TECHNIQUE FOR MEASUREMENT OF NON-SYMBIOTIC NITROGEN FIXATION IN SOME MANITOBA SOILS USING ^{15}N

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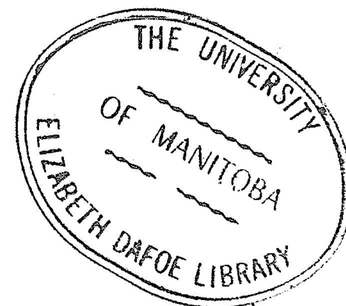

by

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ABSTRACT

An apparatus was designed in which a soil sample can be aerated by a continuous air flow and modified to allow aeration by diffusion as well. Studies were conducted with this apparatus comparing non-symbiotic nitrogen fixation under continuous air flow with aeration by diffusion under aerobic and anaerobic conditions. Fixation of nitrogen was determined by using a gas mixture enriched with ^{15}N.

The preliminary studies indicated very small amounts of nitrogen fixation under aerobic conditions with aeration by continuous flow for an Altona soil. Significant ^{15}N enrichment of the soil was measured under aerobic conditions with soil aeration by continuous flow and diffusion on a non-enriched Portage I soil while no significant enrichment was measured under anaerobic conditions with either method of soil aeration.

Nitrogen fixation studies with a modified apparatus using 53.2 atom % ^{15}N resulted in appreciably higher ^{15}N enrichment of the soil but only when energy was supplied. Under anaerobic conditions (80% A and 20% N_2), with 0.5% glucose plus 0.5% cellulose as an energy source, the diffusion method of aerating the soil sample resulted in 0.54 and 0.48 ppm of nitrogen being fixed per day in two separate studies with a Portage III soil, whereas the continuous air flow method resulted in 0.20 and 0.06 ppm of nitrogen being fixed per day under the same conditions. Under "semi aerobic" (60% A, 20% N_2 and 20% O_2 with no supplemental oxygen added during 14 day incubation period) and aerobic (60% A, 20% N_2 and 20% O_2) conditions, with added energy, 0.09 and 0.03 ppm nitrogen were fixed per day where the sample was aerated by diffusion.

Under these conditions, no fixation took place where the sample was aerated by continuous air flow.

When no energy source was added, no significant nitrogen fixation occurred in the Portage III soil under either "semi aerobic", aerobic or anaerobic conditions regardless of method of aeration.

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I INTRODUCTION

The ability of certain free living organisms to fix molecular nitrogen has been well established in pure culture studies. These studies have shown that many of these organisms exist in soils and therefore are capable of adding nitrogen to soils. Since nitrogen is one of the major nutrients and also the most costly nutrient required for good crop growth, the significance of this process to agriculture is apparent. In the past, difficulties in measuring nitrogen gains due to non-symbiotic fixation have hindered the evaluation of the quantities of nitrogen added to soils in field and laboratory studies. Despite the availability of the sensitive tracer ^{15}N technique in recent years to measure the nitrogen gains in soils attributable to non-symbiotic nitrogen fixation, the agricultural significance still has not been clearly established. A search of the literature revealed a very limited application of this technique in laboratory studies involving nitrogen fixation in soils. Presumably the cost of the tracer ^{15}N and associated equipment required, such as a mass spectrometer and high vacuum apparatus have been a deterent to the use of this technique. A review of the literature also revealed a lack of standard techniques for using tracer ^{15}N for laboratory nitrogen fixation studies in soils.

To date, no studies have been conducted in Manitoba to ascertain the significance of non-symbiotic nitrogen fixation in soils. In view of the large amount of nitrogen required for annual crop production and the need for a better understanding of nitrogen transformation in soils, it seemed worthwhile to study non-symbiotic nitrogen fixation in Manitoba soils. The objectives of the present study were:

(1) To design an apparatus allowing soil aeration by continuous flow and by diffusion.

(2) To evolve a simple method of introducing an artificial atmosphere, including tracer N-15, into a confined apparatus.

(3) To study non-symbiotic nitrogen fixation in some Manitoba soils as affected by method of aeration and readily available energy supply.

II LITERATURE REVIEW

A. Occurrence and Abundance of Asymbiotic Nitrogen Fixers:

The ability of certain free living organisms to convert molecular nitrogen into combined nitrogen has been well established since Winogradsky first demonstrated nitrogen fixation in a strain of butyric acid bacilli and since Beijerinck discovered the aerobic nitrogen fixing bacteria of the *Azotobacter* genus. Since that time, many additional organisms have been isolated that are capable of fixing molecular nitrogen. Lindstrom, Burris and Wilson (39) demonstrated nitrogen fixation by the photosynthetic non-sulfur purple bacterium *Rhodospirillum rubrum*. With the application of N-15 tracer techniques, Lindstrom *et al.*(41) showed fixation by photosynthetic bacteria of the genera *Chromatium* and *Chlorobacterium*.

Lindstrom *et al.*(40), also showed fixation by non-sulfur purple bacteria of the genera *Rhodopseudomones* and *Rhodospirillum*. Sisler and ZoBell (64) observed nitrogen fixation by a species of autotrophic, anaerobic sulfate reducing bacteria of the genus *Desulfovibrio*. Hamilton and Wilson (25), using the ^{15}N tracer technique confirmed *Aerobacter aerogenes* as a nitrogen fixer under anaerobic conditions. Hino and Wilson (28) reported nitrogen fixation by a facultative aerobe which they reported as being similar in general morphological and biochemical properties to *Bacillus polymyxa*. Nitrogen fixation in the aerobic *Pseudomonas* spp., was reported by Anderson (2), and Paul and Newton (53). Proctor and Wilson, using ^{15}N uptake detected fixation in six strains of *Pseudomonas* and eight of *Achrombacter* (58). While results of nitrogen fixation by actinomycetes have been mostly negative, Metcalfe and Brown (4) studied two species of *Nocardia* which possessed the ability to fix

nitrogen. Nitrogen fixation has now been shown to take place in at least twenty species belonging to seven different genera of blue green algae or Cyanophyceae (34).

Although numerous organisms have been isolated and demonstrated the ability to fix molecular nitrogen *in vitro*, the distribution and density of many of these organisms in soil has not been clearly established. The classical *Azotobacter* have global distribution although they are by no means ubiquitous (34). Becking (5) in a global survey reported *Azotobacter* in 33 percent of 392 samples. The density of *Azotobacter* in soil under normal conditions is exceedingly low in comparison to the high counts of bacteria usually obtained in soils by cultural methods (34). Numbers of *Azotobacter* range from 10 to 1.6×10^6 per gram of soil with most figures in the range of 10^3 per gram of soil (10, 21, 32, 42, 44, 66). The clostridia, of which the *Clostrium butyricum* species is the largest group, is practically ubiquitous (34). Bredermann (8) examined 134 soil samples of global distribution and found butyric acid clostridia in all except five samples.

Reliable densities of clostridia are exceedingly difficult to obtain (62). Dilution counts or anaerobic plate counts have usually shown figures from sporadic to 10^5 per gram with some reports exceeding 10^8 per gram under favorable conditions (34).

While species of the other genera that are known to fix nitrogen are present in soils, little is known of their distribution and numerical significance in soils.

B. Requirements for Nitrogen Fixation.

1) Energy - A readily available source of energy or carbon is

essential for non-symbiotic nitrogen fixation. In *in vitro* studies, glucose or mannitol are generally considered favorable nutrients for *Azotobacter*. However, it is unlikely that sufficient quantities of these compounds are present in soils. Winogradsky (70) pointed out that the natural carbon sources for *Azotobacter* in soils probably are simple compounds such as ethanol, butanol, acetate, propionate, butyrate, etc. that arise as fermentation products of various high-molecular substances (*i.e.* cellulose and hemi-cellulose). The range of carbon sources utilized by the clostridia are simple sugars, including pentoses, di- and poly-sacharides, pectic substances, but no organic acids except possibly lactate.

The bulk of the organic matter added to the soil is dead plant residue that consists chiefly of celluloses, hemi-celluloses, and lignin (34). Although cellulose may undergo decomposition by a variety of micro-organisms, in soil the main organisms responsible appear to be the aerobic cytophages, cellvibrios, fungi and actinomycetes and some facultative aerobic bacteria (34). *Azotobacter* apparently is unable to use the metabolic by-products, or the quantities of by-products produced are too small, of such aerobic cellulose decomposers as *Sporocytophaga*, *Cytophaga* (34), *Cellvibrio*, *Cellulobacillus*, *Streptomyces* sp., *Micromonospora chalcea* and *Trichoderma viride* (31). Significant nitrogen fixation under aerobic conditions may be expected by *Azotobacter* with cellulose when in association with facultative aerobes such as *Corynebacterium* (31, 36).

Aerobic decomposition of straw in soil apparently does not yield sufficient simple compounds for appreciable aerobic nitrogen fixation to take place (17, 35). Contrary to these reports however, Rice *et al.* (60),

using a soil-straw mixture with one and five percent straw added, found significant amounts of nitrogen fixed under aerobic conditions with the moisture status of the soil-straw mixture at field capacity, although greater amounts were fixed under water-logged conditions with added straw.

The clostridia appear quite adept at entering into association with some aerobic cellulose decomposers to fix significant quantities of nitrogen, especially when the incubation atmosphere is alternated between atmospheric air and nitrogen gas (67). Rice *et al.*(60) found maximum fixation using a thin soil-straw layer of 3 to 4 mm saturated with water but incubated in air. They suggested that aerobic cellulolytic organisms converted the straw to simpler intermediates which were then utilized by the nitrogen fixing clostridia.

2) Oxygen - The partial pressure of oxygen has a dual effect on the obligatory aerobes. Growth rates are increased markedly by improved aeration (1, 68) although growth may take place at partial pressures of oxygen as low as 0.008 atm (63, 65). Tschapek and Giambiagi (63) and Garbosky (20) reported that the oxygen demand of *Azotobacter chroococcum* decreased with decreasing organic nutrient concentration of the medium, thus behaving like a micro-aerophile. This phenomenon appears to be true of aerobic bacteria generally (72).

While improved aeration increases the growth rate of *Azotobacter* and other aerobes, high partial pressures of oxygen (pO_2 = 0.2 atm) tend to decrease the efficiency of nitrogen fixation (amount fixed per gram energy consumed) because oxygen then competes with gaseous nitrogen for the available hydrogen needed for fixation (51).

More recently Dalton and Postgate (16) have confirmed this phenomenon. They postulated two mechanisms whereby cell free extracts of

Azotobacter vinelandii protected the components of the nitrogenase. Firstly, some steric arrangement of the components results in oxygen tolerance by excluding oxygen from the oxygen-sensitive sites or by stabilizing the complex so that oxygen could not damage it, and secondly, respiration is used to scavenge oxygen from the vicinity of the nitrogen fixing sites. The second postulation, that respiration protects the oxygen-sensitive sites, was first suggested by Philips and Johnson (56), because under excess oxygen, Azotobacter vinelandii consumed sugar at rates greater than those necessary for energy requirements.

Maximum nitrogen fixation, where nitrogen gas was kept at a constant partial pressure of 0.16 atm, was found by Parker and Scutt (51) at a partial pressure of oxygen of 0.1 atm. Myerhof and Burk (46) found a decreasing growth rate but an increasing economy of fixation with decreasing partial pressures of oxygen down to about 0.001 atm. Other workers (49, 63) also found increased nitrogen fixing efficiency at low partial pressures of ($pO_2 = 0.02 - 0.04$ atm) but decreased efficiencies at higher partial pressures ($pO_2 = 0.2 - 0.4$ atm) with fixation ceasing completely at an oxygen partial pressure of 0.6 atm (49).

The effect of oxygen on the facultative aerobes is even more severe than for the aerobic organisms. In Bacillus polymyxa, an oxygen content of 1 percent (0.01 atm) completely stops nitrogen fixation (24), while Pengra and Wilson (54) report that nitrogen fixation stopped at $pO_2 = 0.05$ atm in Aerobacter aerogenes. Hamilton and Wilson (25) also found inhibition of nitrogen fixation by Aerobacter aerogenes grown aerobically. On the other hand, Jensen (33) found this species to fix nitrogen equally well under aerobic or anaerobic conditions.

The effect of oxygen gas on the clostridia and other anaerobic

nitrogen fixers would appear to be a lessening of growth at low pressures but nothing definite is known about the specific effect on nitrogen fixation (34) although recently workers have shown that nitrogen fixation by cell free extracts of C.pasteurianum and B.polymyxa is strongly inhibited by oxygen (12, 13, 23).

3) Reaction - Growth of Azotobacter in soils is usually favoured by a pH in the range of 7.2 to 7.6 with either nitrogen gas or combined nitrogen, although they have been found in soils with a reaction as low as 6.0 (34). The clostridia, on the other hand, can tolerate a wider pH range, but optimum growth is usually also found in near neutral soils (34). Optimum nitrogen fixation activity in enzyme extracts of C.pasteuranium, however, is favored by a pH in the range of 6.3 to 6.5 (41).

4) Mineral Requirements - The mineral requirements of the various aerobic and anaerobic nitrogen fixers has not been studied in great detail although some elements have received considerable attention. Bartels (7), first discovered the requirement of Azotobacter chroococcum for molybdenum, which has since led to the conclusion that it is essential for the nitrogen fixation process for many other aerobic and anaerobic organisms (6, 19, 24, 37, 55, 59, 68). Some of these organisms can replace molybdenum with vanadium in the nitrogen fixation process (6, 37, 68).

Nicholas et al.(47) suggests that small amounts (0.001 ppm) of cobalt may be necessary for the growth of A. vinelandii although it may not be specific for the nitrogen fixation process.

The requirement for iron in the nitrogen fixation process was shown by Grau and Wilson (24) for B.polymyxa while additions of calcium did not influence fixation by this organism, although other workers (11)

have shown a requirement for calcium for most organisms. Greaves and Anderson (22) showed a requirement for sulfate-sulfur by *Azotobacter chroococcum*. Phosphate requirement by *Azotobacter* has long been known and confirmed by many workers (32, 43, 71). However, critical values do not appear to have been established.

C. Quantities of Nitrogen Fixed in Soils.

Increases in nitrogen in field soils have often been reported but seldom has the evidence been such that gains in nitrogen can be unquestionably attributed to non-symbiotic nitrogen fixation (50). The reliability of field studies is debatable due to the fact that fluctuations in nitrogen content of the soil occur as a result of leaching, upward movement of soluble inorganic nitrogen, atmospheric sources of fixed nitrogen and denitrification (17). Thus in order to get an accurate estimate of nitrogen fixation, these processes must be quantified, a task not easily accomplished accurately in field studies. As a result, most nitrogen fixation studies are conducted under laboratory conditions in small closed systems where conditions can be controlled.

Laboratory studies with soils enriched with energy materials indicate that 10 to 20 mg of nitrogen are fixed per gram of energy equivalent to glucose (34). Thus when enriched, efficiency of nitrogen fixation in soils appears to be the same *in terra* as *in vitro* (34). Jensen (30), in experiments with straw, in "moderate moist nitrogen-poor soil" or pure sand kaolin mixture found no appreciable nitrogen fixation although *Azotobacter* multiplied vigorously. He concluded that the lack of nitrogen fixation was due to insufficient soluble organic material from the straw as addition of Ca-lactate caused significant nitrogen fixation. Water saturated sand-soil plus oat straw (1 - 5% straw) mixture showed

significant ($P < 0.01$) nitrogen gains of 16, 73 and 93 ppm when incubated 28, 150 and 250 days, respectively (30). Barrow and Jenkinson (4) reported similar results in that no significant nitrogen fixation was encountered in mixtures of soil and straw unless the mixture was water saturated.

Delwiche and Wijler (17), using the ^{15}N tracer technique, failed to detect nitrogen fixation in various non-enriched soils or soils amended with straw, grass or grass roots. However, they did detect significant nitrogen fixation when the soil was enriched with glucose or sucrose or when an inverted sod disc without added energy was incubated under aerobic conditions. Gains of nitrogen reached or exceeded 40 lbs. per acre six inches only when glucose or sucrose was added as a substrate. Less than 4 lbs. of nitrogen per acre six inches was fixed in the other 46 of 50 samples studied.

Rice and Paul (60) found fixation equivalent to 42 to 52 Kg/ha in soils at field capacity and 13 to 150 Kg/ha in water-logged soils when the soil was amended with one percent straw or less. Fixation was measured using the ^{15}N tracer technique. When soils amended with 5 to 20 percent straw were incubated under water-logged conditions, both the ^{15}N and Kjeldahl techniques showed fixation rates of 500 to 1000 Kg/ha. They concluded that atmospheric aerobic conditions provided favorable environment for aerobic organisms which stimulated decomposition of the straw to provide energy material for anaerobic nitrogen fixing organisms flourishing in the water-logged soil.

Chang and Knowles (14), using the ^{15}N tracer technique, agreed with the results of Delwiche and Wijler (17) that significant fixation under aerobic conditions was only measured when glucose was added.

However, they measured significant fixation under anaerobic conditions with a variety of organic and inorganic soils with and without glucose added. Fixation rates ranged from 1.63×10^{-4} to 29.2×10^{-4} mgms per day per gram dry soil.

Knowles (38) using the data of Chang and Knowles (14), corrected for temperature, estimated annual fixation rates for soils incubated under aerobic conditions with glucose added of 5.0 to 34.6 kg/ha/year. Under anaerobic conditions, without glucose, fixation rates of 0.1 to 44.0 Kg/ha/year were measured. With glucose added, fixation rates were estimated at 39 to 73 Kg/ha/year.

Laboratory studies by Porter and Grable (57) showed nitrogen fixation by both photosynthetic and non-photosynthetic organisms by sod mats incubated in an aerobic atmosphere containing ^{15}N. In a 10 day incubation period, mats in the dark fixed from 0.76 to 1.90 Kg/ha and illuminated mats fixed from 3.72 to 6.96 Kg/ha.

D. Techniques for Measuring Non-symbiotic Nitrogen Fixation.

1) Chemical Measurement of Nitrogen Gains - The classical method of measuring nitrogen fixation in soils has been the measurement of total nitrogen before and after incubation, usually by the Kjeldahl method. There are several factors which make this method of measuring nitrogen fixation not too satisfactory: (a) Only a net difference is measured, so that processes such as denitrification may obscure the total amount of nitrogen actually fixed; (b) The quantities of nitrogen fixed non-symbiotically in soils usually are quite small and therefore not easily detected by the Kjeldahl method unless extensive replication is employed. Bremner (9) pointed out that if a soil containing 0.1 percent nitrogen is incubated in normal air, an increase of one percent in the total nitrogen