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ANAEROBIC NITROGEN FIXATION IN SOME IOWA SOILS

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Data secured by various investigators have proven quite conclusively that the aerobic nitrogen-fixing bacteria of the genus Azotobacter are not active or even present in soils more acid than pH 6.0. Many of the soils in Iowa are more strongly acid than this, and while no Azotobacter have been found in them, some nitrogen fixation certainly occurs. It appears, therefore, that some other organisms or groups of organisms must be responsible.

Anaerobic nitrogen-fixing bacteria of the genus Clostridium have been shown to be capable of living in soils which are quite acid, even to a pH of 5.0, and hence the question arises whether these organisms are present and active in Iowa soils which are too acid in reaction for Azotobacter growth and how they compare in nitrogen-fixing power with the Azotobacter.

Winogradsky (5) first isolated anaerobic nitrogen-fixing bacteria from acid soils in 1894-1895. His work was followed by that of Omeliansky and Solounskoff (3), Bredemann (1) and Truffaut and Bezssonoff (4), and there seems sufficient evidence of the occurrence and importance of these organisms to warrant a study of them in some extensively developed Iowa soils.

EXPERIMENTAL

In the experiments reported in this paper two soil types were studied, Carrington loam, a nearly neutral soil obtained from the Agronomy Farm of the Iowa Agricultural Experiment Station, and Grundy silt loam, an acid soil obtained from an experimental field in Clarke County, Iowa. In the latter case samples were taken from plots variously treated with lime.

Number of Anaerobes in Carrington Loam and Grundy Silt Loam

Four different methods were tested in the determination of the number of anaerobes in these two soils. The first method consisted of inoculating plates of Winogradsky's (5) nitrogen-free medium with known dilutions of soil in sterile water. More of the same medium was then introduced to cover the surface. The

plates were incubated in an anaerobic jar at 24° C. for 7 days, and the number of anaerobes per gram of soil was calculated. In the second method one-eighth gram of finely powdered soil was sprinkled over plates of the same medium, a layer of medium added and the plates incubated as before. The third method involved mixing soil dilutions in sterile water with melted nitrogen-free agar, which was then poured into petri dishes and after the medium had solidified a layer of the same medium was added, and the plates incubated as before. The plates in these three methods were incubated in an anaerobic jar.[1] The fourth method, used in the bacteriological laboratories at the Iowa State College, consisted of using glass tubing about 12 inches long and one and one-half centimeters in diameter as incubation tubes. Dilutions of soil were made in sterile agar and drawn into the glass tubes. They were then sealed at each end in a flame. The tubes had been previously calibrated and found to contain 1 cc. in each 3½ inches. The tubes were then incubated at 24° C. for 7 days and by counting the number of colonies appearing in 3½ inches of the tube, the number of anaerobes per gram of soil was calculated.

The numbers of organisms ranged from 7,000 to 143,000 per gram of soil in the Carrington loam and from 5,000 to 155,000 organisms in the Grundy silt loam. The numbers obtained by the fourth method, which is believed to be the most accurate, were 36,000 for the Carrington loam and 54,950 for the Grundy silt loam. It seems not only possible but entirely probable that in the case of methods 1 and 3 some oxygen was available for the microorganisms thereby permitting the growth of aerobic forms. The lowest figures were obtained in the case of method 2. This was probably due to the fact that many organisms were clinging to one particle of soil and formed only one colony. With the fourth method all the oxygen was unquestionably excluded, thereby permitting no growth other than that of anaerobic organisms. This method was used in later counts made in this work.

Number of Anaerobes in Grundy Silt Loam Variously Limed

In the following tests samples were taken from variously treated plots on the Grundy silt loam, both the surface soil, 1 to 8 inches, and the subsoil, 12 to 18 inches, being sampled. The treatments of these plots were as follows:

Plot No. 1 — No lime, check.

[1] The anaerobic jar was prepared by introducing one gram of pyrogallic acid and 10 cc. of tenth normal sodium hydroxide for every 100 cc. of air space.

No. 4 — 3 tons quarry-run limestone per acre (one lime requirement).
No. 7 — 6 tons quarry run limestone per acre (two lime requirement).
No. 8 — No lime, check.
No. 11 — 3 tons 100-mesh limestone per acre (one lime requirement).
No. 12 — 3 tons hydrated lime per acre (one lime requirement). Same CaO equivalent was applied as in limestone, 560 pounds of hydrated lime was equivalent to 900 pounds of quarry-run limestone.

The pH of the soils was determined by the quinhydrone electrode. The results are shown in Table I. It may be noted that the lime had a marked effect in neutralizing the acidity of the soils.

Experiments were conducted to determine the number of anaerobes in the soils from these plots both in the surface soil and subsoil. Sealed glass tubes, as described before, were used for the determination. The results show that the reaction within the pH range studied had no marked effect upon the number of organisms. However, it appears that in this soil there were as many anaerobes in the subsoil as in the surface soil.

Characteristic colonies which developed in the tubes were picked and transferred to nitrogen-free agar slants and kept in an anaerobic jar for study.

Nitrogen Fixation by Anaerobes

The ability of the anaerobes in these soils to fix atmospheric nitrogen was then studied. For the test one-gram samples from the surface and subsoil of these plots were mixed with 150 cc. of Winogradsky's nitrogen-free medium in 8 oz. bottles. This made the cultures in the bottles approximately 3 inches deep, thus insuring anaerobic conditions. For comparison five-gram samples of the

Table I — pH of the Soil from Plots on Grundy Silt Loam

Sample number	pH
1. Surface Soil	5.3
1. Subsoil	5.3
4. Surface Soil	6.0
4. Subsoil	5.6
7. Surface Soil	5.9
7. Subsoil	5.4
8. Surface Soil	5.4
8. Subsoil	5.6
11. Surface Soil	6.0
11. Subsoil	5.4
12. Surface Soil	6.6
12. Subsoil	5.3

same soils were mixed with 50 cc. of Fred and Waksman's (2) nitrogen-free mannitol solution for aerobic nitrogen fixation and placed in 500 cc. Erlenmeyer flasks, the layer of medium being only 3/16 inch deep, insuring favorable conditions for aerobic fixation. These cultures were incubated at 24° C. for 21 days and analyzed for total nitrogen by the Davisson-Parsons method and the Kjeldahl method. The results are shown in Table II, the figures representing milligrams of nitrogen fixed per culture and also per gram of soil.

Table II — Aerobic and Anaerobic Nitrogen Fixation in Solution Cultures Inoculated with Grundy Silt Loam

Plot	Aerobic		Anaerobic	
	Per Culture (5 gms. Soil)	Per 1 gm. of Soil	Per Culture (1 gm. of Soil)	Per 1 gm. of Soil
	Mgms.	Mgms.	Mgms.	Mgms.
1. Surface Soil	3.3371	0.6674	2.6268	2.6268
1. Subsoil	1.6112	0.3222	1.4010	1.4010
4. Surface Soil	11.5933	2.3186	0.6954	0.6954
4. Subsoil	0.9106	0.1821	0.9106	0.9106
7. Surface Soil	13.5196	2.7039	1.9381	1.9381
7. Subsoil	8.4060	1.6814	1.2342	1.2342
8. Surface Soil	3.9928	0.7986	1.4010	1.4010
8. Subsoil	2.7669	0.5534	2.0218	2.0218
11. Surface Soil	11.5586	2.3117	1.7766	1.7766
11. Subsoil	0.2198	0.0439	1.9964	1.9964
12. Surface Soil	12.0486	2.4097	1.7045	1.7045
12. Subsoil	1.8213	0.3643	2.9188	2.9188

Relatively large amounts of nitrogen were fixed in the aerobic cultures from the surface soil which had received lime. With the aerobic subsoil cultures only in No. 7 where 6 tons of lime was added, was there an appreciable amount of nitrogen fixed. There is a direct correlation between the efficiency of the aerobes in fixing nitrogen and the reaction of the soil. In the two check plots with pH of 5.3 and 5.4 there was very little nitrogen fixation, the same was true in the subsoil of all the plots except No. 7. In the surface soil, the larger amounts of nitrogen were fixed in the cultures inoculated with soil having the highest pH. This seems to indicate that the reaction is a limiting factor in aerobic nitrogen fixation.

Just as much nitrogen was fixed in the anaerobic cultures as in the aerobic cultures. In the majority of cases, however, there was more nitrogen fixed in the anaerobic subsoil cultures than in those from the surface soil. There is no direct correlation between the number of anaerobic organisms in the plots and the amounts of nitrogen fixed, however. In some cases the larger amounts were fixed in the soils from the plots having the largest number of

anaerobes, and in other cases the reverse was true. Furthermore, there appears to be no direct influence of reaction within the pH range tested, upon the amounts of nitrogen fixed anaerobically.

Since there were such large quantities of nitrogen fixed in the aerobic cultures it seemed desirable to determine whether or not Azotobacter were present in these soils. Spontaneous culture plate tests were made and the results showed that few Azotobacter were present in the more acid soils. However, comparatively large numbers were noted in the surface soil of the plots that had received lime.

To determine the nitrogen fixing power of pure cultures of anaerobes isolated from the two soils, heavy inoculations from 12 cultures were made into deep solutions of Winogradsky's nitrogen-free medium in 8 oz. bottles. The cultures were incubated at 24° C. for 21 days and analyzed for total nitrogen by the Kjeldahl method. Large variability was noted in the ability of the various cultures to fix nitrogen. Up to 2.0 milligrams of nitrogen were fixed in the cultures from both soils, three of the cultures giving no fixation.

The anaerobes which fixed more than 0.5 milligram of nitrogen were identified and classified. It was found that of eight cultures from the Grundy silt loam, one was *Clostridium pasteurianum*, one *Clostridium tetanomorphum*, one *Clostridium putrificum*, one *Clostridium spermoides*, and four were *Clostridium aerofoetidum*.

Of the six cultures from Carrington loam, one was *Clostridium multifermentans*, one *Clostridium tetanomorphum*, three were *Clostridium aerofoetidum*, and one was a non-spore-forming Gram negative rod which was not identified.

Summary and Conclusions

1. The use of sealed glass tubes containing dilutions of soil in nitrogen free agar proved most efficient in counting anaerobes in the soil. Approximately 36,000 anaerobes per gram of soil were found in Carrington loam with a pH of 7.2, and 55,000 per gram of soil in Grundy silt loam with a pH of 5.3 to 5.4.

2. In most cases there were as many anaerobes at 12 to 18 inches depth as at 1 to 8 inches and occasionally much larger numbers; this was not generally true with aerobes. Anaerobes were present in relatively large numbers both in the neutral and the acid soils studied. Within the pH range in the soils tested, there was little effect of reaction on the number of anaerobes.

3. Aerobes and anaerobes were about equally efficient in fixing nitrogen in solution cultures inoculated with one gram or five grams of soil. Aerobic fixation was not so great in cultures inoculated with subsoil samples while in most cases anaerobic fixation was

even greater in these than in the cultures of surface soil. From 0.69 to 2.91 milligrams of nitrogen were fixed anaerobically per 100 cc. of solution culture in the tests of surface and subsoil samples as compared with 0.04 to 2.70 milligrams in the aerobic cultures. These results indicate that anaerobic nitrogen fixation is of some value in the Grundy silt loam.

4. To determine the presence or absence of Azotobacter in the limed plots on Grundy silt loam, spontaneous culture plates were prepared according to Winogradsky's method. The results show that a few Azotobacter are present in the soils which had received lime, but the content was too small to account for the high nitrogen fixation obtained in the aerobic solution cultures. A large part of the nitrogen fixed in these cultures was undoubtedly due to the presence of other organisms.

5. Appreciable amounts of nitrogen were fixed by most of the pure cultures of anaerobes isolated from the two soils studied in these investigations.

6. The results reported indicate that there are comparatively large numbers of anaerobes in certain acid soils in Iowa and that they may play an important role in the process of nitrogen fixation in these soils either in conjunction with aerobes in slightly acid soils or acting alone in strongly acid soils.

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