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THE SPONTANEOUS CULTURE METHOD FOR STUDYING THE NON-SYMBIOTIC NITROGEN-FIXING BACTERIA OF THE SOIL

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During the years which have elapsed since the discovery of the non-symbiotic nitrogen-fixing bacteria there have been extensive investigations on the characteristics and activities of this group of organisms. They have been found to be of great importance from the fertility standpoint, adding appreciable amounts of nitrogen to the soil annually where the conditions are favorable for their development. In many cases too, a correlation has been found between the ability of a soil to support a vigorous Azotobacter growth and its crop producing power.

One of the greatest difficulties in the study of this group of microorganisms has been the lack of suitable methods. As a result of a recognition of this fact much attention has been directed to the study of methods and a number have been proposed. One of the earliest involved merely the measurement of the increase in nitrogen in a sample of soil when held under optimum conditions for a definite length of time, usually about 30 days.

The Remy method, which has probably been more widely used than any other, consists in the use of a suitable medium containing dextrose or mannite as a source of energy, inoculated with fresh soil in the proportion of about 10 grams per 100 cc. of solution. The culture is then incubated for 30 days after which the gain in nitrogen, due to the action of the microorganisms, is determined. This method has proved useful in many cases, but there are some serious objections to it as has been pointed out by Winogradsky (3). Probably the biggest objection lies in the fact that the soil organisms are placed in an environment which is entirely different from that to which they have been accustomed in the soil. This change in environment naturally influences the physiological activities of the bacteria and undoubtedly has a pronounced effect upon their ability to fix atmospheric nitrogen. The results obtained by the

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use of this method have often been found to be inconsistent and in many cases they have led to erroneous conclusions.

In order to secure a better measure of the activities of the nitrogen-fixing organisms in the soil Christensen (1) proposed a modification of the method just described. Portions of the soil to be tested were added to mannite solutions containing lime, and to similar solutions without lime. The purpose of this test was to ascertain the occurrence of the Azotobacter in the soil. A similar series of mannite solutions to which soil had been added was sterilized and reinoculated with pure cultures of Azotobacter. This series of tests was designed to determine the suitability of the soil for the growth of Azotobacter both before and after lime had been added to it. In this way Christensen proposed to test the lime requirement of soils. The method was also extended by him to include the testing of soils for phosphorus deficiencies by varying the phosphate content of the nutrient solution. If either lime or phosphorus were lacking in the soils tested it was assumed that there would be a more vigorous development of Azotobacter and a greater fixation of nitrogen where the optimum amounts of lime and phosphate were added. This method was found to indicate quite accurately the lime and phosphorus needs of soils.

Although this method has been found to be of considerable value in studying the nitrogen-fixing bacteria of the soil and a distinct improvement over the earlier methods, it is open to some of the same objections as noted above.

More recently Winogradsky (2), (3), (4), at the Pasteur Institute, has taken up the study of the nitrogen-fixing bacteria, examining the organisms in their natural habitat and measuring their activities in the soil itself, by varying only those factors, the effects of which are under consideration. As a result of his researches he has developed what he has called the spontaneous culture method for studying these non-symbiotic nitrogen-fixing organisms. An outline of the method will be given here and some data secured by its use will be presented.

The method involves the addition to the soil of starch or mannite, at the rate of two to five grams per hundred grams of soil. This widens the carbon-nitrogen ratio and stimulates the growth of organisms that have the ability of utilizing atmospheric nitrogen. Organisms that cannot secure their nitrogen from the air are unable to compete with the nitrogen-fixers and as a result their growth is largely suppressed and the majority of the organisms

developing are of the nitrogen-fixing type. In order that the growth of the nitrogen fixers may be determined without the aid of a microscope Winogradsky suggested that the soil, containing the carbohydrate, be mixed with water to form a thick paste and placed in a half of a petri dish. The surface is then smoothed by means of a moistened glass slide. After about 48 hours incubation in a moist chamber at 28° to 30°C., colonies of nitrogen-fixing bacteria begin to develop on the surface of the soil. They are very distinct and quite similar to the colonies formed on agar plates. Almost pure cultures of nitrogen-fixing bacteria may be obtained by making transfers from these colonies.

Winogradsky found that different soils showed wide variations in the development of colonies on the plates. With soils which were known to be deficient in lime or phosphorus, fewer colonies developed than with soils well supplied with these constituents. He therefore conceived the idea of adding small amounts of calcium carbonate and potassium or sodium phosphate to the soil cultures along with the carbohydrate in order to supply lime and phosphorus in case they are lacking in the soil which is being tested. By comparing the colony development on the plates receiving these additions with the control cultures which received only carbohydrate, the lime and phosphate needs of the soil could be determined. Winogradsky also made comparisons between the yields of crops and the development of the nitrogen-fixing bacteria and he obtained a close correlation in all his tests.

In the work reported here the method as developed by Winogradsky has been used to study the activities of the nitrogen-fixing bacteria in variously treated soils from the Agronomy Farm of the Iowa Agricultural Experiment Station.

Soils from the continuous corn plots at the Agronomy Farm were selected, as they were known to support a fairly vigorous Azotobacter growth. Corn has been grown continuously on these plots since 1913 and they have received the following fertilizer treatments:

Plot 905 — Check — no treatment.
Plot 906 — Farm manure applied at the rate of eight tons once every four years.
Plot 907 — Farm manure and lime. The manure applied as on plot 906, and ground limestone in amounts sufficient to neutralize the acidity as indicated by lime requirement tests.
Plot 908 — Lime applied as required according to lime requirement tests.
Plot 909 Check — no treatment.

The hydrogen-ion concentration of the soils from these plots

was determined at the time of sampling. The results are presented in table I.

Table I — Results of Hydrogen-Ion Concentration Determinations on Soils from Continuous Corn Plots

Plot	905	906	907	908	909
pH	5.60	5.98	7.11	6.23	5.57

Samples of fresh soil were taken from each of these plots and brought to the laboratory for testing. Three 100 gram samples of each soil were weighed out and treated as follows:

1. Potato starch (5%) – check.
2. Starch + lime (0.4%).
3. Starch + lime + di-sodium phosphate (0.6%).

After the materials were thoroughly mixed with the soil sufficient water was added to make a thick paste. This was then packed into duplicate halves of petri dishes and the surface was smoothed by means of a moist spatula. The plates were then placed in moist chamber culture dishes and incubated at 28°C.

After three days incubation a slight Azotobacter growth was apparent on the soil from plot 907 which had received applications of lime and manure in the field. The other soils showed no visible growth at that time.

After four days the number of Azotobacter colonies had increased considerably on the soil from plot 907 and a few colonies had developed on the soils from plots 906 and 908.

At the end of five days incubation the Azotobacter development was very definite and pronounced on some of the soils while on others there was no visible Azotobacter growth. The results have been tabulated and are presented in table II.

Where soil 905 was used there was a good growth of Azotobacter on the plates where lime and phosphate were added, a few small colonies where lime alone was added and none on the plates receiving starch without lime or phosphate. There was some mold growth on the plates where no lime was used. This was also true in most cases for the other soils. There was very little mold growth on the plates receiving lime.

With soil 906 which had been manured in the field there was some Azotobacter growth on the plates treated with lime and phosphate but not as much as there was on the corresponding plates made from soil 905. There was no Azotobacter growth on the plates treated with starch and lime.

The best growth of Azotobacter was secured with the soil from

Table II — Results of the Development of Spontaneous Cultures of nitrogen-fixing Bacteria on Soils from Continuous Corn Plots After Five Days Incubation at 28°C.

Soil No.	Check Plates (Starch Only)	Plates Receiving Starch + Lime	Plates Receiving Starch + Lime + Phosphate
905 Check	No Azotobacter growth. A few mold colonies.	A few colonies of Azotobacter on one plate and none on the other. Not so vigorous growth as on soil treated with lime and phosphorus.	A good development of colonies on both plates. Colonies large and vigorous. As good as on soil 907 with lime and phosphorus but slower to develop.
906 Manure	No Azotobacter colonies, but a few molds.	A few colonies on one plate and none on the other. Colonies slow to develop and small.	A few colonies on each plate. Earlier development than on the lime treated soils, larger and more viorous.
907 Manure + Lime	Good Azotobacter growth but slower in developing than on plates with lime and phosphorus.	Good growth on one plate while only a few colonies on the other. Colonies smaller than on lime and phosphorus plates and slower to develop.	Excellent colony development. Best of any plates. Developed sooner, were larger, more hyaline, and whitish in color. No molds.
908 Lime	A few colonies on each plate, slow to develop and small.	A few colonies on each plate, slow to develop and small.	More colonies developed than on the plates not receiving phosphate, were larger, earlier to develop and more vigorous.
909 Check	No Azotobacter colonies. Numberous molds.	No Azotobacter colonies. No molds.	Small mold colonies beginning to develop. No Azotobacter.

plot 907. The plates treated with starch alone showed a good growth but the colonies were slower to develop than where the lime and phosphate were added. On the latter plates there was more Azotobacter growth than on the plates from any of the soils and the colony development was also much earlier. The colonies were larger and more hyaline than on the plates where no phosphate was added to the soil.

The excellent growth of Azotobacter on the plates from soil 907 may be attributed to the fact that this soil had received applications of manure and lime in the field. These treatments undoubtedly made conditions more nearly optimum for the growth of Azotobacter than in the other plots where lime or manure alone had been applied or where no fertilizer treatment had been made. It is very significant that even in this soil which had been fertilized with manure and lime, the growth of the Azotobacter was stimulated considerably by further applications of lime and of phosphorus in the laboratory. This seems to indicate that this soil is still deficient

in lime or in phosphorus or in both for the maximum growth of Azotobacter.

With soil plot 908 there was a very slight growth of Azotobacter on the plates receiving only starch but a much larger growth was secured by the addition of lime and a phosphate.

With soil 909 there was no growth of Azotobacter on any of the plates, even where lime and a phosphate were applied. On the check plates of this soil there was considerable mold growth but on the plates treated with lime there was none, and where lime and phosphate had been added together only a few small colonies.

The character of the growth of the non-symbiotic nitrogen-fixing bacteria as described above is shown in Plate 1.

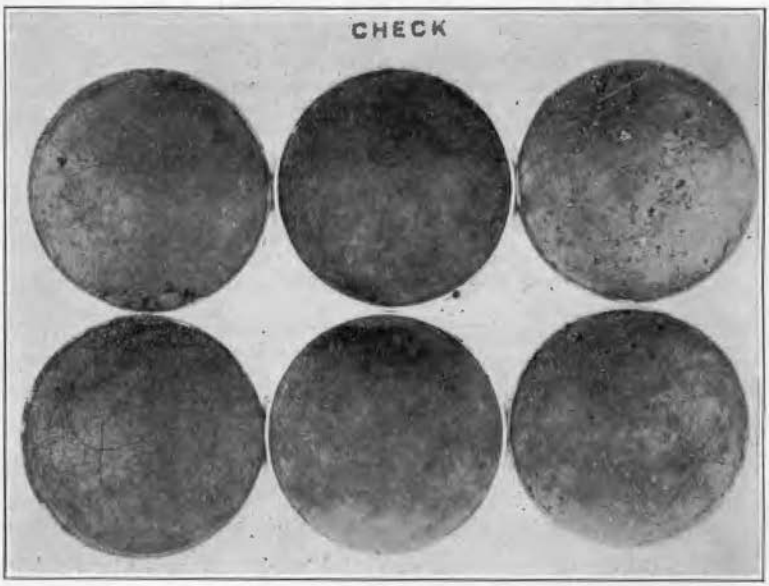

Plate 1. Character of growth of non-symbiotic nitrogen-fixing bacteria upon soil plates. The two plates at the left were untreated, those in the center were treated with lime, and those on the right were treated with lime and mono-sodium-phosphate.

A number of points seem to be quite definitely indicated from this test. First of all it was shown very clearly that the soil treated with lime and manure in the field supported a much more vigorous growth of Azotobacter than the soils treated with either lime or manure alone and the Azotobacter growth on the latter soils was more vigorous than in the soils receiving no fertilization. In fact there were no active Azotobacter organisms present in one of the check soils.

The results show further that the growth of the Azotobacter

in these soils was stimulated considerably by the addition of lime and a phosphate to the soils in the plate tests. This was true in all soils that contained the organisms.

Before drawing conclusions from these tests it will be of interest to consider the natural fertility of these soils and to note the crop yields. Topographically plot 905 is most favorably located while plot 909 is the least favorably located, there being a gradual slope from 905 up to 909. There has undoubtedly been some movement, therefore, of plant food and basic constituents, by washing and leaching, from plot 909 and the other higher-lying plots down to the lower plots. Then too the soil in plot 905 contains a larger proportion of fine soil particles and more organic matter while the soil in plot 909 is quite sandy and low in organic matter in places. The water holding capacity of soil 905 is, therefore, much greater than that of the soil from plot 909 and this certainly has a distinct influence on the crops produced. There is a gradual change in the soil conditions between plots 905 and 909, hence the other plots of the series also vary somewhat in characteristics.

Soil 905 is naturally a better soil than 907 or any of the other soils of the series and this makes the influence of the fertilizer treatments appear less than it actually is. Without fertilizer treatments on any of the soils there probably would have been a gradual decrease in crop yield from plots 905 to 909.

If these variations in the soils be taken into consideration when comparing the Azotobacter growth on the various soils the conclusions may be modified considerably. For example if soil 905 had shown an Azotobacter growth equally as vigorous as soil 907, it would necessarily be assumed that the applications of manure and lime to plot 907 had stimulated the Azotobacter growth and that the use of those fertilizers on that particular soil was desirable. If the Azotobacter growth proved more vigorous on plot 907 than on 905, as was the case in this work, it should certainly be concluded that the application of lime and phosphate had even more effect than indicated by the actual results secured in the test.

Winogradsky has assumed that soils most favorable for the development of Azotobacter would also be most favorable for the production of crops. In this work then it would be assumed that the soil on plot 907 would be more favorable for the production of crops than any of the others in this series, while the soil on plot 909 would be the least productive, the other soils coming in between these two extremes. This was actually the case as shown by the yield of corn secured in 1927 on these plots:

Plot 905 — 33.3 bushels of corn per acre.
Plot 906 — 34.7 bushels of corn per acre.
Plot 907 — 37.3 bushels of corn per acre.
Plot 908 — 32.0 bushels of corn per acre.
Plot 909 — 25.9 bushels of corn per acre.

The results of this spontaneous culture test seem to indicate quite definitely that the soils in the series of plots studied are in need of manure and lime for the best growth of Azotobacter and that the growth of these organisms is stimulated further by the addition of lime and a phosphate even to those soils which received applications of lime and manure in the field. The crop yields obtained on these plots indicate the beneficial effects of lime and manure and there appears, therefore, to be a close correlation between crop yields on these soils and their ability to support a vigorous Azotobacter growth. The soils whose fertility permits of the production of the largest crops also support the most vigorous develpment of the non-symbiotic nitrogen-fixing bacteria, and the soil giving the lowest crop yield actually contained none of these bacteria.

Although the work reported in this paper is of a preliminary nature, it appears quite probable that the spontaneous culture test may serve as a biological method for comparing the fertility of soils. Until further study is made of the method, however, it would be folly to make definite recommendations for the management of a particular soil, based wholly upon the results obtained in its use. Like all other methods, it will probably have its limitations and may not be applicable in all cases even if the effects of various factors are known. Its greatest value will probably be in its use for testing soils for their lime and phosphate needs. Possibly it may serve as a test for a lack of such other constituents as organic matter, potassium, sulfur or other essential plant food elements. In this connection it should be remembered, however, that the Azotobacter are more sensitive to deficiencies of lime and phosphorus than of other constituents and hence the method may not be efficient in other cases.

Considerably more study is necessary before this method can be generally adopted for testing soil needs. Studies should be conducted on several different soils of known crop producing power and of known fertilizer needs. These data should then be correlated with the Azotobacter growth, and the results interpreted on a statistical basis. After such studies have been conducted, the test may prove to be of great practical value to the soil bacteriologist in testing soils for their fertilizer requirements, especially for lime

and phosphorus. This method certainly has many advantages as it is simple in manipulation, it requires only a comparatively short time to obtain results, and most important of all, it permits of the study of the nitrogen-fixing bacteria in their natural habitat and under practically the same environmental conditions to which they are accustomed in the soil in the field.

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