NOTE TO USERS

THE GEOGRAPHIC DISTRIBUTION OF AZOTOBACTER AND RHIZOBIUM MELILOTI IN NEBRASKA SOILS IN RELATION TO CERTAIN ENVIRONMENTAL FACTORS

by

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A THESIS

Presented to the Faculty of

The Graduate College in the University of Nebraska

In Partial Fulfillment of Requirements

For the Degree of Doctor of Philosophy

Department of Agronomy

Lincoln, Nebraska

1940

ACKNOWLEDGMENT

The author wishes to express his appreciation to Dr. F. D. Keim, Dr. T. H. Geedding, Dr. M. D. Weldon, Dr. H. F. Rhoades, Dr. G. L. Poitier, Dr. C. E. Georgi, and Dr. P. A. Downs for their helpful advice and constructive criticism; and to Henry Smith, Maurice Miller, Orvin Meierhenry, and Gerald Gerloff for their aid in securing and analysing samples.

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INTRODUCTION

Since Winogradsky first isolated an anaerobic non-symbiotic nitrogen-fixing organism and Hellriegel and Wilfarth showed that nodules on legumes are due to bacterial infection (37), an abundance of literature has appeared concerning the activity, occurrence, and distribution in soils of the various microorganisms capable of fixing atmospheric nitrogen. This is evidence of the keen interest taken in these organisms and shows the belief in their importance. As a result of this interest and work much evidence has accumulated to support the opinion that fixation of atmospheric nitrogen in the soil is of marked economic importance in maintaining soil fertility.

In this investigation a survey of Nebraska soils has been made in order to determine the distribution of the aerobic non-symbiotic nitrogen fixers as well as the aerobic symbiotic nitrogen fixers of the genera Azotobacter and Rhizobium respectively. In connection with this survey, some of the characteristics of these soils which may effect this distribution were studied.

It is estimated that a normally active flora of Azotobacter, in some cultivated soils, may fix as high as 30 to 40 pounds of nitrogen per acre each year. It seemed desirable, then, to determine their distribution in Nebraska soils, in an effort to establish whether or not the soil flora includes these organisms and possibly indicate why continued fair yields of small grain are reported on much of the land not fertilized and without a legume in the rotation.

Each year Nebraska farmers plant an estimated 3,000,000 pounds of alfalfa seed and 7,500,000 pounds of sweet clover with very little use of pure culture inoculation. Large areas of the state are coming under irrigation where legumes will be used that have not been extensively grown previously. In addition, little is known about legume organisms in areas now supporting large acreages of legumes. With such facts in mind it seemed advisable to determine the presence of Rhizobium melilloti in Nebraska soils in an attempt to establish some factual basis for inoculation recommendations to those desiring to secure new stands of sweet clover or alfalfa. When a legume is included in a rotation it is usually desired, not only to produce a maximum crop yield, but also to benefit the soil. Only when an effective strain of Rhizobium is present in sufficient numbers to produce efficient nodulation on all the host plants in early stages of growth, can these desirable objectives be obtained.

Up to this time practically no research has been reported on the microflora of the soils of Nebraska. Hence there is little basis for predicting the activity of these organisms under environmental conditions as they exist here. It is hoped that this work will not only supply some information of value in directing future agricultural activities, but also will be of aid in stimulating and directing further biological and chemical studies.

EXPERIMENTAL PROCEDURE

Samples

During the fall of 1938, a total of 316 soil samples was collected from 38 important soil series in 78 of Nebraska's 93 counties. They were selected to include the predominant soil series of each county and were

distributed as shown in Fig. 3. None of the samples was taken from fields containing alfalfa or sweet clover. The majority of the samples was from cultivated fields containing small grain stubble or corn stalks. Aseptic technique was employed to insure against gross contamination. Each sample, representing a composite of about ten borings taken to a depth of six inches, was placed in a new 16 oz. drug carton which had been previously treated with a mixture of paraffin and Opalwax*.

The description of each soil type is given in the soil survey reports from the various counties (29). The soil type and legal description of each location is included in Table 1 of the appendix.

Determination of Azotobacter Presence

The presence of Azotobacter was determined in these samples by the soil plaque method principally as described by Winogradsky (44, 45, 46) but with some modification. It consisted of mixing 50 gm of soil with 2.5 gm corn starch, 0.3 gm dipotassium phosphate, 0.2 gm calcium carbonate and 0.5 ml of 0.5 per cent ammonium nitrate, and enough molybdate solution, containing 0.5 ml of 1 per cent ammonium molybdate per liter of water, to make a well puddled paste. This paste was placed into glass caster cups 11 x 42 mm inside dimensions, and smoothed on the surface with a moist chamber for 48 hours, at which time raised, viscous, macroscopic colonies on the surface indicated the presence of Azotobacter. This method was decided upon after numerous trials of several methods and combinations. Molybdate in small concentrations was found to stimulate the growth of Azotobacter in accordance with the observations of several workers.

*Opalwax is a DuPont synthetic wax with a high melting point. It was used to prevent the melting of the wax mixture at high summer temperatures.

(36, 2, 3, 6). The nitrate was used to stimulate the growth of larger colonies and has been found and concluded by some (16, 47, 13) to be beneficial or at least not harmful to Azotobacter when present in small quantities. Many of the more sandy soils failed to show surface colonies until sterilized clay soil was added to the mixture. As suggested by Sackett and Stewart, the addition of Kaolin (26), allows the formation of a smooth surfaced plaque on which macroscopic colonies may appear if the organisms are present.

Determination of Rhizobium Presence

In order to determine the presence of legume bacteria a method was used much the same as described by Wilson (40, 41). The alfalfa plants were grown in closed two quart fruit jars fitted with bottle rubbers and caps containing an opening which was plugged with cotton as shown in Fig. 1. One thousand grams of air-dry fine sand was sterilized in each jar under 20 pounds steam pressure for three hours. The seeds were scarified in concentrated sulfuric acid, washed free of acid, soaked in mercuric chloride solution (1:1000) and then washed free of chloride with sterile distilled water. Ten grams of the soil to be tested was added to each jar along with the seeds and 100 ml of a modified Crone's solution (4). About ½ inch of coarse dry sterile sand was placed over the seeds to reduce the incidence of "damping off" organisms.

The jars were placed to a depth of three inches in tanks of running water maintained at a temperature of 18-20° C. These tanks were located in the greenhouse. Artificial light supplemented daylight (Fig. 1). It was not possible to test all the soils at one time so lights were used to

insure a uniform length of day during all seasons. About 30 days was allowed for the growth of the nodules in every case. After this period of time the plants were carefully washed out and examined for nodules.

Fig. 1. Jars and tanks used in growing alfalfa for the Rhizobium studies.

Soil Reaction

The results of many workers show that in many cases, the reaction of the soil and the presence and growth of nitrogen-fixing organisms is very closely associated. It was believed that reaction data on these samples might show a relationship to the distribution of the organisms.

After a rather extensive study of legume bacteria in soils Wilson (40, 41) concluded that the disappearance of the organisms from the soils, seemed to be correlated with increasing acidity. He also found that soils which had been limed contained sufficient legume bacteria to produce adequate nodulation.

Bryan (5) found that nodule formation is influenced by the reaction of the medium. In fact Fred, Baldwin and McCoy (9) have offered numerous

references and reviews to indicate how profoundly aerobic nitrogen fixing organisms are affected by the reaction of their environment.

Burk et al., Gainey, Fred and Davenport, Martin, and others (6, 8, 10, 11, 12, 14, 20) have found a close relationship between Azotobacter presence and activity, and reaction. Krishna (19) concluded that soil reaction is the dominant factor influencing nitrogen fixation in soils. A pH value of six has been maintained as being critical.

Turk (34) found Azotobacter present and active in soils with pH values as low as 5.44. He noted no relationship between quantities of nitrogen fixed in aerobic culture solutions and pH values of the soils.

In order to determine pH, electrometric methods were used on all samples. The quinhydrone method with a saturated calomel half-cell and a Leeds and Northrup potentiometer was used in all cases where the pH was below eight. A glass electrode apparatus (Cameron) was employed to test the samples with a more alkaline reaction. All pH determinations were made on a water-soil ratio of 2.5 : 1.

Soluble Phosphorus.

The determination of soluble phosphorus should not be overlooked since it is an essential element and has been found, by numerous investigators, to be important and intimately associated with nitrogen fixers.

Truesdell (33) working with alfalfa, found phosphate beneficial to both plant and organism. In working with soybeans, Wilson (39) found that phosphates in soil cultures increased nodule production. Helz and Whiting (15) in field trials found phosphorus fertilizers increased nodulation when used in amounts not inhibitory to germination. In pot experi-

ments. Thornton (32) produced an increase in number of nodules on inoculated plants by incorporating fresh chaff with soil. This increase was augmented by further addition of phosphates. Sewell and Gainey (26) found that in acid soils low in calcium, nodulation of alfalfa was benefited more by superphosphate than lime. However, the two in combination were best. They also found the supply of available phosphorus to be very important in determining the degree of inoculation obtained with alfalfa grown in a normally acid soil. Walker and Brown (38) showed that rock phosphate enabled the soil of Agronomy Farm at Ames to support a larger number of alfalfa and red clover organisms. However, the phosphorus in combination with manure, lime etc. gave a still larger number of viable legume organisms.

Opinions as to the requirement and sensitivity of Azotobacter for phosphorus seem to be variable. Niklas, et al. (23) found phosphorus as well as calcium to be essential. Burk and Lineweaver (7) found phosphorus not essential for growth of Azotobacter either in fixed or free nitrogen except possibly at untested concentrations below 0.1 to 1 ppm. of phosphate. At concentrations above this, it was very stimulating to the rate of growth. Turk (34) found lime, phosphorus, and potassium, either alone or in combination to aid nitrogen-fixers in most cases. Martin, Walker, and Brown (20) in a survey of Iowa soils found the presence of Azotobacter to be closely associated with pH and available phosphorus, but more closely to pH, however in a study of Arizona soils, Martin (21) found little correlation between activity of Azotobacter and available phosphorus content.