Conversion of Atmospheric Nitrogen to Soil with the Application of Terreplenish.

A. Introduction

(1) Effects to be measure is increased nitrogen in the soil from atmospheric nitrogen with a soil-applied diluted mixture of Terreplenish and water. The primary ingredients of Terreplenish is Azotobacter and Pseudomonas heterotrophic bacteria. *Azotobacter* is a genus of free-living diazotrophic bacteria whose resting stage is a cyst. It is primarily found in neutral to alkaline soils, in aquatic environments, and on some plants. It has several metabolic capabilties, including atmospheric nitrogen fixation by conversion to ammonia. Their unique system of three distinct nitrogenase enzymes makes these bacteria potentially critical in supplying plant available nitrogen for growing crops. *Azotobacter* . have the highest metabolic rate of any organisms.

(2) The trials were conducted at Beaver Creek Gardens of Poplar Grove Illinois. between August 16th, 2013 and September 25th, 2014.

(3) Solutions in the Land, LLC was contracted in 2011 to present to write efficacy trial protocols, conduct trials and review the purblished literature on the active ingredients in the product made by Feed Earth Now, called Terreplenish.

(4) Solutions in the Land, LLC profile:

Ronald G. Doetch, Managing Partner, Principal Investigator, University of Illinois, BS Agronomy, 1969, Wisconsin Integrated Cropping System Trials Advisory Board, 1996-2010, University of Wisconsin, Arlington Research Farm, Agriculture Efficacy Trials, 1970- present.

Cal Pickrum, Research Intern

R Douglas Galbraith, Research Intern

B. The Product Terreplenish

(1) Application Guidelines

For Pre Plant Application: Apply 7-10 days ahead of seeding.
For Post Plant Application: Apply after emergence. Applications may be repeated every 7 days or every 3 in fast maturing crops< 45 days

Dilution Guidelines

Suggested dilution 25- 50 parts water to 1 part Terreplenish; this container makes 25 gallons of solutions. Do not mix stronger than one 64oz container to 5 gallons of water.

Re-entry time - ZERO

Safe for pet and child exposure immediately after application.
Tank mixing with synthetic & chemical fertilizers is discouraged.

Chemical analysis

Numerous trace amino acids, carboxylic acids, peptides, alcohols, ammonium salts -0.1%, complex sugars, esters, aldehydes; concentrate pH before dilution 4.0; Microorganisms> 108 CFU/ml,1%: Lactobacillus casei.

Caution

Avoid spills and prolonged direct contact with the skin and wash with clean water

immediately to prevent injury irritation. Do not ingest. The fermented products repeatedly have been shown to be free pathogens. Keep out of sunlight. Avoid prolonged exposure to oxygen by replacing cap after each use.

(2) Claim: Soil-applied Terreplenish will convert atmospheric nitrogen to fixed soil nitrogen as a result of the life processes and rapid reproduction of the Azotobacter bacteria.

C. Materials, Methods and Procedures

In 1901, Dutch microbiologist M. W. Beijerinck, using an enrichment culture technique with a medium devoid of a combined nitrogen source, discovered an aerobic microorganism capable of fixing molecular nitrogen to which the name of Azotobacter chroococcum was given. The genus Azotobacter comprises large, Gram-negative, primarily found in neutral to alkaline soils, obligately aerobic rods capable of fixing N2 nonsymbiotically. Azotobacter is also of interest because it has the highest respiratory rate of any living organism. In addition to its ecological and physiological importance, Azotobacter is of interest because of its ability to form an unusual resting structure called a cyst. Azotobacter cells are rather large for bacteria, many isolates being almost the size of yeast, with diameter of 2-4μm or more. Pleomorphism is common and a variety of cell shapes and sizes have been described. Some strains are motile by peritrichous flagella. On carbohydrate containing media, extensive capsules or slime layers are produced by free-living N2 fixing bacteria. Azotobacter is able to grow on a wide variety of carbohydrates, alcohols and organic acids. The metabolism of carbon compounds is strictly oxidative and acids or other fermentation products are rarely produced. 2 All members fix nitrogen, but growth also occurs on simple forms of combined nitrogen: ammonia, urea and nitrate (Brock et al., 1994).

The bacterium used in this study was Azotobacter chroococcum, which was used for agricultural purposes by Catek, Havana, Cuba. Stock cultures of A. chroococcum was maintained on N-free agars and stored at 4°C. Olive Mill Wastewater OMW used in this study was obtained from a continuously operating olive factory at Morova in 2002. OMW was sterilized separately and added to the basal medium with its whole content that is; with its suspended and dissolved solids. Then pH of the medium was adjusted to 7 .0 The growth medium was inoculated with approximately 106 cell/ml A. chroococcum cells grown at preculture medium for 22 h. Preculture medium was the N-free medium and it was inoculated with one loopfull of bacterial cell transferred from the solid medium. Preculture was cultivated at 35°C and 150 rpm. Growth was quantified with aerobic plate count method. The samples taken from the growth medium was diluted to appropriate concentration. Dilution solution was the N-free medium except the carbon source. The diluted cell 23 suspensions were inoculated over the solid N-free medium. And the plates were incubated at 35°C for 40 h then the white colored colonies were counted.

Nitrogen fixation capacity of A. chroococcum was quantified indirectly by measuring the products of nitrogen fixation activity; extracellular protein and ammonia concentrations. Extracellular protein concentration was measured with Modified Lowry Method (Hartree, 1972). The samples taken from growth medium were centrifuged at 15000 rpm for 15 min at 0°C and the supernatant was analyzed with Lowry Method. The reagents used in Lowry Method are given in Appendix A and the standard curve preparation and procedure of this method is described in Appendix B. Standards were prepared with bovine serum albumin (BSA). Absorbances of the samples were read by taking the blank as the uninoculated medium itself, since the salts contained in the medium interact with the reagents causing

overestimation. The ammonia concentration was measured with Nesslerization Method following the procedures described in Standard Methods for the Examination of Water and Wastewater. The reagents and the procedure of Nesslerization Method were described in Appendix C and D, respectively. Absorbances of the samples were measured at 400 nm against the blank solution prepared as the uninoculated 24 initial medium, since the salts contained in the medium interact with the reagent causing overestimation.

D. Results

As a study to further fix soil nitrogen with A. chroococcum, this nitrogen-fixing bacteria was cultivated in chemically defined nitrogen-free medium. Effect of several factors on growth and nitrogen fixation capacity of A. chroococcum, such as pH, temperature, aeration, inorganic salts and combined nitrogen was evaluated. Growth is measured as viable counts by plate counting. Although this method takes longer time, the other two methods that were faster, counting cells with Thoma Chamber and optical density measurement, were not appropriate. While counting with Thoma Chamber both the viable and dead cells are counted and OMW contains a high dead cell load, the results obtained with these methods had lead into errors. Also measuring growth with optical density is not suitable for the OMW containing medium due to the dark color of OMW. Ammonia and extracellular protein are the nitrogenous secretions of the nitrogen fixation activity of Azotobacter in nitrogen free or deficient medium, which are available to the plants or other organisms. That is why in this study nitrogen fixation is evaluated using the ammonia and extracellular protein concentrations as criteria in growth medium under diazotrophic conditions.

The dynamics of growth and nitrogen fixation at different physiological conditions and nutrient requirements of A. chroococcum in chemically defined N-free medium was determined. The maximum cell concentration were obtained when A. chroococcum was grown at neutral pH, 35°C, 150 rpm and in medium supplemented with manganese salt at 0.01% concentration as 4.8x107 cfu/ml. The maximum nitrogen fixation products were attained when A. chroococcum was grown at pH 8, 35°C, 150 rpm and in medium supplemented with manganese salt at 0.01% concentration as 10.4 mg/l extracellular protein and 1.5 mg/l ammonia.

Conclusions – In greatly varied medium similar to agricultural soil conditions, A. chroococcum secretes some substances available for plants, such as protein and ammonia.

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