5 percent) = 0.8843

TABLE III

Average Daily Evolution of Carbon as Carbon Dioxide

| Treatment (Unsterilized) | Average daily evolution of C as $CO_2$ (mgm.) | % added carbon recovered as $CO_2$ |
|---|---|---|
| 2.0% mannitol | 19.14 | 44.74 |
| 1.365% sodium benzoate | 18.66 | 44.04 |
| 2.0% straw | 9.18 | 38.34 |
| 5.0% straw | 15.56 | 14.01 |
| 10.0% straw | 24.57 | 11.48 |
| Check | 1.46 | |

The total amount of carbon recovered from the sodium benzoate and mannitol treatments was 44.04 percent and 44.74 percent respectively, indicating that decomposition has proceeded nearly to completion. The percentage recovery from the straw treatments falls off rapidly as

the percentage of straw increases indicating, much as to be expected, that under these conditions the efficiency of decomposition decreases as the percentage of straw increases.

It will be noted from Fig. 2 that the curve for the mannitol treatment was higher than the sodium benzoate curve for the first 7 days, after which the positions were reversed. It seemed desirable to know whether or not this variation the first 7 days presented a real difference. This was determined by applying "Students" method of paired comparisons (Table IV).

TABLE IV

Comparison of Carbon Dioxide Evolution of Mannitol and Sodium Benzoate During the First 7 Days of Incubation(mgm.)

| Days | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
|---|---|---|---|---|---|---|---|
| Sodium Benzoate | 5.59 | 15.16 | 21.61 | 28.54 | 26.65 | 23.85 | 23.11 |
| Mannitol | 7.80 | 21.38 | 34.13 | 36.20 | 32.63 | 30.61 | 26.45 |
| Difference | 2.21 | 6.22 | 12.52 | 7.66 | 5.98 | 6.76 | 3.34 |

Mean difference = 6.384 $\sigma$ = 3.078 $\sigma_D$ = 1.256
"t" = 5.082

With the above analysis giving the highly significant "t" value of 5.082 one may conclude that mannitol presents a source of carbon which is readily available and is initially decomposed more rapidly than is the sodium benzoate but that the latter becomes available more slowly or is attacked by a more specific group of organisms but that the effect at the end of the 20 days is practically the

same.

The carbon dioxide curves of the three straw treatments were so similar in trend that it was considered worthwhile to run a coefficient of correlation on the percentage of straw present vs the average daily evolution of carbon dioxide. The average daily evolution of carbon dioxide from the check plot was subtracted from each of the straw treatments so the figures given present the variation in carbon due to the straw treatments alone. (Table V).

TABLE V

Coefficient of Correlation
Percent Straw in the Soil vs Average Daily $CO_2$ Evolution

| % straw in soil | Average daily $CO_2$ evolved | N = 3 |
|---|---|---|
| 2 | 7.724 | r = 0.9997 |
| 5 | 13.964 | r for significance = 0.9970 |
| 10 | 23.234 | |

This correlation is seen to be significant. Although the correlation is high the number of determinations is so small that it would be rather hazardous to make any definite predictions from these data. They indicate, however, that between the limits of 2 percent and 10 percent straw there is a relationship between the amount of straw present in the soil and the average daily evolution of carbon dioxide.

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It is seen from Fig. 2 that the soils which were sterilized and inoculated with Azotobacter vinelandii failed to show any development of the organism. The organism could not be recovered from the soil after incubation even though the soil was heavily inoculated twice during the 20-day incubation period. It seems possible that as a result of its being carried on artificial medium for a considerable length of time, the physiological characteristics of the organism have been so altered as to render it incapable of living in the soil. It is also possible that sterilization has so altered the soil conditions as to make it unfavorable for the growth of this particular organism.

The Azotobacter counts (Table VI and Figs. 5-7) were analyzed statistically. The straw treatments and unsterilized check being analyzed by the variance method and the mannitol treatment and the sodium benzoate treatment by the method of paired comparisons. The treatments were divided in this manner because the mannitol and sodium benzoate treatments differed so greatly from the straw treatments in the number of Azotobacter present that the ungrouped data were not of sufficient homogeniety to be treated in one analysis.

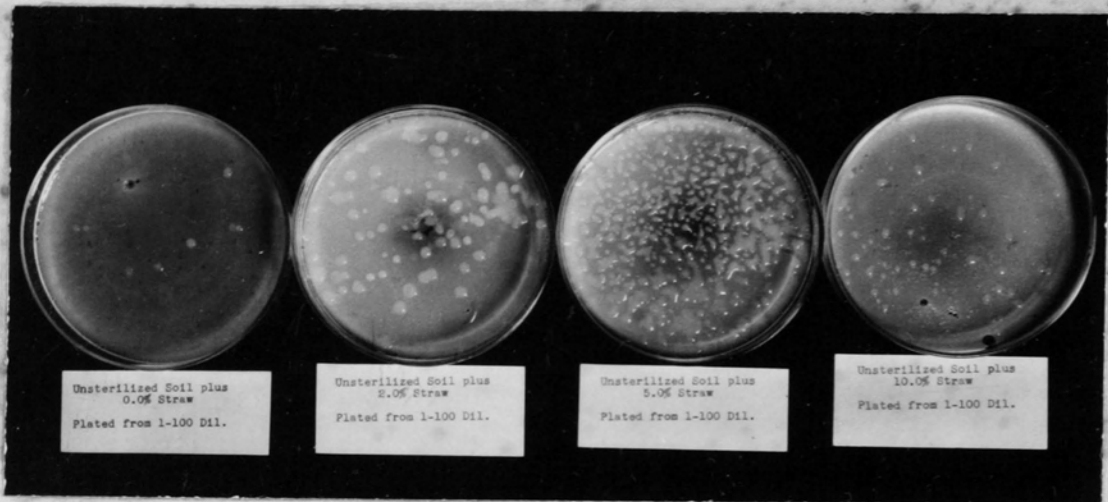



Fig. 5. Azotobacter Plates Poured From Straw Treated Soils.

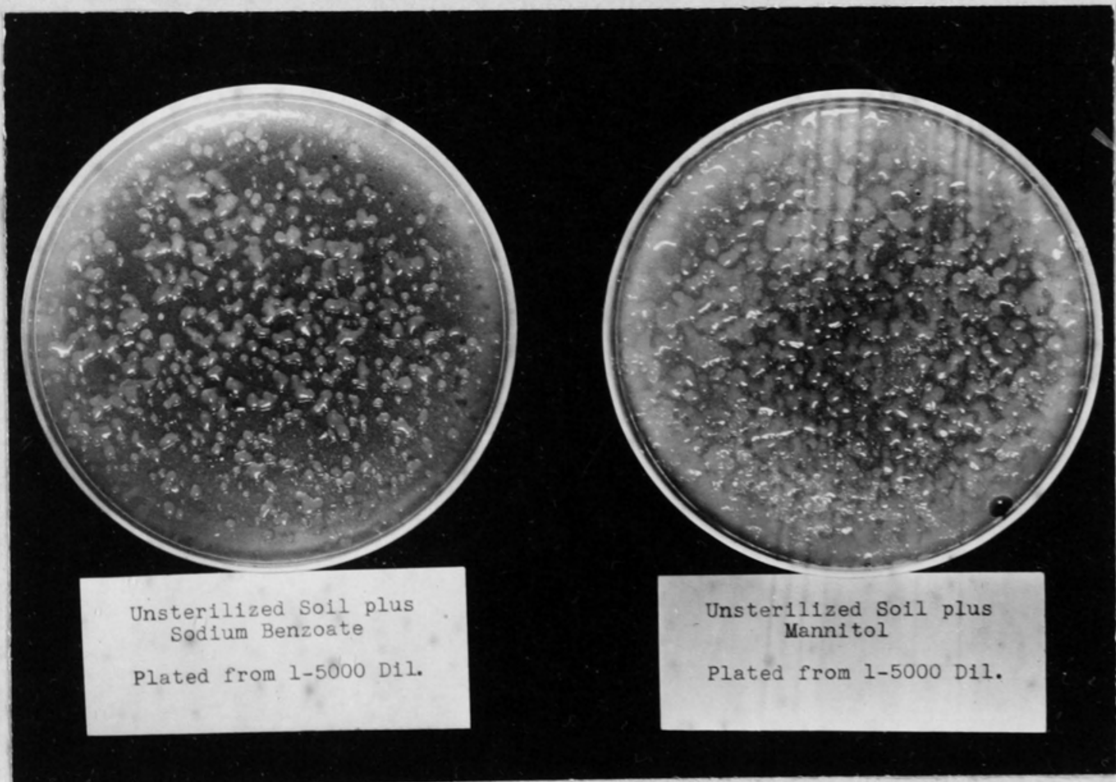



Fig. 6. Azotobacter Plates Poured From Sodium Benzoate and Mannitol Treated Soils.

TABLE VI

Averages of Azotobacter Counts per gm. Soil

| Treatment | Before Incubation | After Incubation | Gain During Incubation |
|---|---|---|---|
| 1. 2% mannitol-sterile + Az. vinelandii | 0 | 0 | 0 |
| 2. 2% mannitol -unsterilized | 300 | 5,967,000[2] | 5,966,700 |
| 3. 1.365% Na-benzoate -sterile + Az. vinelandii | 0 | 0 | 0 |
| 4. 1.365% Na-benzoate -unsterilized | 300 | 2,500,000[2] | 2,499,700 |
| 5. Soil sterilized + Az. vinelandii | 0 | 0 | 0 |
| 6. 2% straw-unsterilized | 300 | 8,000[1] | 7,700 |
| 7. 5% straw-unsterilized | 300 | 45,250[1] | 44,700 |
| 8. 10% straw-unsterilized | 300 | 5,550[1] | 5,200 |
| 9. Soil-untreated-unsterilized | 300 | 300[1] | 0 |

[1] $\sigma$ = 2149. $\sigma_D$ = 1519. Level of significance (5 percent) = 3435
$\sigma_{\bar{x}}$ = 1074.5

[2] $\sigma$ = 500,000 $\sigma_D$ = 352,800 "t" = 9.688

Thus it is seen that the increase in Azotobacter due to treatment represents a significant increase in all cases and that the 5 percent straw treatment shows significantly higher counts than does either the 2 percent or the 10 percent treatment. The 2 percent straw treatment does not, however, show significantly higher counts than the 10 percent treatment. The mannitol treatment

shows significantly higher counts than the benzoate treatment. These variations in Azotobacter counts indicate that for the multiplication of Azotobacter, mannitol provides a much better source of energy than does sodium benzoate and that both of these present a much better source than does the decomposing straw in any of the concentrations tried. A straw concentration of 5 percent provided a better environment for the multiplication of Azotobacter than did a concentration very far above or very far below this percent. It is shown that the application of straw to the soil introduces a source of energy that is utilized by Azotobacter, and results in a pronounced increase in the Azotobacter flora.

The coefficient of correlation was determined for the average daily evolution of carbon dioxide vs the $\log_{10}$ of the Azotobacter counts at the end of the incubation period to determine whether or not this experiment showed any relationship between the rate of decomposition as measured by carbon dioxide evolution and the growth of Azotobacter (Table VII).

TABLE VII

Coefficient of Correlation

(A) Average Daily $CO_2$ Evolution vs
(B) $Log_{10}$ of Azotobacter Numbers

| Treat-ment | 2% Mannitol | 1.365% Na-benzoate | 2% Straw | 5% Straw | 10% Straw | Check |
|---|---|---|---|---|---|---|
| A | 19.148 | 18.877 | 9.198 | 15.852 | 24.700 | 1.4665 |
| B | 6.7757 | 6.3979 | 3.8031 | 4.6532 | 3.74036 | 2.4712 |

r = .5915   r for significance = .811

There is no significant correlation shown to exist between the average daily evolution of carbon dioxide and the $log_{10}$ of the Azotobacter numbers. The actual number of Azotobacter was also used to determine whether or not any relationship could be shown between these two factors. This test, however, gave an r value of only .3517. This experiment, then,fails to show any significant relationship between rate of decomposition of organic matter and numbers of Azotobacter cells.

All of the soils, both treated and untreated, were analyzed for nitrogen at the beginning and at the end of the incubation period to determine what changes had resulted due to the various treatments. These analyses are given in Table VIII.

TABLE VIII

Nitrogen Content* in p.p.m.

| Soil Treatment | Before Incubation | | | After Incubation | | | Change During Incubation | | |
|---|---|---|---|---|---|---|---|---|---|
| | Total Nitrogen | $NH_3$-N | $NO_3$-N | Total Nitrogen | $NH_3$-N | $NO_3$-N | Total Nitrogen | $NH_3$-N | $NO_3$-N |
| 1. 2% mannitol -sterilized + Az. vinelandii | 1444. | 19.4 | 3.3 | 1445. | 38.8 | 7.5 | + 1 | +19.4 | + 4.2 |
| 2. 2% mannitol -unsterilized | 1459. | 6.3 | 6.3 | 1588. | 0.0 | 0.0 | +129 | - 6.3 | - 6.3 |
| 3. 1.365% Na-benzoate-sterilized + Az. vinelandii | 1441. | 22.5 | 5.6 | 1441. | 40.6 | 8.8 | 0 | +18.1 | + 3.2 |
| 4. 1.365% Na-benzoate -unsterilized | 1446. | 5.6 | 10.6 | 1540. | 0.0 | 0.0 | + 94 | - 5.6 | -10.6 |
| 5. Soil-sterilized + Az. vinelandii | 1524. | 22.5 | 6.9 | 1519. | 43.1 | 10.0 | - 5 | +20.6 | + 3.1 |
| 6. 2% straw -unsterilized | 1601. | 7.5 | 10.0 | 1682. | 0.0 | 0.0 | + 81 | - 7.5 | -10.0 |
| 7. 5% straw -unsterilized | 1746. | 18.1 | 28.1 | 1820. | 0.0 | 0.0 | + 74 | -18.1 | -28.1 |
| 8. 10% straw -unsterilized | 1925. | 29.3 | 50.0 | 2089. | 0.0 | 0.0 | +164 | -29.3 | -50.0 |
| 9. Soil-untreated -unsterilized | 1460. | 0.0 | 5.0 | 1540. | 0.0 | 0.0 | + 80 | 0 | - 5.0 |

* Each figure represents the average of a single determination on each of the four replicates.

The change in total nitrogen during incubation was analyzed by the analysis of variance and gave the following:

$\sigma_{+} = 28.77 \qquad \sigma_{\bar{x}} = 14.38 \qquad \sigma_{D} = 20.33$

Level of significance (5 percent) = 41.96

From the table it is seen that there has been a significant amount of nitrogen fixed in all flasks except those treated with Az. vinelandii. In each case where nitrogen fixation has taken place both the ammonia nitrogen and nitrate nitrogen has been reduced to zero. It is also to be noted that there has been a significant amount of nitrogen fixed due to treatment in only two cases, i.e. the 2 percent mannitol treatment and the 10 percent straw treatment, with a fixation of 129 p.p.m. and 164 p.p.m. of nitrogen respectively. Since these two treatments represent the extremes in Azotobacter numbers and highest values in average daily carbon dioxide evolution, correlation coefficients were run to show the relationship between these three factors (Table IX).

TABLE IX

Correlation Coefficients Between Azotobacter Number, Amount of Nitrogen Fixed, and Average Daily $CO_2$ Evolution

| Factors | Correlation (r) | Correlation Necessary for Significance |
|---|---|---|
| Azotobacter numbers vs | | |
| Nitrogen fixed | .2483 | .811 |
| $CO_2$ evolution | .3517 | .811 |
| $Log_{10}$ Azotobacter numbers vs | | |
| Nitrogen fixed | .1425 | .811 |
| $CO_2$ evolution | .5915 | .811 |
| Nitrogen fixed vs | | |
| $CO_2$ evolution | .7330 | .811 |

It is readily seen from the table that there is no significant correlation between any of the factors tested. It may be concluded then that the rate of decomposition of organic matter as measured by carbon dioxide evolution bears no significant relationship to the growth of Azotobacter. Increase in the numbers of Azotobacter in the soil was not accompanied by a corresponding increase in the amount of nitrogen fixed. In fact, the largest amount of nitrogen fixed (10 percent straw) was accompanied by the smallest increase in the number of Azotobacter. It does not seem likely that the nitrate nitrogen present has exerted any pronounced variation in the results obtained, for in the case of the 10 percent straw treatment the nitrate nitrogen is present in the greatest initial quantity and nitrogen fixation was

greatest, but this relationship does not exist in the other straw treatments. In the case of the 2 percent mannitol where nitrogen fixation is second highest the nitrate nitrogen is present in a very small amount. It would seem, therefore, that the nitrate nitrogen present has introduced no corresponding variation in nitrogen fixation.

It is entirely possible that the Azotobacter count at the end of the 20-day incubation period does not give a true picture of the Azotobacter activity during incubation, but it is doubtful that this final count departs far enough from the actual condition to account for the discrepancies that exist between Azotobacter numbers and the amount of nitrogen fixed.

## Series 2

This series of tests was run to determine whether or not the Azotobacter of a soil, normally containing this bacterium, could be stimulated to greater activity by the application of certain salts known to exert a stimulatory effect on their development in artificial media. It has been shown in the previous series that the addition of 5 percent straw to this soil brings about a large increase in Azotobacter numbers. This series was also designed to determine what influence inorganic nutrients in combination with 5 percent straw might exert on the Azotobacter numbers.

One hundred grams of Agronomy Farm soil were placed in 500 cc. Erlenmeyer flasks and the various inorganic nutrients were added to this soil in the water which was added to bring the moisture content up to 25 percent. The flasks were treated as follows:

Nos. 1-4 0.2 p.p.m. Mo as $Na_2MoO_4.2H_2O$.
Nos. 5-8 500 p.p.m. P as $Ca(H_2PO_4)_2.H_2O$.
Nos. 9-12 500 p.p.m. K as KCl.
Nos.13-16 40 p.p.m. Fe as $FeSO_4.7H_2O$.
Nos.17-20 Check - 100 gms. soil untreated.
Nos.21-24 Same as Nos. 1-4 + 5 percent wheat straw.
Nos.25-28 Same as Nos. 5-8 + 5 percent wheat straw.
Nos.29-32 Same as Nos. 9-12 + 5 percent wheat straw.

Nos. 33-36 Same as Nos. 13-16 + 5 percent wheat straw.

Nos. 37-40 Same as Nos. 17-20 + 5 percent wheat straw.

The flasks were plugged with cotton and placed in the 28° C. constant temperature room where they were allowed to incubate for four weeks. The moisture content was maintained at 25 percent by keeping the flasks at a constant weight by the addition of distilled water. The number of Azotobacter cells per gram of soil was determined by pouring dilution plates at the beginning of the experiment and at the end of 1 week, 3 weeks and 4 weeks. Four plates were poured from each flask, making a total of 16 plates for each treatment. The average number of Azotobacter cells per treatment for the different periods of incubation is given in Table X and shown graphically in Fig. 7.

An analysis of variance was applied to the data. The skeleton and pertinent information derived from this analysis is given in Tables XI and XII. (Sub-treatments in this analysis refer to straw and no straw).

TABLE X

Averages* of Azotobacter Counts per gm. Soil

| Sub-treatment | Treatment: Mo. | P. | K. | $Fe^{++}$ | Check | Average |
|---|---|---|---|---|---|---|
| | 0 Weeks | | | | | |
| No Straw | 180 | 190 | 220 | 265 | 210 | 213 |
| Straw | 145 | 120 | 140 | 115 | 100 | 124 |
| Average | 162.5 | 155.0 | 180.0 | 190.0 | 155.0 | 168.5 |
| | 1 Week | | | | | |
| No Straw | 285 | 125 | 180 | 160 | 230 | 196 |
| Straw | 465 | 530 | 780 | 420 | 300 | 499 |
| Average | 375.0 | 327.5 | 480.0 | 290.0 | 265.0 | 347.5 |
| | 3 Weeks | | | | | |
| No Straw | 185 | 150 | 110 | 95 | 220 | 152 |
| Straw | 155 | 155 | 180 | 140 | 160 | 158 |
| Average | 170.0 | 152.5 | 145.0 | 117.5 | 190 | 155 |
| | 4 Weeks | | | | | |
| No Straw | 265 | 130 | 155 | 210 | 215 | 195 |
| Straw | 290 | 595 | 385 | 205 | 155 | 326 |
| Average | 277.5 | 362.5 | 270 | 207.5 | 185.0 | 260.5 |
| Average over month | 246 | 249 | 268 | 201 | 198 | |

General Average for Sub-treatments.

No straw = 190 Level of significance = 63.2

Straw = 275

* Each figure represents the average of 4 plates poured from each of the 4 replications.

Fig. 7 The Influence of Inorganic Nutrients and Straw on Azotobacter Numbers

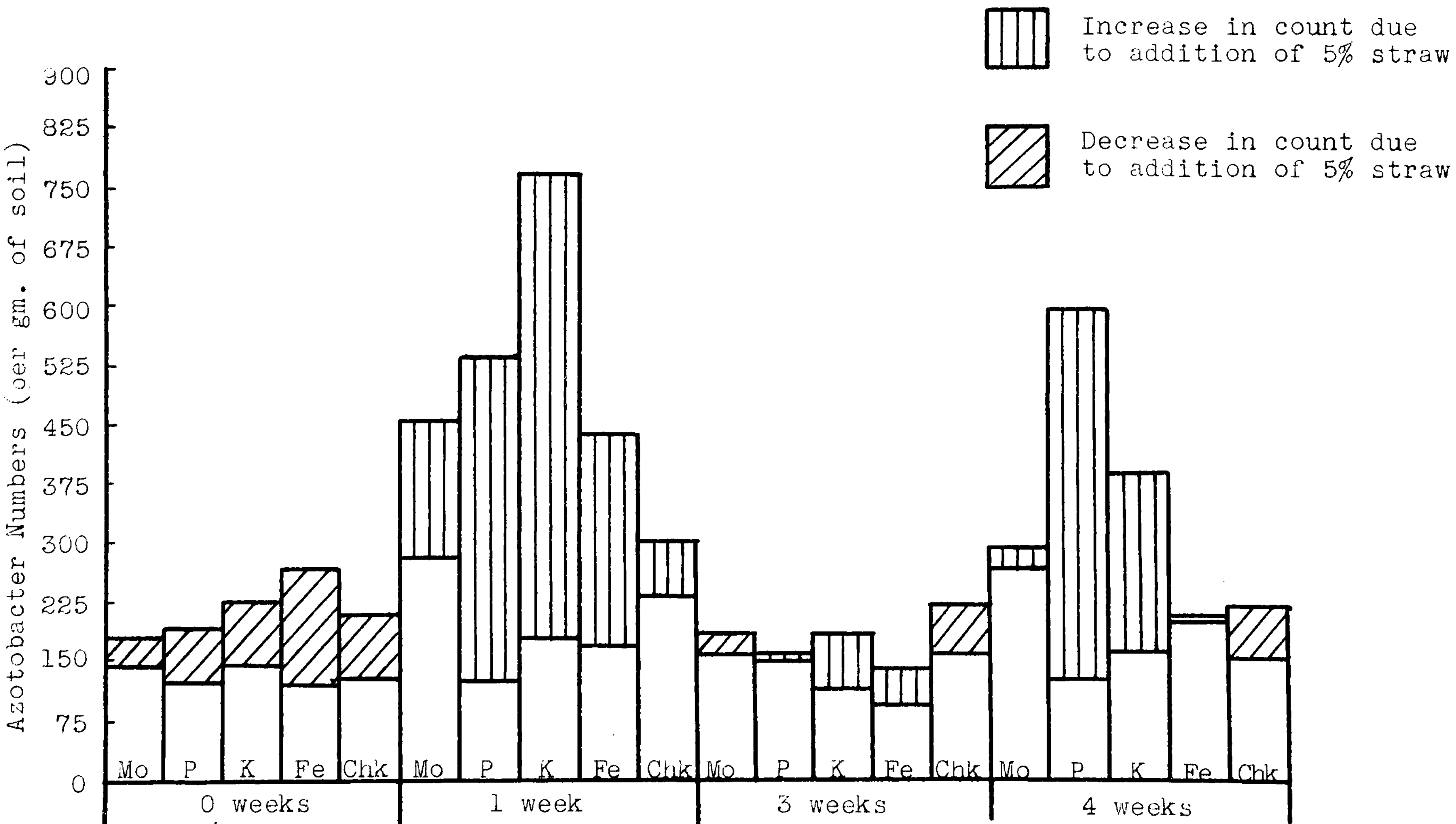


TABLE XI

Analysis of Variance of Azotobacter Numbers

| Variance due to: | Degrees of Freedom | F | $\sigma_d$ |
|---|---|---|---|
| Replicates | 3 | 0.35 | |
| Time | 3 | 20.22** | |
| Sub-treatments | 1 | 19.35** | |
| Treatments | 4 | 2.02 | |
| Treatments x time | 12 | 1.52 | |
| Treatments x sub-treatments | 4 | 6.00** | |
| Treatments x time x sub-treatments | 12 | 1.92 | |
| Time x sub-treatments | 3 | 18.16** | |
| Error | 117 | | 126.4 |
| Total | | | |

$\sigma_{\bar{x}}$ = 22.35 $\sigma_D$ = 31.6 Level of significance (5 percent) = 63.2

** Exceeds 1 percent point.

TABLE XII

Interaction of Treatments and Sub-treatments

| Interactions | Cross Difference | Constants |
|---|---|---|
| Mo - Sub-treatments | 72 | $\sigma_{\bar{x}}$ = 28.2 |
| P - Sub-treatments | 260* | $\sigma_{DD}$ = 56.4 |
| K - Sub-treatments | 235* | |
| Fe - Sub-treatments | 76 | Level of significance (5 percent) = 112.9 |

Note: Cross differences greater than the level of significance are significant.

It will be seen from these data that the inorganic nutrients have brought about no significant variation in the number of Azotobacter in the soil. There is, on the other hand, a significant variation in the Azotobacter numbers with regard to time. There has been a significant increase in the Azotobacter numbers at the end of one week's incubation. At the end of three weeks the numbers have fallen off until there is practically the same count as at the beginning of the experiment. At the end of four weeks the count has again increased to a level significantly above the original count. This variation in numbers is probably due to the logarithmic growth and death cycles which occur as utilizable carbonaceous material is made available. When the organic matter has been broken down to a point where it may be used as a source of energy by the Azotobacter, there is a rapid growth of Azotobacter. This period is followed by a period of rapid decrease in numbers due to the rapid utilization of the available carbonaceous material. This depression in numbers of Azotobacter is followed by a second increase as more energy material is made available by the decomposition of the organic matter present in the soil. This second increase is not as great as the first and it is entirely possible that this cyclic fluctuation would continue, each peak being lower than the preceding one, until available

organic matter again becomes the limiting factor.

The sub-treatment, or the 5 percent straw, also brought about a significant increase in the number of Azotobacter present over the treatments in which no straw was added.

It will also be noted from Table XI that there was a significant interaction between the treatments and the 5 percent straw treatment. The interactions of molybdenum, phosphorus, potassium, and ferrous iron with straw were tested separately to determine wherein the significant interaction took place. An analysis of these interactions is shown in Table XII. It will be seen from this analysis that the molybdenum and the iron brought about no significant interaction. Both the phosphorus and the potassium, on the other hand, interacted with the straw-treated soils and brought about a significant increase in the Azotobacter count. This means that both phosphorus and potassium, in the presence of the straw treatment, brought about a greater increase in the Azotobacter population. than any of the three treatments alone. It would seem, then, that a limiting factor in the growth of Azotobacter in this soil is available carbonaceous material and that phosphorus and potassium are only of secondary importance. Iron and molybdenum cause no measureable alteration in the development of Azotobacter, either in the presence or the absence of the carbonaceous material used.

## Series 3

This series of tests was run on virgin dryland soil, known to be extremely low in Azotobacter cells. It was sought to determine what factors were limiting for the development of this organism. It is known that certain inorganic salts act as nutrients for Azotobacter. The application of these salts to the soil should, in case they are limiting factors, allow the Azotobacter to develop. The sample was obtained by scraping off the surface vegetation and taking the first four inches of soil. This soil sample was brought into the laboratory where it was dryed, thoroughly mixed, and screened through a ten mesh screen.

The experiment was set up by placing 200 grams of the soil in 500 cc. Erlenmeyer flasks. These flasks were treated as follows:

Part 1

| | |
|---|---|
| Nos. 1-4 | 0.2 p.p.m. Mo as $Na_2MoO_4 \cdot 2H_2O$ |
| Nos. 5-8 | 500 p.p.m. P as $Ca(H_2PO_4)_2 \cdot H_2O$ |
| Nos. 9-12 | 500 p.p.m. K as KCl |
| Nos. 13-16 | 40 p.p.m. Fe as $FeSO_4 \cdot 7H_2O$ |
| Nos. 17-20 | Check |
| Nos. 21-24 | 1% mannitol |

The moisture content of the soil was maintained at 20 percent by keeping flasks at a constant weight by the addition of distilled water. The flasks of soil were

incubated in the 28° C. constant temperature room for four weeks. The number of Azotobacter cells in the soil was determined at weekly intervals by plating out on Wenzl's modified medium. The counts are given in Table XIII. The soil was tested for the presence of Azotobacter at the beginning of the experiment by plating out on Wenzl's medium in a dilution of 1-10 and also by sprinkling a weighed amount of the soil directly on the surface of the medium. None of these plates showed Azotobacter development, though in the case of the sprinkled plates as much as 0.18 gm. of soil was added to the plates.

TABLE XIII

Azotobacter Numbers in Dryland Pasture Soil (per gm. Soil)

| Treatment | Original Soil | 1 week | 2 weeks | 3 weeks | 4 weeks |
|---|---|---|---|---|---|
| 1. 0.2 p.p.m. Mo | 0 | 0 | 0 | 0 | 0 |
| 2. 500 p.p.m. P | 0 | 0 | 0 | 0 | 0 |
| 3. 500 p.p.m. K | 0 | 0 | 0 | 0 | 0 |
| 4. 40 p.p.m. Fe | 0 | 0 | 0 | 0 | 0 |
| 5. 1% mannitol | 0 | 1,830 | 9,125 | 12,000 | 13,550 |
| 6. Check | 0 | 0 | 0 | 0 | 0 |

It is seen from this table that the addition of the inorganic nutrients brought about no alteration in the Azotobacter flora of this soil. The mannitol, on the other hand, brought about a large increase in the number of Azotobacter cells present. Since it is doubtful that

this dryland soil is totally lacking in Azotobacter cells, it may be assumed that an available source of energy is the limiting factor for the growth of Azotobacter. When this energy material was supplied in the form of mannitol there was an immediate and pronounced increase in the number of Azotobacter cells present. The number of organisms continued to increase during the entire four weeks of incubation.

Part 2

With the above results in mind it seemed desirable to know whether or not an available source of carbon was the only limiting factor for the development of Azotobacter in this soil, or if one or more of the inorganic nutrients played a secondary role in the development of this organism. This was determined by adding both mannitol and the inorganic nutrients to the soil and inoculating with a crude culture of Azotobacter. The experiment was set up by placing 200 gms. of soil in Erlenmeyer flasks and treating as follows:

Nos. 1-4 0.2 p.p.m. Mo as $Na_2MoO_4.2H_2O$ and 0.5% mannitol
Nos. 5-8 500 p.p.m. P as $Ca(H_2PO_4)_2.H_2O$ and 0.5% mannitol
Nos. 9-12 500 p.p.m. K as KCl and 0.5% mannitol
Nos. 13-16 40 p.p.m. Fe as $FeSO_4.7H_2O$ and 0.5% mannitol
Nos. 17-20 0.5% mannitol

These flasks were inoculated with 1 cc. of a suspension of Azotobacter isolated shortly before from the

Agronomy Fam soil. The flasks were incubated under the same conditions as temperature and moisture as those in Part 1. Azotobacter counts were made at the end of one, two and three weeks. These counts are given in Table XIV. The statistical analysis of the counts is given in Table XV.

TABLE XIV

Azotobacter Counts* in Inoculated Dryland Soil (per gm.Soil)

| Soil Treatment | Time | | | Means |
|---|---|---|---|---|
| | 1 Week | 2 Weeks | 3 Weeks | |
| 1-0.2 p.p.m.Mo+ 0.5% mannitol | 54,500,000 | 62,875,000 | 69,125,000 | 62,166,000 |
| 2-500 p.p.m.P + 0.5% mannitol | 42,625,000 | 25,125,000 | 23,625,000 | 30,458,000 |
| 3-500 p.p.m.K + 0.5% mannitol | 81,375,000 | 67,125,000 | 61,750,000 | 70,083,000 |
| 4-40 p.p.m. Fe+ 0.5% mannitol | 65,125,000 | 61,500,000 | 50,500,000 | 59,041,000 |
| 5-Check 0.5% mannitol | 48,125,000 | 47,250,000 | 41,625,000 | 45,666,000 |
| Means | 58,350,000 | 52,775,000 | 49,325,000 | |

* Each figure is the average of two plates poured from each of the four replicates.

TABLE XV

Analysis of Variance of Azotobacter Counts in Dryland Soil

| Variance due to: | Degrees of Freedom | F | $\sigma_{\Delta}$ |
|---|---|---|---|
| Replicates | 3 | 0.29 | |
| Treatments | 4 | 24.35** | |
| Time | 2 | 3.46* | |
| Treatments x time | 8 | 1.96 | |
| Error | 42 | | 10,948,000 |
| Total | 59 | | |

$\sigma_{\bar{x}}$ for treatments = 3,160,500 $\sigma_{\bar{x}}$ for time = 2,448,000

$\sigma_{D}$ for treatments = 4,469,500 $\sigma_{D}$ for time = 3,462,000

Level of significance =9,032,000 Level of significance = 6,996,000

* Exceed 5 percent point ** Exceeds 1 percent point

It will be seen from these data that the inorganic nutrients added to the dryland soil in combination with mannitol exerted a differential effect on the number of Azotobacter cells which were added to the soil. The molybdenum, potassium, and iron treatments all have a significantly higher count than does the check. This may be interpreted to mean that these three inorganic salts have had a stimulatory effect on the Azotobacter flora. The phosphorus treatment, on the other hand, brought about a significant decrease in the Azotobacter cells in the soil. The depressing effect of phosphorus under certain conditions has been noted before but no adequate explan-

ation of the phenomenon is forthcoming.

It will also be noted from Table XIV that there was a gradual decrease in mean Azotobacter count over the three weeks incubation period. There are, however, cases within individual treatments where the opposite is the case, e.g. the molybdenum treatment.

It may be concluded, then, that this dryland soil furnished very little energy material for Azotobacter growth and as the mannitol supply is exhausted there is a gradual decrease in the Azotobacter count. This decrease in count may be partly compensated for by the increased development of the Azotobacter in the presence of the inorganic nutrients.

<u>Series 4</u>

This series of experiments was set up to determine the extent to which the decomposition products of cellulose could act as a source of energy for Azotobacter. This was tested with various concentrations of cellulose, both in the absence of added nitrate nitrogen and in the presence of added nitrate nitrogen. Sterilized Agronomy Farm soil was used in the test. One hundred grams of the soil was placed in 250 cc. Florence flasks. The flasks were treated as follows:

Nos. 1-4 0% cellulose. No nitrate.

Nos. 5-8 1% cellulose. No nitrate.

Nos. 9-12 5% cellulose. No nitrate.

Nos. 13-16 10% cellulose. No nitrate.

Nos. 17-20 0% cellulose + 365 p.p.m. $NaNO_3$

Nos. 21-24 1% cellulose + 365 p.p.m. $NaNO_3$

Nos. 25-28 5% cellulose + 365 p.p.m. $NaNO_3$

Nos. 29-32 10% cellulose + 365 p.p.m. $NaNO_3$

Nos. 33-36 Check flasks

The moisture content of the flasks was adjusted to 25 percent. To each of these sterilized flasks was added 1 cc. of a suspension of a cellulose decomposing bacterium designated in this laboratory as cellulose decomposer No. 12. The flasks were connected to the carbon dioxide apparatus in the constant temperature room and the carbon dioxide evolved was determined daily for 16 days. At the end of this 16-day period the flasks were inoculated with a crude culture of Azotobacter and the carbon dioxide evolved was determined daily for the next 16 days. The averages of the carbon dioxide evolved during the 32-day incubation period are given in Table XVI and shown graphically in Figs. 8 and 9. The carbon dioxide from the check flasks was subtracted from that evolved by the treated flasks, hence the data given represent the carbon dioxide evolution due to treatment

Figs. 8 and 9 show that there has been a variation in the amount of carbon dioxide evolved by the various cellulose treatments and that there has been much more

AVERAGES* OF CARBON DIOXIDE EVOLVED (In mg.)

| Soil Treatment | Time in days | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 |
| 1. 0% cellulose + cellulose decomposer | 5.16 | 5.36 | 3.59 | 2.39 | 1.59 | 1.42 | 1.07 | 1.00 | 0.62 | 0.76 | 0.32 | 0.59 | 0.52 | 0.49 | 0.36 | 0.26 |
| 2. 1% cellulose + cellulose decomposer | 4.81 | 5.86 | 3.35 | 2.35 | 1.89 | 1.42 | 1.37 | 1.20 | 1.10 | 1.07 | 0.62 | 0.67 | 0.77 | 1.04 | 1.34 | 1.17 |
| 3. 5% cellulose + cellulose decomposer | 3.80 | 4.75 | 3.82 | 3.69 | 3.44 | 3.24 | 3.26 | 3.22 | 3.40 | 3.32 | 3.15 | 3.29 | 3.25 | 3.52 | 3.54 | 3.52 |
| 4. 10% cellulose + cellulose decomposer | 2.48 | 3.00 | 3.10 | 3.42 | 3.20 | 3.09 | 3.12 | 3.40 | 3.46 | 3.77 | 3.37 | 3.50 | 3.64 | 3.74 | 3.55 | 3.55 |
| 5. 0% cellulose + 365 p.p.m. $NaNO_3$ + cellulose decomposer | 3.62 | 7.69 | 5.66 | 2.99 | 2.49 | 2.00 | 1.44 | 1.16 | 1.22 | 0.79 | 1.30 | 0.70 | 0.54 | 0.59 | 0.45 | 0.19 |
| 6. 1% cellulose + 365 p.p.m. $NaNO_3$ + cellulose decomposer | 3.24 | 6.80 | 5.55 | 3.19 | 3.44 | 3.97 | 4.65 | 5.75 | 5.62 | 6.71 | 6.80 | 6.46 | 6.47 | 6.45 | 6.66 | 5.94 |
| 7. 5% cellulose + 365 p.p.m. $NaNO_3$ + cellulose decomposer | 3.32 | 7.40 | 6.69 | 4.92 | 6.50 | 7.74 | 8.07 | 9.57 | 9.76 | 9.81 | 9.96 | 9.39 | 9.37 | 8.92 | 9.24 | 8.75 |
| 8. 10% cellulose + 365 p.p.m. $NaNO_3$ + cellulose decomposer | 2.55 | 6.25 | 6.96 | 6.79 | 7.71 | 9.25 | 10.00 | 9.55 | 9.47 | 8.97 | 9.46 | 8.10 | 8.76 | 7.39 | 7.92 | 6.07 |

| Soil Treatment | Time in days | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | 30 | 31 | 32 |
| 1. 0% cellulose + cellulose decomposer | 1.17 | 0.96 | 0.64 | 0.56 | 0.71 | 0.45 | 0.40 | 0.56 | 0.46 | 0.51 | 0.64 | 0.30 | 0.27 | 0.40 | 0.87 | 0.21 |
| 2. 1% cellulose + cellulose decomposer | 2.81 | 2.39 | 2.12 | 2.07 | 2.39 | 2.24 | 2.21 | 2.65 | 2.45 | 2.94 | 3.07 | 1.97 | 1.72 | 2.82 | 1.80 | 1.80 |
| 3. 5% cellulose + cellulose decomposer | 5.40 | 4.67 | 4.22 | 4.07 | 4.04 | 4.32 | 3.72 | 3.91 | 3.60 | 3.61 | 3.95 | 2.51 | 3.26 | 2.79 | 2.81 | 2.74 |
| 4. 10% cellulose + cellulose decomposer | 4.89 | 4.07 | 3.62 | 3.41 | 3.34 | 3.55 | 3.65 | 3.40 | 3.45 | 3.51 | 4.29 | 2.80 | 3.37 | 3.56 | 3.39 | 3.41 |
| 5. 0% cellulose + 365 p.p.m. $NaNO_3$ + cellulose decomposer | 1.06 | 0.64 | 0.51 | 0.61 | 0.52 | 0.30 | 0.24 | 0.45 | 0.25 | 0.10 | 0.19 | 0.27 | 0.09 | 0.19 | 0.22 | 0.25 |
| 6. 1% cellulose + 365 p.p.m. $NaNO_3$ + cellulose decomposer | 8.19 | 6.91 | 6.22 | 5.67 | 6.14 | 6.05 | 5.30 | 4.99 | 4.66 | 4.45 | 4.30 | 4.44 | 3.40 | 3.10 | 3.35 | 2.74 |
| 7. 5% cellulose + 365 p.p.m. $NaNO_3$ + cellulose decomposer | 9.57 | 8.57 | 8.11 | 8.26 | 6.41 | 6.07 | 6.41 | 5.55 | 5.30 | 4.42 | 4.50 | 5.22 | 3.87 | 4.54 | 3.91 | 3.50 |
| 8. 10% cellulose + 365 p.p.m. $NaNO_3$ + cellulose decomposer | 7.97 | 7.15 | 7.01 | 7.07 | 5.61 | 6.20 | 6.02 | 5.44 | 5.10 | 4.79 | 5.31 | 5.64 | 4.32 | 4.35 | 3.87 | 3.25 |

Fig. 8 Average daily evolution of carbon as carbon dioxide

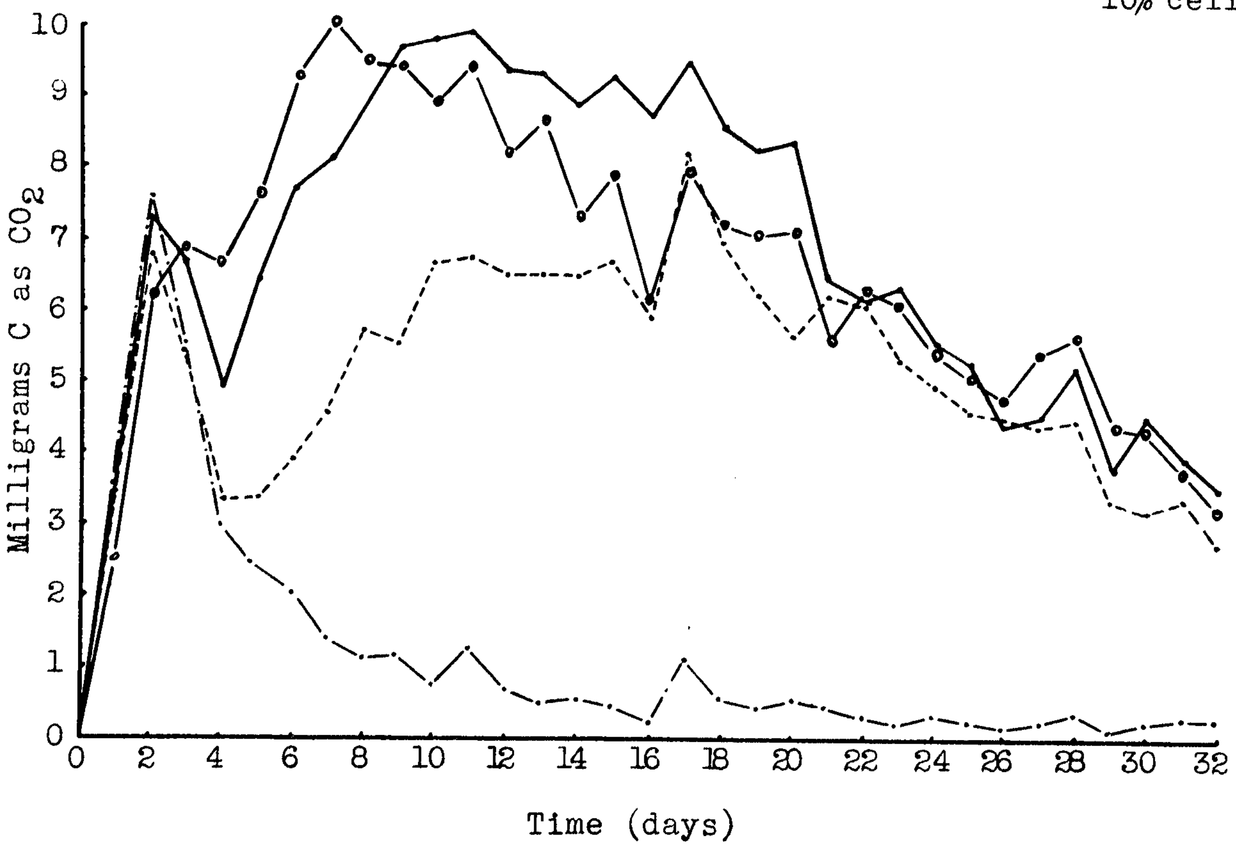
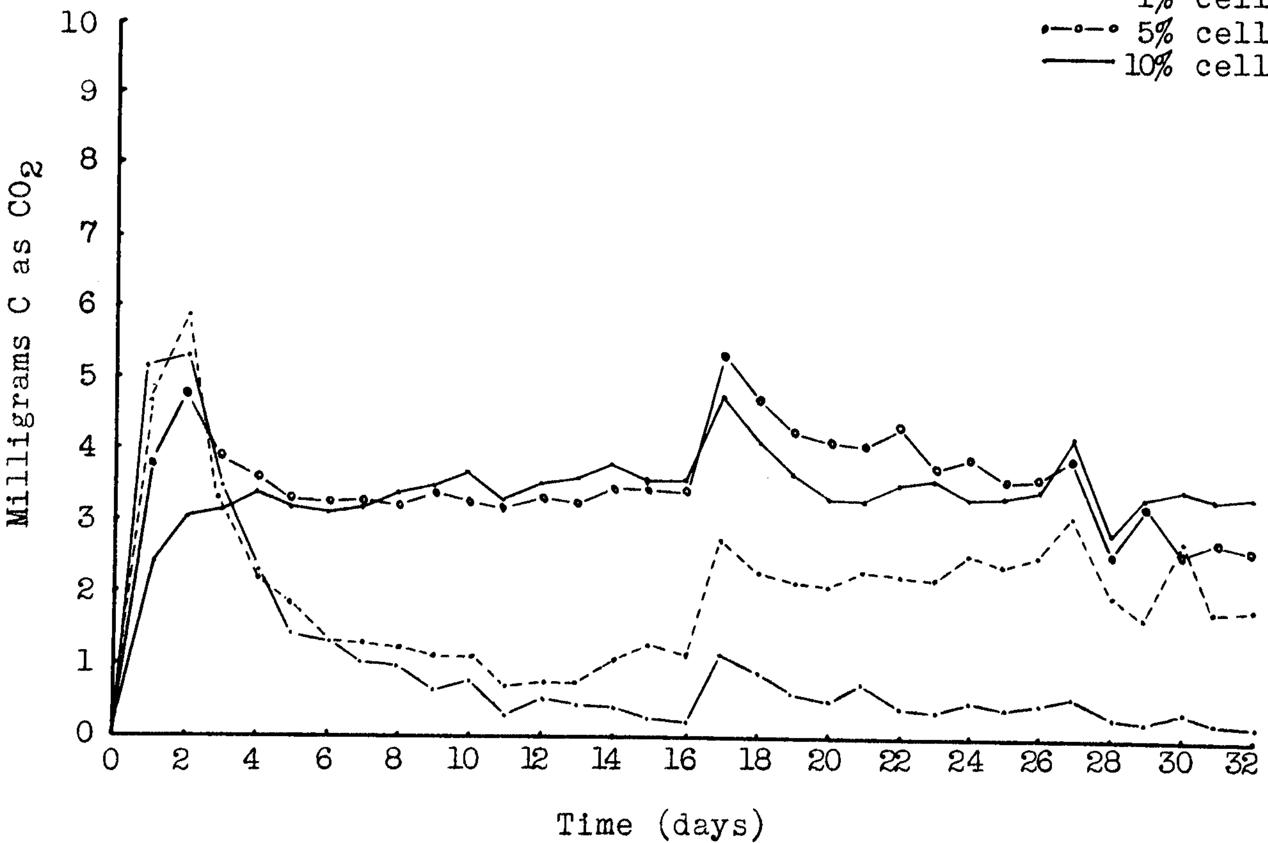


Fig. 9 Average daily evolution of carbon as carbon dioxide



carbon dioxide given off by the flasks to which nitrate has been added. The data for carbon dioxide evolution were analyzed statistically to determine wherein significant differences lay. This analysis is shown in Tables XVII and XVIII.

TABLE XVII

Analysis of Variance of Carbon Dioxide Evolution

| Variance due to: | D.F. | Sums of Sqs. | | F |
|---|---|---|---|---|
| Replicates | 3 | 4.71 | 1.58 | 7.62** |
| Cellulose | 3 | 2,816.28 | 938.76 | 45,570. ** |
| Time | 15 | 265.68 | 17.71 | 85.97** |
| Periods | 1 | 180.82 | 180.82 | 877.76** |
| Nitrates | 1 | 1,545.82 | 1,545.82 | 7,503.98** |
| Cellulose x time | 45 | 173.66 | 3.86 | 18.73** |
| Cellulose x time x periods | 45 | 473.84 | 10.53 | 51.11** |
| Cellulose x time x periods x nitrates | 45 | 144.43 | 3.21 | 15.58** |
| Cellulose x time x nitrates | 45 | 51.00 | 1.13 | 5.48** |
| Cellulose x periods | 3 | 60.90 | 20.30 | 98.54** |
| Cellulose x periods x nitrates | 3 | 45.60 | 15.20 | 73.78** |
| Cellulose x nitrates | 3 | 466.60 | 155.53 | 755.00** |
| Time x periods | 15 | 162.94 | 10.86 | 52.71** |
| Time x periods x nitrates | 15 | 297.93 | 19.86 | 96.40** |
| Time x nitrates | 15 | 58.77 | 3.91 | 18.98** |
| Periods x nitrates | 1 | 164.16 | 164.16 | 79.68** |
| Error | 765 | 157.66 | 0.206 | |
| Total | 1,023 | 7,070.805 | | |

$\sigma$ = 0.453

** Exceeds 1 percent point.

TABLE XVIII

Constants for Carbon Dioxide Evolution

| | $\sigma_{\bar{x}}$ | $\sigma_D$ | Level of significance (5%) |
|---|---|---|---|
| Cellulose | 0.026 | 0.036 | 0.072 |
| Nitrate | 0.018 | 0.025 | 0.049 |

Average Daily Evolution of Carbon as Carbon Dioxide

| | | | |
|---|---|---|---|
| 0% cellulose | 1.13 mg. | No nitrates | 2.56 mg. |
| 1% cellulose | 3.67 mg. | 365 p.p.m. $NaNO_3$ | 5.03 mg. |
| 5% cellulose | 5.30 mg. | | |
| 10% cellulose | 5.06 mg. | | |

Interaction of Cellulose and Nitrate

| Interactions | Cross Difference |
|---|---|
| 1% cellulose<br>No nitrate and nitrate | 2.98 |
| 5% cellulose<br>No nitrate and nitrate | 2.85 |
| 10% cellulose<br>No nitrate and nitrate | 3.04 |

Level of significance (5 percent) = 0.16.

The above tables show that all primary causes of variance and all interactions tested were significant. Since cellulose treatments and nitrate treatments were the only ones of primary interest, they were the only ones analyzed further to determine wherein the significance lay. This completed analysis is given in Table XVIII. All cellulose treatments are shown to differ significantly from each other. The amount of carbon dioxide evolved increased with an increase in cellulose content up to 5 per-

cent cellulose. The amount of carbon dioxide given off from 10 percent cellulose treatment was, however, less than that given off from the 5 percent cellulose treatment. It would seem, then, that a decomposition equilibrium was reached at approximately 5 percent cellulose and that an increase in cellulose far above this percentage did not bring about a corresponding increase in the rate at which the cellulose was decomposed.

The rate of decomposition of the cellulose was strongly influenced by the presence of nitrates, the average amount of carbon dioxide given off in the presence of nitrates being about twice that given off in the absence of added nitrates. This was largely to be expected since the nitrate nitrogen originally present in the soil was probably not enough to supply the needs of the cellulose decomposers.

It will also be noted in Table XVIII that there was a significant interaction between the nitrate and cellulose treatments in all concentrations of cellulose. This tends to confirm the hypothesis that an available supply of nitrogen becomes a limiting factor in the decomposition of cellulose, because this significant interaction is interpreted to mean that there is more carbon dioxide given off when both cellulose and nitrate are added together than when each is added separately.

The number of Azotobacter cells present in the soil of the treated flasks was determined at the end of the experiment. The averages of these counts per treatment are given in Table XIX. The analysis of these counts is given in Table XX.

TABLE XIX

Averages of Azotobacter Counts in Cellulose and Nitrate Treated Soil

| Treatment | No Nitrate | 365 p.p.m. $NaNO_3$ | Mean |
|---|---|---|---|
| 0% cellulose | 741,700 | 233,700 | 487,700 |
| 1% cellulose | 574,200 | 472,500 | 523,300 |
| 5% cellulose | 698,500 | 962,500 | 830,500 |
| 10% cellulose | 322,000 | 662,500 | 492,200 |
| Mean | 584,100 | 582,800 | |

TABLE XX

Analysis of Variance of Azotobacter Numbers in Cellulose and Nitrate Treated Soils

| Variance due to: | Degrees of Freedom | F | $\sigma_\Delta$ |
|---|---|---|---|
| Replicates | 3 | 1.5697 | |
| Treatments | 7 | 5.496** | |
| (Cellulose) | (3) | 5.383** | |
| (Nitrates) | (1) | 0.0003 | |
| (Cellulose x nitrates) | (3) | 7.441** | |
| Error | 21 | | 201,600 |
| Total | 31 | | |

$\sigma_{\bar{x}}$ for cellulose = 71,300

$\sigma_D$ for cellulose = 100,800

Level of significance for cellulose = 209,700

These tables show that the Azotobacter count was markedly influenced by the treatment the soil received. Treatment as given in Table XX refers to both nitrate and cellulose treatments. This was broken down into its component parts to determine what factors were contributing to this significant difference. It is seen from the table that cellulose and the interaction of cellulose with nitrates were the two factors which brought about the variation. Nitrates alone caused no significant differences in the Azotobacter count. The interaction of nitrates with cellulose means that the two in combination, under the conditions of this experiment, provided a more favorable environment for Azotobacter development than did either working alone. The role played by the nitrate in bringing about this increased development of Azotobacter was probably an indirect role. It has been shown that more decomposition of cellulose took place in the presence of the nitrates, and since the energy material for Azotobacter was derived from the decomposition of cellulose, it is probable that this interaction provided a more favorable environment for Azotobacter development. The nitrate nitrogen added was probably all utilized by the cellulose decomposers during the first 16 days of incubation, so it is quite probable that the nitrate nitrogen acted only indirectly by increasing cellulose decomposition and thus

providing more decomposition products which were utilized by Azotobacter.

## SUMMARY

This investigation into the relationship of environmental factors to the growth and development of Azotobacter was undertaken in an attempt to bring about a more thorough understanding of the role Azotobacter may play in soils. Throughout the investigation, soil both untreated and modified in certain known ways, was the medium used to test the responses of Azotobacter to these controlled modifications. The purpose of this procedure was to maintain as nearly as possible the natural habitat of this organism. Agronomy Farm soil and virgin dryland pasture soils were the two soils used for the investigation.

Sodium benzoate, mannitol, wheat straw, and cellulose (ground filter paper) were tested as a source of energy for Azotobacter.

A pure culture of Azotobacter vinelandii was used in one instance, but proved unsatisfactory. Thereafter a crude culture of Azotobacter, isolated from the Agronomy Farm soil, was used wherever Azotobacter inoculation was necessary.

The effect of such nutrients as molybdenum, phosphorus, potassium, and iron was determined both in the presence and in the absence of an added source of energy.

The decomposition of the organic matter added to the

soils was followed by determination of the amount of carbon liberated as carbon dioxide.

All Azotobacter counts were made by plating out the soil on a modified Wenzl's medium.

Where possible, all data obtained were analyzed statistically to facilitate the reduction of the amount of data and to describe them in such a way that certain conclusions might be drawn concerning the data.

## CONCLUSIONS

1. Mannitol presented a source of carbon which was readily available and was initially more rapidly decomposed than was sodium benzoate. The point to which decomposition proceeded, as measured by carbon dioxide evolution, at the end of 20 days was virtually the same with both organic nutrients.

2. The average daily evolution of carbon dioxide from soils treated with 2 percent, 5 percent, and 10 percent wheat straw was strongly correlated with the percentage of straw present.

3. Mannitol brought about a larger increase in the Azotobacter count of the Agronomy Farm soil than did sodium benzoate.

4. The decomposition products of wheat straw furnished a source of energy for Azotobacter. Five percent straw in the soil gave the largest increase in Azotobacter

count.

5. There was no significant correlation between the increase in Azotobacter count and the amount of nitrogen fixed. There was no significant correlation between the rate of decomposition of the organic matter as measured by carbon dioxide evolution and the increase in Azotobacter numbers, nor was there any significant correlation between carbon dioxide evolution and the amount of nitrogen fixed.

6. The Kjeldahl method of nitrogen analysis is not of sufficient accuracy to be used with any large degree of success in a study of nitrogen-fixation in the soil.

7. The addition of molybdenum, potassium, phosphorus or iron to the Agronomy Farm soil brought about no alteration in the Azotobacter count.

8. Phosphorus and potassium, when added to the Agronomy Farm soil in conjunction with wheat straw, brought about a larger increase in Azotobacter counts than did the straw alone.

9. An available source of energy is probably the limiting factor in the development of Azotobacter in both the soils tested.

10. In tests in which dryland pasture soil was inoculated with Azotobacter it was found that molybdenum, potassium, and iron in conjunction with mannitol as a

source of energy, gave higher Azotobacter counts than did the mannitol alone. Phosphorus and mannitol gave a lower count than did the mannitol alone.

11. The decomposition products of cellulose furnished a source of energy for Azotobacter. The addition of 365 p.p.m. of $NaNO_3$ to the cellulose-soil mixture brought about a more complete decomposition of the cellulose, which in turn, furnished more utilizable carbonaceous material for Azotobacter. Five percent cellulose was the concentration at which Azotobacter showed the greatest increase in number.

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THESIS ABSTRACT

SOME FACTORS INFLUENCING THE
ACTIVITY OF AZOTOBACTER IN SOILS

by

Lynn L. Gee

This investigation into the relationship of environmental factors to the growth and development of Azotobacter in soils was undertaken in an attempt to bring about a more thorough understanding of the role this microorganism plays in the soil. Throughout the investigation soil, both untreated and modified in certain known ways, was the medium used to test the responses of Azotobacter to these controlled modifications. The purpose of this procedure was to maintain as nearly as possible the natural habitat of this organism. Agronomy Farm soil and virgin dryland pasture soil were the two soils used for the investigation.

Sodium benzoate, mannitol, wheat straw, and cellulose (ground filter paper) were tested as sources of energy for Azotobacter.

A pure culture of Azotobacter vinelandii was used in one instance, but it proved unsatisfactory. Thereafter a crude culture of Azotobacter, freshly isolated from the

Agronomy Farm soil, was used wherever Azotobacter inoculation was necessary.

The effect of such inorganic nutrients as molybdenum, phosphorus, potassium, and iron was determined in both types of soil used and in the presence and absence of an added source of carbon.

The decomposition of the organic matter added to the soil was followed by daily determinations of the amount of carbon evolved as carbon dioxide.

The Azotobacter counts were made by plating out on Wenzl's medium to which a small amount of sodium molybdate was added.

All incubation was done in the 28° C. constant temperature room.

Where possible, all data were analyzed statistically to facilitate the reduction of the amount of data and to describe them in such a way that certain conclusions might be drawn concerning the phenomena through which the data was obtained.

As a result of this investigation the following conclusions were drawn:

1. Mannitol presented a source of carbon which was readily available and was initially more readily decomposed than was sodium benzoate. The point to which decomposition proceeded, as measured by carbon dioxide evolution, at the end of 20 days

was virtually the same with both nutrients.

2. The average daily evolution of carbon dioxide from soils treated with 2 percent, 5 percent, and 10 percent wheat straw was strongly correlated with the percentage of straw present.
3. Mannitol, when applied to the Agronomy Farm soil, brought about a larger increase in the Azotobacter count than did sodium benzoate.
4. The decomposition products of wheat straw furnished a source of energy for Azotobacter. Five percent wheat straw in soil gave the largest increase in Azotobacter count.
5. There was no significant correlation between the increase in Azotobacter count and the amount of nitrogen fixed. There was no significant correlation between the rate of decomposition of the organic matter, as measured by carbon dioxide evolution, and the increase in Azotobacter numbers. Nor was there any significant correlation between carbon dioxide evolution and the amount of nitrogen fixed.
6. The Kjeldahl method of nitrogen analysis is not of sufficient accuracy to be used with

any high degree of success in a study of nitrogen-fixation in the soil.

7. The addition of molybdenum, potassium, phosphorus, and iron to the Agronomy Farm soil brought about no alteration in the Azotobacter count.

8. Phosphorus and potassium when added to the Agronomy Farm soil in conjunction with wheat straw brought about a larger increase in Azotobacter counts than did the straw alone.

9. An available source of energy is probably the limiting factor in the development of Azotobacter in both soils tested.

10. In tests in which dryland pasture soil was inoculated with Azotobacter, it was found that molybdenum, potassium, and iron in conjunction with mannitol as a source of energy, gave higher Azotobacter counts than did the mannitol alone. Phosphorus and mannitol gave a lower count than did the mannitol alone.

11. The decomposition products of cellulose furnished a source of energy for Azotobacter. The addition of 365 p.p.m. of sodium nitrate

to the cellulose-soil mixture brought about a more complete decomposition of the cellulose. This resulted in the formation of a larger quantity of carbonaceous material which could be utilized by Azotobacter, and a corresponding increase in the Azotobacter flora of the soil. Five percent cellulose was the concentration at which Azotobacter showed the greatest increase in number.

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