STUDIES ON THE FIXATION OF NITROGEN IN OKLAHOMA SOILS BY NON-SYMBIOTIC BACTERIA

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INTRODUCTION

Fixation of nitrogen by non-symbiotic bacteria is a problem which has been studied by many investigators. By employing solutions or cultures containing essential nutrients, a suitable carbohydrate and no available nitrogen, measurable quantities of nitrogen soon accumulate after inoculation with nitrogen fixing bacteria and incubation for a definite period. It is commonly assumed that the carbohydrate supplies energy for the growth of the organisms which change free nitrogen into amino or amide forms which are further synthesized into proteins. Chemical analyses for total nitrogen which are made at regular intervals will indicate the rate of accumulation of organic nitrogen when different soil organisms are inoculated into media containing various forms of carbohydrate material.

Review of Literature

Beijerinck (2)[1] discovered Azotobacter in 1901, and his description of two species, Azotobacter chroococcum and Azotobacter agile, was followed by Lipman's (13) description of two additional species, Azotobacter vinelandii in 1903 and Azotobacter beijerinckii in 1904.

Hunt (10) states that "a very large percentage of the total nitrogen fixed by Azotobacter in the first few days of its growth in culture media consists of amino acid nitrogen. This proves that the elementary nitrogen goes through this simple organic form before it is synthesized into protein. It also proves what is perhaps most important, that the nitrogen is fixed by combination with hydrogen and not with oxygen, thus insuring much greater economy of energy. Moreover, and contrary to common belief, not sugar, but products of its decomposition form the true source of energy for the growth of Azotobacter."

Lipman and Burgess (14) have shown that, with a proper supply of energy producing materials, nitrogen will accumulate when all agricultural soils are inoculated into a properly constituted mannite solution. The reaction of the soil, as expressed by its hydrogen ion concentration, is of prime importance in influencing the development of the particular types of nitrogen fixing bacteria. When the pH is above 6.0 active fixation of nitrogen occurs until a pH of 8.0 to 8.5 is reached. Azotabacter will not develop in soil when the pH is less than 6.0. Nitrogen fixing bacteria, such as the clostridium group and perhaps Bacterium aerogenes and Bacterium asterporus will develop in acid soil.

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[1] (2) Reference to literature cited.

Gainey (3) examined 418 soils in Kansas and 199 were found to contain Azotobacter. The soils containing Azotobacter fixed on an average 8.30 milligrams of nitrogen in an agar solution, containing 20 grams of mannite, 10 grams of soil, 200 cc of distilled water, and incubated for 12 days. An equivalent amount of carbohydrate added to the 219 soil samples which did not contain Azotobacter fixed on an average 4.61 milligrams of nitrogen.

Stoklosa (18), has shown that the growth of Azotobacter is limited in soils which are deficient in available phosphorus. He found that the weight of the dry cells of these organisms contained as much as five per cent of phosphoric acid. When an excess of mannite is added to a soil suspension containing Azotobacter, Clostridium, and other nitrogen fixing bacteria a marked increase in bacteria numbers will occur. Since about one unit of available phosphorus is required for every two units of nitrogen fixed or assimilated by the organisms and synthesized into microbial protein, the supply of available phosphorus in the soil may be a limiting factor in nitrogen fixation. The actual amount of nitrogen fixed in the soil, especially by Azotobacter, may then become merely an index of the available phosphorus in the soil.

Waksman and Karunkar (19), have shown that the ability of a soil to fix nitrogen, when an excess of a readily available source of energy is added to it, depends upon the microbial flora of the soil, the physico-chemical conditions of the soil, especially its reaction, and upon the presence of available phosphate and potassium salts.

Winters (22), found that nitrogen fixation in the soil is first accompanied by a decrease in nitrates probably due to the assimilation of these compounds by nitrogen fixing organisms.

Sackett and Stewart (17), have recommended the use of a soil plaque method to study soil deficiencies by using Azotobacter development as an index of phosphorus and potassium availability.

Experimental Results

The following experiments were planned to study the non-symbiotic nitrogen fixation in Oklahoma soils, since wide variations occur due to differences in, (a) topography, (b) age, (c) parent material, (d) climate, and (e) natural vegetation.

In eastern Oklahoma where the annual rainfall varies from 35 to 50 inches a high percentage of acid soils occur, and the quantity of available plant nutrients is low because of extensive leaching. In western Oklahoma where the rainfall varies from 15 to 30 inches annually the percentage of acid soils is low and the quantity of available plant nutrients is high.

Effect of Containers on Fixation of Nitrogen by Non-Symbiotic Bacteria

This experiment was planned to study the effect of open and covered beakers and flasks on the quantity of nitrogen fixed by soil organisms. Richfield very fine sandy loam from Harper County, Oklahoma was used in this study. Five grams of soil, one-half gram of glucose and 100 cc of sterile distilled water, were added to each container. Comparisons were made in triplicate using 250 cc beakers covered and uncovered, and 250 cc Erlenmeyer flasks. The results of this experiment are given in Table I.

Table I. A Study of the Effect of Covered and Uncovered Containers on the Fixation of Nitrogen by Non-Symbiotic Bacteria.

Date	Kind of Containers	Milligrams of Nitrogen Fixed.
2/2/33	Covered Beakers	1.77
	Open Beakers	1.47
	Covered Flasks	0.70
	Open Flasks	0.81
2/11/33	Covered Beakers	1.33
	Open Beakers	1.02
	Covered Flasks	0.61
	Open Flasks	0.91

It is evident that more fixation occurred in the beakers than in the flasks. Since Azotobacter is an aerobic organism it grows near the surface of the suspension. More surface area was exposed to the air in the beakers than in the flasks. Covering the flasks had no appreciable effect on nitrogen fixation.

Hunter (11), has demonstrated that a gain in nitrogen fixation by non-symbiotic bacteria occurs when a current of air is passed through the soil suspension during the incubation period. The largest gain occurred on the fourth day. The results obtained by the aeration method were doubled as compared with nitrogen fixed in non-aerated suspensions. In order to compare the effect of aeration and non-aeration on nitrogen fixation, an experiment was conducted using Richfield very fine sandy loam treated in the following manner: Five grams of soil was placed in a 200 cc Erlenmeyer flask containing one-half gram of glucose and 100 cc of sterile distilled water and the soil suspensions were incubated for

six days at 27° C. Air was drawn through as a result of suction produced by a water pump. All comparisons were made in triplicate and total nitrogen was determined by the Kjeldahl method. The average results of two separate experiments are given in Table II.

Table II. Data on the Effect of Aeration on the Fixation of Nitrogen by Non-Symbiotic Bacteria.

	Aerated		Non-Aerated	
Date	Days Incubated	Average Milligrams of Nitrogen Fixed	Days Incubated	Average Milligrams of Nitrogen Fixed
1/4/33	6	2.20	6	0.672
1/11/33	6	2.20	6	0.652

The evidence shows that more than twice as much nitrogen was fixed in aerated suspensions as compared with non-aerated samples and indicates that the organisms which are responsible for nitrogen fixation in the soil are aerobic in character.

Effect of Different Sources of Carbohydrate on The Fixation of Nitrogen by Non-Symbiotic Bacteria

The purpose of this study was to determine the effect produced by different sources of carbohydrate on the fixation of nitrogen by non-symbiotic bacteria. The carbohydrates used were sugar, starch and wheat straw. The experiments were conducted according to the following procedure: Five grams of Richfield very fine sandy loam soil, one gram of carbohydrate and 100 cc of sterile distilled water were added to 500 cc Erlenmeyer flasks, which were incubated for seven days at a temperature of 28° C. Triplicate determinations were made on each material. The suspensions were not aerated. Data are presented in Table III.

Table III. Data of the Effect of Different Sources of Carbohydrate on the Fixation of Nitrogen by Non-Symbiotic Bacteria.

Days Incubated	Dextrose Milligrams of Nitrogen Fixed	Starch Milligrams of Nitrogen Fixed	Wheat Straw Milligrams of Nitrogen Fixed
7	1.43	0.85	-7.3

The highest value was obtained from the dextrose which is a readily available form of energy for non-symbiotic bacteria. A negative value for nitrogen fixation was obtained when straw was used as a source of energy. This was probably due to the action of denitrifying bacteria which caused the liberation of some gaseous nitrogen from protein material in the straw under the anaerobic conditions which occurred in this experiment. Wheat straw was used in this experiment because it is abundant in western Oklahoma and is returned to the soil when combines are used to harvest the wheat.

A Study of Different Media for the Growth of Non-Symbiotic Bacteria

Winogradsky (21), has recommended the silica gel plate as a medium for the growth of non-symbiotic bacteria. The advantage of this method is due to the fact that silica gel contains no nitrogen which interferes with the vigorous growth of nitrogen fixing organisms. Ten cc of Ashby's solution (1), was added to 25 grams of granular silica gel, which was a commercial product and quite acid in character. The acidity probably interferred with the growth of the organisms, although the gel was washed for twenty-four hours with water before the nutrient solution and soil suspensions were added. Silica gel plates prepared from sodium silicate treated with hydrochloric acid were not studied.

Since sand is composed largely of quartz and contains very little organic matter it was treated with Ashby's nutrient solution, and agar in various combinations. The containers used in this study were covered beakers and petri dishes, the purpose being to compare the two containers, when handled under identical conditions. White quartz sand passing a forty mesh sieve was ignited in an electric furnace for thirty to sixty minutes to destroy organic matter. Fifty grams of sand, free from organic matter, ten cc of Ashby's solution and one cc of soil suspension prepared by mixing one gram of Richfield very fine sandy loam and five cc of water was placed in different containers and incubated for twelve days at 28° C. At the end of this period the amount of total nitrogen was determined. An incomplete set of data was obtained when the sand was used alone. Other combinations which were compared were, (a) Agar, Ashby's solution, and sand, (b) Agar, Ashby's solution, and no sand, and (c) Ashby's solution, sand and no agar. The results are given in Table IV.

Table IV. Effect of Different Media and Kind and Size of Containers on the Fixation of Nitrogen by Non-Symbiotic Bacteria.

Agar, No Sand, and Ashby's Solution			Agar, Sand and Ashby's Solution		
Petri Dishes	Petri Dishes	Covered Beakers	Petri Dishes	Covered Beakers	Covered Beakers
90 mm	150 mm	600 cc	150 mm	600 cc	1000 cc
		Milligrams of Nitrogen Fixed			
0.91	4.20	2.09	0.63	1.33	1.33
0.91	4.12	2.02	1.05	1.99	1.45
0.98	4.26	2.00	1.95	1.26	1.05
Ave. 0.93	4.19	2.94	1.21	1.53	1.28

Table IV. (Continued)

Ashby's Solution, Sand, and No Agar			
Covered Beakers	Covered Beakers	Petri Dishes	Petri Dishes
600 cc	100 cc	90 mm	150 mm
Milligrams of Nitrogen Fixed			
0.90	0.85	1.45	1.77
0.91	0.84	1.47	1.45
----	----	----	1.95
Ave.0.90	0.85	1.46	1.72

--- Samples were ruined.

It is evident from the data presented in table four that the greatest fixation of nitrogen by non-symbiotic bacteria occurred in the large petri dishes (150 mm) containing Ashby's solution in agar but no sand. The values for the beakers were considerably lower in this case than results obtained in large petri dishes. The fixation of nitrogen on the other media and combinations was rather consistent, throughout this experiment. The results indicate that the best medium for the fixation of nitrogen by soil organisms would be Ashby's solution in agar using the large petri dishes as containers.

Soil Deficiency Study

Sackett (17), has recommended a procedure for studying mineral deficiency in soil by measuring the growth of Azotobacter on soil plaques. In order to test the value of this method under Oklahoma conditions, five soils were used in this experiment, namely, Richfield very fine sandy loam, Quinlan very fine sandy loam, Grant very fine sandy loam, Vernon

fine sandy loam, and Oswego silt loam. Each soil was prepared in quadruplicate and incubated for three days at 30° C. At the end of this time Azotobacter colonies are supposed to appear on soils not deficient in minerals. The results obtained from Sackett's procedure in this study were disappointing due to the fact that mold growth completely covered the surface of each plaque. No colonies of bacteria developed and the method was not used in subsequent tests.

Effect of Time of Incubation and Amount of Carbohydrate on Fixation of Nitrogen by Non-Symbiotic Bacteria

Non-Symbiotic bacteria must have a source of energy which is readily available and in addition the nitrogen content of the medium must be low. Murray (15), used straw as a source of carbohydrate for the growth of non-symbiotic bacteria. Other investigators have studied the effect of different organic compounds on species of bacteria belonging to the genus Azotobacter. No attempt was made in this investigation to study any single group of organisms. In order to determine what the quantity of nitrogen fixation would be in relation to the length of incubation and the amount of carbohydrate supplied, an experiment was planned and carried out in the following manner: Agar containing varying amounts of Ashby's solution was added to each petri dish and inoculated with a suspension containing one-half gram of Richfield very fine sandy loam soil. Each test was run in triplicate and total nitrogen was determined on the fourth day. In addition 20, 40, 60, and 80 cc of agar media was placed in petri dishes and analyses were made for total nitrogen on the fourth, eighth, twelfth and sixteenth day after the experiment was started. Mannite was used as a source of carbohydrate. The results of the experiment are given in Table V.

Table V. Effect of Time of Incubation and Amount of Carbohydrate on the Fixation of Nitrogen by Non-Symbiotic Bacteria Grown on Agar Media.

	Milligrams of Nitrogen Fixed on Different Volumes of Media			
Days	20 cc.	40 cc.	60 cc.	80 cc.
4	0.56	1.40	1.33	----
4	0.70	1.40	0.79	0.42
4	----	0.98	0.98	0.42
Ave	0.63	1.28	1.01	0.42
8	2.31	4.62	5.18	6.79
8	2.80	4.69	3.57	6.19
8	3.15	----	5.81	5.60
Ave	2.74	4.65	4.87	6.62
12	2.05	6.30	6.80	10.35
12	2.40	6.65	7.40	10.10
12	1.95	5.65	6.95	10.90
Ave	2.14	6.20	7.05	10.45
16	2.80	5.80	7.00	11.05
16	2.95	----	3.90	11.60
16	3.20	5.75	6.95	11.60
Ave	2.98	5.78	5.95	11.40

---- Samples ruined.

The only conclusion that can be made from the data in Table V is that the amount of nitrogen fixed depends upon the amount of energy supplied and the length of time that it is kept under an environment which is favorable for the growth of the bacteria. The results of this experiment indicate that 80 cc. of the agar media, inoculated with one-half gram of soil and incubated for 12 days at a temperature from 28 to 30° C. will give information in regard to the activity of non-symbiotic nitrogen fixing organisms in different soils. Glucose may be substituted for mannite in Ashby's solution and similar results will be obtained.

Studies on Soil From Series 6200 Experiment Station Farm at Stillwater, Oklahoma

The soil on this area is typical Kirkland very fine sandy loam which has been divided into plots which receive different fertilizer treatments and in addition legumes have been grown on some plots.

This particular series is divided into four strips running north and south and are numbered A, B, C, and D from east to west. Strips running east and west are numbered one, two, three, through twelve, from north to south. Samples were collected on plots numbered eight through twelve for strips numbered A, B, C, and D and also for plots numbered two, three and four in strip number D.

The results of this study are given in Table VI.

Table VI. Results of Nitrogen Fixation Studies on Soil From Series 6200 Experiment Station Farm, Stillwater, Oklahoma.

Strip Number	Plot Number	Soil Treatment	8/16-28/33	9/10-23/33
			Milligrams of Nitrogen Fixed	
A	8 - 12	None	1.82	1.77
B	8 - 12	Phosphorus	1.72	1.73
C	8 - 12	P & K	1.49	3.10
D	8 - 12	P & K & Lime	1.00	2.23
D	2,3,4	Lime only	3.25	4.20

These results indicate that soils which had been treated with finely ground limestone gave greater fixation of nitrogen than other single treatments or combination of treatments. The effect of the lime has raised the pH value of this soil from pH 5.5 to above 6.0 so that conditions would be favorable for Azotobacter to grow.

Study of Nitrogen Fixation in Soil Series From Different Parts of Oklahoma

The scope of this study is represented by 14 different soils collected from farming areas in different sections of the state. The soil number, series name, and location are given in Table VII. There are three soil series from the northeastern part of the state which are representative for this section exclusive of the Northern and Southern Ozarks. One sample was taken about seventy miles southeast of Stillwater, which represents to a fair degree the soils of this particular section of the state. The Foard series represents the typical farming soils of the Southwest; while Vernon, Kirkland, and Yahola soils are found in central Oklahoma.

The 14 soil series listed in table seven represent wide variations in chemical and physical soil characteristics. These soil series were selected from various sections of the state in order to study nitrogen fixation by non-symbiotic bacteria for each particular soil series.

The purpose of this study was to determine the natural ability of the organisms in different soils to fix nitrogen when a suitable source of energy and proper moisture and temperature conditions were provided; and to quantitatively determine the amount of nitrogen fixation by chemical analyses and study this variation during the growing season. Analyses for total nitrogen, total phosphorus, total salts, easily soluble phosphorus, and pH values were made in order to study the relation between the plant nutrient content and the nitrogen fixing ability of soil organisms present in each soil series. These data are given in Table VIII.

In connection with this study a sample of cultivated soil was obtained from Utah. This soil is assumed to have a high nitrogen fix-

ing power and was used in comparison with Oklahoma soils. The Utah soil has maintained a high crop yield for a long period of time without the addition of any commercial fertilizer. The initial supply of the plant nutrients was high in this particular sample of soil. The following is an exact note which accompanied the sample of soil from the Utah Station, "82E., in cropping test, cropped to alternate wheat and fallow since 1903. Spring plowed for fallow, average crop yield 22.3 bushels of wheat per acre."

Table VII. Name and Location of Soil Series Used in the Study of Nitrogen Fixation in Oklahoma Soils by Non-Symbiotic Bacteria.

Soil Number	Name of Series	County
1	Derby	Kingfisher
2	Yahola	Payne
3	Vernon	Payne
4	Vernon	Kingfisher
5	Bates	Rogers
6	Parsons	Mayes
7	Summit	Rogers
8	Richfield	Harper
9	Grant	Alfalfa
10	Oswego	Garfield
11	Kirkland	Payne
12	Quinlan	Woodward
13	Conway	Creek
14	Foard	Kiowa
15	------	From Utah*

* Out of state sample.

The soils used in this study have all been cultivated. A representative sample weighing approximately seventy-five pounds was collected from each area. The nitrogen fixing ability of the organisms in these soils was studied by sampling dry soil and moist soil kept covered in containers in the field every twelve days. The amount of nitrogen fixed when a sample of each soil was added to culture media and the nitrate nitrogen and moisture content of the moist soil were determined. These

data are given in Table IX.

Methods of Analyses

Chemical studies: The soil samples were analyzed for total nitrogen, total phosphorus, total salts, pH values and easily soluble phosphorus by N/5 sulphuric acid extraction (9).

Studies on nitrogen fixation: The following procedure was used to determine the variation in nitrogen fixation by non-symbiotic bacteria, as affected by time of sampling. The details of this procedure are as follows: Petri dishes 15 centimeters in diameter and four centimeters deep were used. Eighty cc of agar which was sterilized at a pressure of 15 pounds for 30 minutes was placed in each petri dish and five cc of each soil suspension was added just before the agar began to solidify. The soil suspension is prepared by shaking ten grams of well pulverized soil with 100 cc of sterile distilled water. Each soil sample was run in duplicate and incubated for twelve days at a temperature ranging from 28 to 35° C. At the end of this time the total nitrogen was determined by the Kjeldahl method recommended for soils. In order to minimize the growth of molds on the cultures the agar-agar should be soaked for twenty-four hours in distilled water to remove the water soluble nitrogen which seems to be responsible for the development of a luxuriant mold growth. Blank determinations for nitrogen were made on 80 cc of the agar media and were subtracted from the total nitrogen obtained when incubated samples were analyzed.

Methods of Taking and Handling Soil Samples

The soils used in this study were divided into two parts. One part was air dried and kept in a quart jar in the laboratory. The other por-

tion of each sample was placed in a tall galvanized container and placed in a trench in the field. A small water proof shed was built over the containers in order to protect them from rain. Distilled water was added once every week. The amount of water added was approximately one-tenth of the weight of the soil. The samples kept under the shed are termed "field samples" in this study. The soils kept in the glass jars are termed "laboratory samples". The field samples were collected every two weeks with a spatula which was sterilized with methyl alcohol after each sample was taken. About 75 to 150 grams of each soil was transferred to clean 250 cc beakers and taken to the laboratory where the soils were weighed and air dried on clean paper. After the soils had been air dried for 48 hours they were re-weighed and the per cent of moisture calculated.

Nitrate nitrogen was determined each time field samples were collected. The nitrate nitrogen in the air dry samples was determined only once since nitrification does not take place in dry soils. Nitrate nitrogen was determined by the phenol-disulfonic acid method.

Soil suspensions were prepared by shaking ten grams of soil with 100 cc of sterile distilled water. A five cc portion was removed with a sterile pipette and transferred to 30 cc of agar media placed in a 15 cm petri dish and incubated for twelve days at a temperature of 28 to 35° C. At the end of this period the agar media on which the bacteria developed was transferred to a 500 cc Kjeldahl flask and the total nitrogen determined in the usual manner.

The agar media used in this study was prepared as recommended by Ashby (1) and the composition of the media was as follows:

Distilled Water	1000.00 cc	NaCl	0.2 gm
K_2HPO_4	0.2 gm	$CaSO_4 \cdot 2H_2O$	0.1 gm
Glucose	15.0 gm	$CaCO_3$	2.0 gm
$MgSO_4 \cdot 2H_2O$	0.2 gm	Agar-agar	15.0 gm

Table VIII. Total Phosphorus, Easily Soluble Phosphorus, Total Nitrogen, Total Salts, and pH, of Soils Selected to Study Nitrogen Fixation by Non-Symbiotic Bacteria. (Values given in pounds per acre foot 6 2/3" deep.)

Soil Series	Total Phosphorus	Easily Soluble Phosphorus	Total Nitrogen	Total Salts	pH
Bates	555	12	2435	240	7.01
Conway	200	2	1485	288	6.77
Derby	240	24	505	296	6.20
Foard	455	160	1990	320	7.25
Grant	520	230	1790	288	6.96
Kirkland	220	28	2350	240	5.80
Oswego	300	40	1790	320	6.48
Parsons	520	28	3330	280	5.55
Quinlan	300	320	1090	304	7.85
Richfield	580	128	1595	328	7.65
Summit	440	32	2885	280	5.75
Utah*	4712	1600	1740	240	8.30
Vernon (3)	240	12	1960	320	5.94
Vernon (4)	440	160	3165	416	8.20
Yahola	260	56	1345	528	5.75

* Out of state sample.

The values reported on chemical analyses given in table eight reveal that the sample of soil from Utah is very high in both total and easily soluble phosphorus. The Utah soil sample also has a high pH value of 8.25 and is about average in nitrogen and total salt content as compared with the Oklahoma soils.

It is evident from the data presented in table eight that the soils studied are quite variable in chemical composition. For example, the two Vernon soils have wide chemical values which can probably be explained due to Vernon number four being classed as a Pedocal and Vernon number three, a Pedalfer.

The values reported for total salts have the most consistent value for any chemical constituents reported.

The lowest pH value was 5.55 in the Parsons series and the highest value was the Utah sample which had a pH of 8.25, closely followed by the number four Vernon series with a pH value of 8.20.

The easily soluble phosphorus content of the soil series had the widest variation for any of the chemical values reported. The Conway series having two pounds per acre, the sandy Quinlan series 320 pounds per acre, and the Utah soil 1,600 pounds per acre. Total phosphorus varied in the Oklahoma soils from 200 to 580 pounds per acre. The analyses show that the easily soluble phosphorus content of the Quinlan series is slightly higher than the total phosphorus content, but the variation is within the limits of analytical error.

The Derby series which is aeolian in origin and undeveloped pedologically, is very low in total nitrogen, the value being slightly above 500 pounds per acre, whereas, the Kirkland, Parsons, and Summit series all have approximately 3,000 pounds of total nitrogen per acre.

Table IX. Effect of Seasonal Variation on Nitrogen Fixation by Non-Symbiotic Bacteria

Name of Series	Milligrams of Nitrogen Fixed							
	May 7-19		May 22-June 5		June 6-18		June 19-July 1	
	Field	Lab.	Field	Lab.	Field	Lab.	Field	Lab.
Bates	0.00	0.77	2.38	4.48	5.53	4.80	1.40	5.80
Conway	0.98	2.30	1.82	2.03	3.74	4.90	1.41	2.52
Derby	0.00	0.00	4.34	4.34	5.51	3.64	2.45	1.75
Foard	2.80	4.62	4.97	6.58	5.07	5.14	6.15	5.20
Grant	0.00	0.00	3.50	4.24	3.74	4.34	6.30	2.50
Kirkland	1.78	2.31	3.18	3.71	2.94	4.83	0.42	4.00
Oswego	0.21	0.21	2.17	3.50	3.50	3.81	1.33	4.85
Parsons	0.14	0.35	1.33	4.34	4.62	5.07	2.10	3.45
Quinlan	6.37	4.34	7.14	8.61	4.90	5.60	8.05	10.75
Richfield	6.02	6.02	8.40	3.85	5.67	5.04	7.28	8.15
Summit	0.14	1.82	4.62	4.00	4.13	5.04	1.92	3.64
Utah						4.97		1.18
Vernon(3)	1.33	2.55	0.77	4.27	4.76	3.88	2.00	3.22
Vernon(4)	1.26	1.82	4.41	7.98	4.51	3.70	7.65	9.08
Yahola	0.28	0.63	2.94	5.38	5.18	4.51	3.20	4.18

Table IX. (Continued)

Seasonal Variation in Nitrogen Fixation

Name of Series	Milligrams of Nitrogen Fixed							
	July 2-14		July 16-28		July 29-Aug.10		Aug. 12-23	
	Field	Lab.	Field	Lab.	Field	Lab.	Field	Lab.
Bates	2.25	2.30	2.80	1.81	2.64	0.93	2.85	2.40
Conway	1.40	2.45	1.02	0.16	----	----	3.00	2.50
Derby	0.91	2.30	2.67	0.35	0.79	0.00	1.90	1.10
Foard	4.75	5.10	3.92	1.33	5.30	0.99	3.40	4.40
Grant	3.85	2.97	0.23	1.19	5.28	3.56	3.40	2.70
Kirkland	1.40	3.08	1.08	1.74	2.11	2.57	1.45	4.20
Oswego	1.47	2.80	0.95	0.69	3.98	4.88	2.35	3.40
Parsons	3.70	1.61	2.67	0.95	2.66	2.38	3.00	4.60
Quinlan	2.52	3.57	4.00	0.36	6.85	6.40	3.65	5.00
Richfield	2.66	4.98	6.10	5.10	8.70	6.65	4.90	4.65
Summit	3.70	4.40	0.36	2.27	3.30	3.37	4.10	3.10
Utah	****	2.88	****	0.16	****	0.45	****	4.10
Vernon(3)	3.30	6.15	2.01	0.42	2.38	0.00	2.60	2.00
Vernon(4)	4.25	1.89	5.98	4.45	8.85	6.51	3.15	2.15
Yahola	3.85	4.40	5.45	2.14	2.31	2.03	3.40	2.80

---- Samples ruined.

**** Insufficient amount of soil to run field test.

Table IX. (Continued)

Seasonal Variation In Nitrogen Fixation

Name of Series	Milligrams of Nitrogen Fixed							
	Aug.24-Sept.5		Sept. 6-18		Sept. 18-30		Oct. 1-13	
	Field-	Lab.	Field-	Lab.	Field-	Lab.	Field-	Lab.
Bates	2.70	4.30	2.60	2.50	3.30	1.90	1.45	2.85
Derby	3.40	4.35	2.20	2.70	3.10	1.25	1.85	2.75
Foard	3.70	5.80	5.95	4.90	4.90	3.30	3.00	1.00
Kirkland	1.10	0.70	3.15	5.40	3.25	4.20	1.25	1.00
Manchester	2.35	0.70	1.70	9.35	-----	10.10	0.55	3.55
Oswego	3.65	0.90	1.35	4.30	2.60	5.95	3.05	1.00
Parsons	2.70	2.20	3.00	1.55	2.95	2.90	1.25	0.95
Quinlan	11.30	5.25	8.95	5.80	10.00	10.30	6.75	3.85
Richfield	10.40	5.80	10.35	7.05	5.40	6.55	4.05	3.85
Summit	5.90	3.35	1.95	1.25	3.80	4.25	2.00	1.85
Sapulpa	1.15	1.10	2.30	1.10	3.05	3.20	2.75	1.00
Utah	****	3.25	****	1.90	****	2.90	****	1.00
Vernon(3)	2.40	2.65	1.70	3.95	4.15	2.50	1.50	1.75
Vernon(4)	8.35	10.60	10.55	6.05	10.35	8.20	6.35	5.25
Yahola	2.80	2.20	2.70	2.40	3.80	1.90	1.75	2.45

----- Samples ruined.

**** Insufficient amount of soil to run field test.

Table X. Seasonal Variation in Nitrate Nitrogen Content In Field Soils

Name of Series	Nitrates (NO_3) In Parts Per Million					
	5/6/33	5/19/33	6/5/33	6/18/33	7/1/33	7/28/33
Bates	1.00	12.52	20.00	8.35	16.70	10.02
Derby	0.83	7.89	12.50	11.88	12.50	10.02
Foard	0.87	13.40	16.70	11.70	18.35	13.36
Kirkland	0.74	8.80	15.87	7.52	14.20	18.37
Manchester	1.20	14.20	20.00	12.50	14.20	10.02
Oswego	0.80	99.18	13.36	8.35	13.35	10.02
Parsons	2.60	23.35	20.00	8.35	12.50	10.02
Quinlan	1.20	8.35	17.50	12.53	22.50	16.70
Richfield	0.43	6.70	12.50	16.70	11.70	9.19
Summit	4.50	34.00	22.70	17.50	170.00	85.00
Sapulpa	0.74	8.00	11.70	13.36	15.05	11.69
Vernon(3)	0.57	5.87	20.88	8.35	16.70	13.36
Vernon(4)	0.84	8.40	14.19	9.19	20.05	20.88
Yahola	1.00	10.00	13.36	8.35	12.50	15.03

Table X. (Continued)

Seasonal Variation in Nitrate Nitrogen Content in Field Samples

Name of Series	Nitrates (NO_3) In Parts Per Million						
	7/28/33	8/10/33	8/23/33	9/5/33	9/18/33	9/30/33	10/13/33
Bates	20.04	10.85	15.03	33.40	23.40	23.50	44.30
Derby	10.02	20.04	9.19	96.86	20.88	18.00	25.85
Foard	43.42	20.04	23.78	45.09	38.40	20.00	51.80
Kirkland	19.20	10.02	11.69	53.44	26.80	15.00	67.00
Manchester	22.55	25.05	16.70	63.46	21.80	30.00	50.00
Oswego	28.69	8.35	30.06	18.37	10.00	20.00	75.30
Parsons	10.85	10.85	6.68	10.02	8.35	10.05	11.70
Quinlan	20.88	16.70	30.06	91.85	30.00	31.00	76.00
Richfield	20.04	8.35	11.69	16.70	11.69	6.68	15.10
Summit	68.75	87.67	150.30	133.60	51.00	30.00	92.00
Sapulpa	20.04	20.88	13.36	28.39	27.60	13.50	21.80
Vernon(3)	15.03	33.40	10.85	110.22	33.40	27.00	75.20
Vernon(4)	18.37	11.69	8.35	13.36	21.70	23.50	18.40
Yahola	13.36	20.04	14.19	33.40	31.80	22.50	48.50

Table XI. Per cent of Water in the Field Soils at Time of Sampling.

Name of Series	5/5/33	5/19/33	6/5/33	6/18/33	7/1/33	7/14/33
			(Air Dry Basis)			
Bates	12.65	12.80	18.20	12.05	7.82	27.20
Derby	5.10	6.40	8.20	6.95	4.81	9.10
Foard	12.20	16.60	20.30	20.30	20.10	27.80
Kirkland	9.80	13.90	16.30	10.80	8.95	19.00
Manchester	7.65	12.00	16.80	10.20	6.16	21.20
Oswego	8.85	13.50	20.20	11.92	8.30	20.50
Parsons	18.00	20.40	26.00	21.80	12.51	35.80*
Quinlan	36.60	14.45	17.20	14.80	12.30	19.30
Richfield	14.90	21.20	20.35	18.30	7.50	25.50
Summit	15.60	23.25	36.00	12.10	18.75	27.40
Sapulpa	8.60	11.25	13.40	12.20	7.45	19.10
Vernon(3)	9.50	15.90	17.50	17.50	12.90	28.00
Vernon(4)	13.35	17.25	18.75	18.75	12.06	33.05
Yahola	8.70	13.90	14.70	14.70	13.00	23.05

* Water added 4 days previous to sampling.

Table XI. (Continued)

Per cent of Water in the Field Samples

Name of Series	7/28/33	8/10/33	8/23/33	9/5/33	9/18/33	9/30/33	10/14/33
	(Air Dry Basis)						
Bates	28.80	12.40	16.80	18.25	11.55	7.70	10.90
Derby	13.30	11.00	9.75	12.80	7.05	6.08	5.10
Foard	29.60	27.40	26.40	33.00	26.20	19.40	16.90
Kirkland	22.20	13.00	17.80	21.40	11.60	9.15	11.20
Manchester	17.50	12.80	16.30	17.35	9.80	8.60	9.00
Oswego	19.80	11.00	11.50	19.00	9.75	10.50	8.55
Parsons	33.60	29.80	30.85	29.00	25.85	17.70	17.45
Quinlan	20.06	15.20	14.50	17.55	11.50	7.60	10.70
Richfield	17.70	21.30	23.40	22.40	30.80	7.55	11.90
Summit	26.80	25.60	24.00	24.00	37.10	15.60	16.90
Sapulpa	19.15	16.90	22.50	19.40	13.80	11.20	11.60
Vernon(3)	27.40	18.25	25.00	25.00	21.00	20.06	14.35
Vernon(4)	47.50*	28.30	42.50	38.00	29.40	22.70	18.00
Yahola	23.60	13.90	20.60	20.40	16.10	13.80	13.51

* Water added four days previous to time of taking sample.

Table XII. Averages of Non-Symbiotic Nitrogen Fixation, Nitrates, and Water Content of Field Soils at Time of Sampling.

Name of Soil Series	Milligrams of Nitrogen Fixed in Soil Samples		Nitrates in p.p.m.* field soils	Per cent of Water in the Field Soils
	Field	Laboratory		
Bates	2.49	1.89	17.35	15.60
Conway	1.89	1.94	15.85	14.35
Derby	2.43	2.05	19.73	9.13
Foard	4.50	4.03	24.38	20.75
Grant	2.58	3.77	23.34	12.72
Kirkland	1.93	3.14	20.66	22.63
Oswego	2.22	3.06	18.91	13.31
Parsons	2.51	2.53	11.18	24.58
Quinlan	6.16	6.14	11.34	18.67
Richfield	7.23	5.81	28.85	16.28
Summit	2.99	3.20	72.85	23.32
Utah	-----	1.13	-----	-----
Vernon(3)	2.41	2.70	28.53	19.45
Vernon(4)	6.31	5.64	14.54	26.12
Yahola	3.14	2.92	18.77	16.15

* p.p.m. = parts per million.

----- No values reported.

These data reveal that seven soils out of fifteen had a higher nitrogen fixation by non-symbiotic bacteria under field conditions. The Richfield and Vernon (number four) soil series had the largest fixation for any field sample. In addition to these two soils the Quinlan, Foard, Yahola, Bates and Derby all had a higher nitrogen fixation in the field samples than in the laboratory samples, and ranked in the order given. The greatest value obtained from any field sample was 7.23 milligrams of nitrogen fixed by non-symbiotic bacteria, and the greatest value for any laboratory sample was 6.14 milligrams of nitrogen fixed. The lowest

value reported for the field samples was 1.89 and for the laboratory samples 1.13. This gives a maximum variation of 5.34 milligrams of nitrogen fixation in the field samples and 5.01 milligrams in the laboratory samples.

The highest fixation of nitrogen by non-symbiotic organisms in the soil samples occurred in soils high in available phosphorus which had a pH above 7.0. The soil sample from Utah, however, gave a very low nitrogen fixation value and it had a pH value of 8.25. In soils having a pH value of 5.75 fixation probably was due to the activity of bacteria other than the Azotobacter group.

The soils which gave the highest fixation values are found in western Oklahoma, and are high in both easily soluble and total phosphorus.

The data in table nine show that six of the 14 field samples reached their peak of seasonal nitrogen fixation between the dates of June 1 and 15, while two groups of four soils each reached their maximum fixation from June 15 to August 1, and the last group reached their peak between September 1 and 15. The lowest values for the field samples as a whole were made during the first week of May, and as late as July 15, for two samples.

Seven of the 15 laboratory samples reached their maximum fixation between June 1 and 15, and including the same samples as reported early under field samples. Likewise, there were two groups of four soils each that gave a maximum fixation between July 1 and 10, and from September 1 to 25. The lowest values reported were the same as reported under field samples.

The nitrogen accumulation increased rapidly in all soils about the

first of June and continued rather consistently until the latter part of September and to the first half of October. This would indicate that the season of activity for non-symbiotic bacteria is limited. The highest nitrogen fixation value occurred in these soils during the summer months and in September. Under field conditions the activity of these organisms would be limited by available moisture and food supply.

The nitrate nitrogen content was high in nearly all soils on July 28, and all of the soils except the Summit reached a maximum on September 5. There was also a decided increase in nitrate nitrogen on October 13 over July values. The nitrate and water content of the different soils do not seem to correlate in any way with nitrogen fixation by non-symbiotic bacteria.

S U M M A R Y

Studies on the fixation of nitrogen in Oklahoma soils by non-symbiotic bacteria were made to determine the amount of nitrogen which was synthesized under favorable conditions by the growth of non-symbiotic bacteria. Chemical analysis for total nitrogen determined at regular intervals indicates that a rapid fixation of organic nitrogen will occur when soil organisms are inoculated into media containing glucose, mannite or starch.

In a series of laboratory experiments using Richfield very fine sandy loam it was found that more nitrogen fixation occurred in covered 250 cc beakers than in either open or covered 250 cc Erlenmeyer flasks. Since Azotobacter is an aerobic group of organisms which grow on or near the surface of the media, it is logical to assume that better results would be obtained in beakers because more surface area was exposed to the air in the beakers than in the flasks. Covering the flasks had no effect on nitrogen fixation.

The effect of aeration on the fixation of nitrogen by non-symbiotic bacteria show that more than twice as much nitrogen was fixed in aerated suspensions as compared with the non-aerated samples.

The effect of different sources of carbohydrate on the fixation of nitrogen by non-symbiotic bacteria were studied and the highest value was obtained from dextrose which is a readily available form of energy for non-symbiotic bacteria. The value for starch was considerably lower than for dextrose. Wheat straw gave a negative value of seven and three-tenths milligrams of nitrogen which was probably due to anaerobic conditions, which in the presence of a large amount of straw caused the liberation of some nitrogen in the gaseous form as a result of the action of dentrifying bacteria.

A study of the effect of different media on the growth of non-symbiotic bacteria indicated that a commercial source of silica gel was not satisfactory for the growth of bacteria because it was too acid. Media prepared from: (a) Agar, Ashby's solution and quartz sand, (b) Agar, Ashby's solution and no sand, (c) Ashby's solution, sand and no agar were studied and the data on this experiment clearly indicate that the largest fixation of nitrogen by non-symbiotic bacteria occurred in large petri dishes containing Ashby's solution in agar without quartz sand. Nitrogen fixation in beakers was considerably lower in this experiment than results obtained in large petri dishes. The fixation of nitrogen on other media was rather consistent throughout the experiment.

The results of a study on the effect of length of incubation period and amount of carbohydrate on fixation of nitrogen by non-symbiotic bacteria shows that, the amount of nitrogen fixed is dependent directly upon the amount of energy supplied and the length of time that the media is kept under proper conditions which are favorable for the growth of bacteria. It is not advisable to use more than 80 cc of agar media or to incubate at a temperature lower than 28° C. or above 35° C. for a longer period than 12 days.

Field studies conducted on soils collected from series 6200 on the Oklahoma Experiment Station farm which had received different fertilizer treatments indicate that a greater nitrogen fixation was obtained from soil treated with finely ground limestone than from any other single fertilizer treatment or combination of treatments. The lime which was applied to this soil has raised the pH value from 5.50 to above 6.0 so that conditions are favorable for the growth of non-symbiotic bacteria.

Studies were also made to determine the variation in nitrogen fix-

ation by non-symbiotic bacteria as affected by time of sampling. The soils used to determine the fixation of nitrogen in Oklahoma soils by non-symbiotic bacteria were divided into two parts. One part was air dried and kept in quart jars in the laboratory. The other portion of each sample was placed in tall galvanized containers and placed in a trench and protected from rain by a board roof. The purpose of this study was to determine the natural ability of these non-symbiotic soil organisms to fix nitrogen when a suitable source of energy was supplied in an adequate amount and the proper moisture and temperature conditions controlled. The results on seasonal studies of nitrogen fixation by non-symbiotic bacteria reveal that seven of the 15 soils gave a higher nitrogen fixation when kept moist under field conditions. Laboratory samples gave practically the same values during this study as was reported for the field samples. The most favorable pH range for nitrogen fixation was between 7.0 and 8.0. The soils which gave the highest fixation values are found in western Oklahoma and are high in both easily soluble phosphorus and other available nutrients.

Nitrogen fixation by non-symbiotic bacteria in these soils was not constant throughout the year. The highest nitrogen fixation values occurred between June 1 and September 15. The assimilation of atmospheric nitrogen by soil organisms working independently of leguminous plants is not always considered in calculating the nitrogen balance in the soil, but it is quite probable that non-symbiotic bacteria play an important part in the maintenance of soil nitrogen particularly in the non acid soils of western Oklahoma.

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