Effect of Microbial Inoculation on Nitrogen Plant Uptake and Nitrogen Losses from Soil and Plant-Soil Systems.

by

Pamela Calvo Velez

A dissertation submitted to the Graduate Faculty of
Auburn University
in partial fulfillment of the
requirements for the Degree of
Doctor of Philosophy

Auburn, Alabama
December 14, 2013

Keywords: PGPR, nitrogen, nitrous oxide,
agriculture, microorganisms, corn

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Approved by

Joseph W. Kloepper, Chair, Professor, Department of Entomology and Plant Pathology
Leonardo de la Fuente, Assistant Professor, Department of Entomology and Plant Pathology
David Held, Associate Professor, Department of Entomology and Plant Pathology
Allen Torbert, Affiliate Professor, Department Department of Crop, Soil and Environmental Sciences

Abstract

Reducing the negative impacts of agricultural fertilizers is a world-wide concern, both from an environmental and human health perspective. One way to reduce these impacts is by enhancing plant uptake, which improves nutrient use efficiency, thereby reducing the amounts of fertilizer needed. The general objective of this research was to evaluate the effect of microbial-based inoculants on nitrous oxide (N_2O) emissions from plant and soil systems and their effect on enhancing plant nitrogen uptake. Specific objectives were: *i)* to evaluate the effect of microbial inoculants (PGPR mixture (BM), Soil Builder™ product (SB), and Soil Builder™ product filtrated (SBF)) on N_2O emissions from soil in a jar incubation study using different types of nitrogen (N) fertilizers; *ii)* to evaluate and confirm the effect of the same microbial inoculants on N_2O emissions when a plant is present in the system in a greenhouse study using different types of N fertilizers; *iii)* to understand the effect of microbial inoculants on corn growth, nutrient uptake, and root morphology of corn evaluated at different growth stages and with different types of N fertilizers; *iv)* to identify the best PGPR *Bacillus* spp. mixture that has with the potential to reduce N fertilization and obtain results comparable to a 100% N recommended fertilization, and *v)* Determine if selected mixture of PGPR increase plant growth and transcript levels of nitrate and ammonium uptake genes of *Arabidopsis thaliana* . Emissions

of N_2O were reduced by 62% on average with SBF and SB in soil treated with calcium ammonium nitrate (CAN) and by 66% on average with SB, SBF, and BM in soil treated with urea ammonium nitrate (UAN). In the greenhouse study, cumulative fluxes of N_2O from pots at 41 DAP showed a significant reduction of 37% (BM) and 23% (SBF) with CAN fertilizer. When UAN was used, reductions of 26% (SB), 28% (SBF), and 49% (BM) were obtained. However, no reduction of N_2O was reported with urea fertilizer. Corn growth parameters (height, shoot fresh and dry weight) and nutrient uptake were increased in corn plants treated with microbial inoculants, and the differences were greatest in plants evaluated at VT stage. Total root length, root volume, and root surface were increased at the V2 and V4 stages in plants with microbial inoculants. Three PGPR mixtures reduced N fertilization on when fresh weight (16%), dry weight (1.5%), and nitrogen uptake (6.64%), were evaluated in cabbage. Greater reduction of N fertilization was observed in pepper where PGPR mixtures allowed reductions of 30.46% (fresh weight), 30.03%, (dry weight) and 26.93% (nitrogen uptake). Plant growth parameters of *A. thaliana* were increase by PGPR mixtures. Transcript levels of 5 of the six nitrate uptake genes and four of the five ammonium uptake genes evaluated in roots were increased by PGPR mixtures. Overall, the results demonstrate that microbial inoculants can reduce emissions of N_2O resulting from some types of nitrogen fertilizers and can increase plant growth and plant nutrient uptake, thereby reducing nitrogen fertilization.

Acknowledgments

I would like to dedicate this dissertation to my Dad, who taught me to always pursue my dreams. He will always be my inspiration.

First, I thank God and my family. Their love provided me inspiration and was the driving force to complete this journey. I would like to express my deepest appreciation to my advisor

Dr. Joseph Kloepper for giving me the great opportunity to grow as a scientist and for believing in me. Without his guidance, encouragement, and infinite help, this dissertation would not have been possible. I would like to thank my dissertation committee for their support and important suggestions.

This dissertation would not have been possible without the help of so many people. I am grateful to my laboratory colleagues and the plant pathology department faculty and staff. A special thanks goes to my dear friend John McInroy for always encouraging me and for his valuable life lessons. Also, I would like to thank my colleagues in the USDA National Soil Dynamics Laboratory who collaborated on this project, specifically Dr. Torbert and Dr. Dexter Watts who offered valuable support and suggestions. Special thanks to the staff in the Plant Science Research Center, especially to Jane Farr for her help with all the greenhouse logistics. Thanks to Dr. Simon Zebelo from his collaboration in this dissertation and for his infinity patience.

I want to express my deepest appreciation to my mom for always being there for me even though she was miles away. She always cheered me up and was with me in every step of this journey. Finally, I like to thank my fiancé Holcer Chavez. Without his love, patience, and infinite support this work would not have been possible.

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List of Abbreviations

PGPR	Plant-Growth Promoting Rhizobacteria
TSA	Triptic Soy Agar
NiUE	Nitrogen Use Efficiency
NUE	Nutrient Use Efficiency
UAN	Urea-ammonium nitrate
CAN	Calcium-ammonium nitrate

Literature Review

I. The Nitrogen cycle

Nitrogen (N) is one of the most important and limiting elements present in plants where it is found in proteins, chlorophyll, and nucleic acids. Plants need to uptake this element from different sources in order to complete their physiological functions. Even though the atmosphere is the principle source of N, plants cannot take up nitrogen directly as N_2 gas. Consequently, only less than 0.005 % of N is found in forms that plants can acquire (Scharenbroch and Lloyd, 2004).

The N cycle plays an important role in terrestrial ecosystems. Most N enters the soil as NO_3^- or NH_4^+ in rainfall or by N fixation. In agriculture fields, high amounts of N enter the cycle by chemical or organic fertilizers (Franco and Munns, 1982). Once the N is in the soil, it interacts with the atmosphere, soil particles, soil solution, microorganisms, and plants. Different N transformations and chemical reactions take place during the N cycle.The most important transformations that take place in the N cycle are mineralization, inmobilization, nitrification, denitrification, fixation, and volatilization. These transformations are highly affected by environmental conditions such as temperature and moisture. Because the environmental conditions are constantly changing, the interactions between all the transformation reactions are very dynamic (Alva, et al., 2005).

The process of mineralization involves a conversion of soil organic matter, crop residues, and manures to inorganic forms. Organic N is converted to NH_4^+ by soil microorganisms. The reverse process, called immobilization, occurs when the microorganisms present in the soil compete with crops for N. In this process nitrate and ammonium are taken up by soil organisms and thereby become unavailable to crops. This immobilization of N is only temporary. When the microorganisms die, the decomposition process again makes N available for crop use.

Mineralization and immobilization are considered to have a major influence on the amount of bioavailable N in soil and are highly affected by environmental variables such as soil moisture, temperature, pH, type of organic materials in the residue, and the C:N ratio in the soil. The C:N ratio is often used as an indication of whether mineralization or immobilization will occur. When surface soil layers have a C:N ratio greater than 30:1, immobilization is highly likely to occur. This is because microorganisms need N to assimilate the available C, In order to complete their N needs they also take inorganic N from the soil. The result of this process is more organic N and less inorganic N present in the soil. When the C:N ratio is below 20:1, N mineralization is likely to occur because N is available and microbes decompose the organic material present in the soil. The result of this process is more inorganic N present in the soil. When the C:N ratio is between 20-30:1, both mineralization and immobilization may occur, but they will generally balance (Alva, et al., 2005, Sullivan, et al., 2002). However, it is important to consider that even though part of the N is often easily mineralized, another part is bound to the lignin fraction so it is very resistant to mineralization (Sullivan, D. et al., 2002)

The nitrification process is considered to control the availability of NO_3^- in soil (Jarvis, 1999). It takes place when autotrophic nitrifying bacteria obtain energy from the oxidation of ammonium or nitrite. Nitrification occurs in two steps. In the first part *Nitosomonas* oxidizes NH_4^+ to NO_2^-. Then *Nitrobacter* oxidizes the NO_2^- to NO_3^-. Nitrification rates are higher when soils are warm and moist. Soil pH has a direct relation to the nitrification rates. When pH decreases, nitrification rates decrease, which is an effect that is highly related to the optimum pH for *Nitrosomonas* and *Nitrobacter* (Shammas, 1986). Denitrification occurs when denitrifying bacteria use nitrate, nitrite, or nitrous oxide as terminal electron acceptors during the oxidation of organic substrates. Nitrate (NO_3^-) is reduced to NO_2^- and then to gaseous forms including N_2O and N_2 that are released to the atmosphere. The amount of denitrification depends on the availability of NO_3^-, soil saturation, soil temperature, and on availability of decomposable organic matter. When low soil oxygen levels are present, O_2 can no longer diffuse. Consequently, soil microorganisms will deplete the O_2 and then begin using the NO_3^-. If a soil is too cold for microbial

activity or if there is too little organic matter available for the microorganisms, the O_2 will not be depleted and denitrification will not occur. This process is more likely to occur in poorly drained clay soils than in well drained sandy soils (Alva, et al., 2005). The process of denitrification is detrimental, since plant-available N is lost to the atmosphere (Sullivan, et al., 2002).

Ammonia gases can be lost to the atmosphere due to the process of ammonia volatilization. This potential loss of ammonia gas (NH_3) depends on the rate of NH_3/NH_4^+ present in the aqueous phase of the soil (Jarvis, 1999). The NH_3/NH_4^+ rate is highly regulated by soil pH. Consequently, volatilization is increased when soil pH is increased. In addition to pH, the volatilization process is affected by other factors such as temperature, wind speed, and whether the fertilizers are incorporated, injected, or surface applied (Alva, et al., 2005). A significant amount of N fertilizer can be lost through the process of volatilization if it is not properly applied. Many commercial fertilizers contain ammonium or convert readily to ammonium, as in the case of urea. Under alkaline pH conditions (pH > 7), a percentage of the ammonium (NH_4^+) can be converted to ammonia gas (NH_3) and escape to the atmosphere. In addition to soil pH, high soil temperature, excessive soil moisture, and strong winds will contribute to such loss.

Biological N fixation is an important process related to the N cycle. Some legumes can form a symbiotic association with specific bacteria to convert atmospheric N_2 to a form plants can use. The plant provides nutrients and other compounds to the N_2-fixing bacteria, and in return, the plant benefits from the N fixed by the microorganisms. The amount of N fixed varies among plants and growing conditions. The symbiotic association is highly specific; thus, the bacterial species that fixes N_2 with soybeans does not effectively fix N_2 with alfalfa (Werner, Mahna, et al., 2005). The site of the N_2-fixing process is a root nodule that forms on the root system and has a pink color if it is actively fixing N_2. Nitrogenase enzyme, which is highly affected by soil and weather conditions, could accelerate the N fixation process (Baldani and Baldani, 2005). Furthermore, the biological fixation process is also highly affected by low soil pH and high levels of soil mineral N.

II. Potential nitrogen losses

Despite the important role of synthetic N fertilizers, it is clear that there are some negative effects of fertilizers when their use results in increased amounts of reactive N in the environment. Nitrogen losses to the environment usually take place when high concentrations of soluble N forms are present in the soil solution in excess of the amount that plants can absorb (Chien, et al., 2009). While most of the N losses occur locally in terrestrial systems, the influence of these emissions spreads regionally and globally as they move through water and air across political and geographic borders. The two most important ways to lose N to the environment are: nitrate leaching and N_2O emissions (FAO, 2008).

2.1. Nitrate leaching

Global estimates of total N losses indicate that leaching, erosion, and runoff constitute approximately 46% of all losses (Motavalli, et al., 2006). Leaching occurs when nitrate (NO_3^-) moves from a terrestrial to an aquatic ecosystem. Nitrate is highly mobile in soil, and the amount that is present in the soil is a reflection of the interactions between removal process (mineralizaton and nitrification) and input of nitrate through fertilization (Jarvis, 1999). Among all nutrients, nitrate is considered to be one of the most important water pollutants today (Bouwman, et al., 2005, Petrovic, 1990). Since pre-industrial times, the flux or discharge of nitrates to coastal waters by rivers around the world has doubled; rising from roughly 21 Tg/yr to over 40 Tg/yr (1 teragram (Tg) = 10^{12} g = 1.1 million US tons) (Green, et al., 2004.).

River export of N ranges from 7 to 13% of total N inputs in developing, industrialized, and transitional countries. According to Bouwman et al. (2005), total N inputs and total river N export have increased since 1970 and are projected to continue to increase through 2030. Nitrate flux from rivers has consequences to human health and to the environment. Consumption of water with high concentration of

nitrates can result two medical conditions: methaemoglobinaemia ('blue-baby syndrome') in infants and stomach cancer in adults. The environmental problems of nitrate flux to lakes and ocean begin with an increase of algae growth, but the more detrimental effect occurs when algae die and the bacteria that decompose them use the available oxygen. Due to the decrease of oxygen levels in the lakes and ocean, fish and other organisms die. An example of the severity of excessive flux of nitrate in the environment is that over 60% of coastal rivers and bays in the US have been moderately or severely degraded by nutrient pollution, especially by nitrate (Motavalli, et al., 2006) . A specific example of the negative results of N fertilizers is the "dead zone" in the Gulf of Mexico where nutrients washing from fertilized farms across the Mississippi basin cause oxygen starvation, leading to an almost lifeless area in the gulf (Malakoff, 1998).

2.2. Nitrous oxide

In the soil, different processes generate N_2O. The most important ones are microbial processes carried out by nitrifying microorganisms (nitrifiers) and denitrifying microorganisms (denitrifiers) (Pathak, 1999). Nitrifiers are autotrophic microorganisms that obtain energy for the CO_2 fixation from ammonia (NH_3) or nitrite (NO_2^-) oxidation. Ammonia oxidizers or primary nitrifiers convert ammonia or ammonium (NH_4^+) to nitrite in the first part of the process. In the second part oxidizers or secondary nitrifiers convert nitrite to nitrate. The main genus responsible for the first part of nitrification is *Nitrosomonas*, and the one responsible for the second part is *Nitrobacter*. Nitrous oxide can be formed during ammonia oxidation through chemical decomposition of intermediates between NH_4^+ and NO_2^-, which occurs due to a special form of chemodenitrification (Chalk and Smith, 1983). Thus, ammonia oxidation in the nitrification process can be a source of N_2O. The final product of nitrification (NO_3^-) is the substrate needed for denitrification process which also produce N_2O production. Denitrifiers are heterotrophic microorganisms that use nitrate (NO_3^-) or nitrogen dioxide (NO_2^-) as alternatives to O_2 as

an electron acceptor in low-oxygen conditions. In this process nitrous oxide (N_2O) is also an intermediate product and can be emitted. Denitrifiers are widely distributed across the bacterial taxa, including *Pseudomonas*, *Bacillus*, *Thiobacillus*, *Propionobacterium* and others (Firestone, 1982). Nitrous oxide is a regular intermediate of denitrification. Some factors that could increase nitrous oxide production from denitrification are: low pH and high concentration of nitrate in the soil. Besides these two important pathways there is evidence that another process called nitrifier denitrification could be responsible for a percentage of nitrous oxide production from soil. Nitrifier denitrification begins with NH_3 or NH_4+ that is oxidized (like in the first part of nitrification) to NO_2^-. The difference is that NO_2^- is converted directly to N_2O and N_2. This process is carried out by autotrophic ammonia oxidizers. It differs from nitrification and denitrification in which different groups or microorganism together convert NH_3 to N_2O (Kuai and Verstraete, 1998). The amount of N_2O lost via nitrifier denitrification may vary between 0-30 % of the total N_2O production (Wrage, Velthof, et al., 2001). In summary it seems that all the processes involved in nitrous oxide production overlap and the occurrence of each one depends on many soil variables. Therefore, the process responsible for nitrous oxide production could change due to changes in soil conditions. It is important to remember that once nitrous oxide is produced, N is no longer available for plants and is rapidly lost to the atmosphere.

Nitrous oxide is not only important because it is a way to lose N from soils but also because it is a potent greenhouse gas. Nitrous oxide is a very stable gas with a lifetime of over 170 years (Pathak, 1999). Although the total global emissions of N_2O are lower than those of CO_2, the global warming potential of N_2O is 300 times greater than CO_2 (Goyal, Tischner, et al., 2005).

The atmospheric concentration of N_2O has steadily increased since 1750. The current concentration of 315 ppb is about 16 % more than that during the pre-industrial era and is increasing at the rate of 0.25 %/yr (Goyal, et al., 2005). Agriculture remains the single biggest source of anthropogenic N_2O (Bouwman et al., 2002). In a model analysis of major U.S. cropping systems, Del Grosso et al.

(2005) found that modern agricultural N_2O emission was more than 2 times that of pre-1940 management and about 6 times that of native vegetation.

The rate of production and emission of N_2O depends primarily on the availability of a mineral N source, the substrate for nitrification or denitrification. However, production and emission also depend on other factors like soil moisture, oxygen concentration in soils, soil p H, soil texture, temperature, fertilizer application, organic manure, and plants (Pathak, 1999). This last factor represents a key point to be considered. Plants influence the nitrate and carbon content of the soil. They have a direct influence on nitrate availability through uptake and assimilation, making nitrate unavailable to denitrification. They also have an indirect effect that occurs when the organic matter from the roots that is mineralized provides nitrate for denitrification process (Pathak, 1999).

The intensification of agriculture and the increase use of synthetic N fertilizers and livestock are mainly responsible for the global increase in N_2O emissions in the last few decades (Del Grosso, et al., 2005, Kroeze, et al., 1999). During the 20th century, an expansion of agricultural land coupled with the intensified use of N fertilizer inputs caused a net increase of global N_2O emissions from 11 Tg N/yr in 1850 to 18 Tg N/yr in 1994 (Kroeze, et al. 1999). As a result, N fertilizers are the major source of nitrous oxide emissions contributing to 68% of the total N_2O emissions (FAO, 2008). The loss of N fertilizer as N_2O is generally in the range of 0.01–3%. This N_2O loss depends on the types of N fertilizer. Among all the N fertilizers, anhydrous ammonia results in the highest percentage of N_2O losses (Pathak, 1999).

Some options to ameliorate the problem of increasing N_2O emissions from agriculture systems include improvement of soil management, efficient fertilization, and implementation of soil erosion controls and conservation practices. However, there is a lack of complete studies that address the actual effect of some agronomic practices on nitrous oxide production. It is necessary to evaluate more systems that could reveal the actual production of nitrous oxide in agriculture fields. Furthermore, studies on nitrous oxide production under different agronomic practices, including fertilizer application, will help in understanding the soil dynamics associated with nitrous oxide production.

III. Nutrient management

3.1. Nitrogen fertilizer

The increases in the world's population and the area of cultivated land have led to increased global consumption of N, which is required to maintain high levels of crop yields. The use of synthetic N fertilizer became possible with the discovery in the early 20th century of the Haber–Bosch process, the N fixation reaction of N gas and hydrogen gas, over a catalyst, which is used to produce ammonia. Due to this new discovery, N consumption in agriculture was increased by twenty-fold from 1950 to 2009. It is projected that by 2012 the world N consumption will reach 139 million tons. The world's main consumers of N are East Asia, South Asia, North America, and West Europe (FAO, 2008).

Currently there are many different types of inorganic commercial N fertilizer. The most common commercial N fertilizers are anhydrous ammonia, urea, N solutions, ammonium sulfate, and ammonium nitrate. Anhydrous ammonia (82% N) is a gas at normal atmospheric temperatures and pressure. It is the slowest of all N fertilizer forms to convert to nitrate. Therefore, it would have the least chance of N loss due to leaching or denitrification. It must be injected into the soil;therefore, it would have no loss due to surface volatilization. The disadvantage of anhydrous ammonia is that it is hazardous to handle and is toxic to living organisms (Vitosh, Johnson, et al., 1995).

Urea (46% N) is widely used in solid and liquid fertilizers. Urea is converted to ammonium carbonate by an enzyme called urease. Due to this process, ammonia volatilization can cause significant losses that range from 50% to 90% (Ontario Ministry of Agriculture Food and Rural Affais and (OMAFRA), 1999). These losses usually occur more when urea is applied to the surface of warm, moist no-till soils. On the other hand, urea has relatively desirable handling and storage characteristics andusually has the lowest cost per pound of N compared to other single-element N fertilizers.

Ammonium nitrate contains 34% N and is used as a source of N in many blends of liquid and dry fertilizers. For soils subject to leaching or denitrification, ammonium nitrate would not be preferred. Ammonium nitrate has no urea in it; therefore, it would be a good choice for surface application where ammonia volatilization is expected. However, pure ammonium is very hygroscopic and can be explosive under certain conditions.

UAN is a N solution (32% N) that is made up of urea and ammonium nitrate. The nitrate in this product is subject to leaching and denitrification as soon as it is placed in the field. The urea components are subject to the same loss mechanisms as urea. To avoid these losses, this type of solution is usually applied using dribble surface bands or rolling coulter injection.

Ammonium sulfate (21% N and 24% sulfur) is a N source with little or no surface volatilization loss when applied to most soils compared to urea. Ammonium sulfate is a good source of sulfur when it is needed. Its disadvantage is that it is the most acidifying form of N fertilizer. It requires approximately 2 to 3 times as much lime to neutralize the same amount of acidity as formed by other common N carriers.

In order to choose the source of N to be applied to one specific crop it is necessary to consider many variables. First, it is very important to know the crop requirements.Then it is necessary to compare these requirements with the information of the natural N present in the soil given by the soil analysis. The soil analysis also provides important information about the type of soil, pH, SOM, texture, water holding capacity, and EC. Other considerations are the environmental conditions and the irrigation system to be used. All these variables will help not only to decide the best type of commercial N fertilizerbut also to establish the correct timing of application. Finally, the main reason to evaluate all the variables before applying N fertilizer is to assure the best way to obtain a high uptake level and reduce possible N losses from the system.

3.2. Nitrogen use efficiency

In the last few years there has been an increased interest in enhancing nutrient use efficiency (NiUE). Improving fertilizer use efficiency in terms of nutrient uptake and crop yield is important to fertilizer producers and users. Many studies on N uptake by crops have indicated that there is often a very low efficiency in uptake of the applied N. The percentage of applied N that is uptaken by crops ranged from 25% to 70%, depending on the crop and the type of soil (Chien, et al., 2009, Roberts, 2008, Sullivan, Hart, et al., 1999). These values demonstrate the need to improve nutrient uptake in the field.

Many ideas have been developing in order to increase NiUE in crop production. The new strategies can be classified as new products and new management practices. For example, new fertilizer products include slow release fertilizers that have physical coatings or chemical compositions that provide slow and controlled release of plant nutrients. Other new products called “stabilized fertilizers” are treated with urease and/or nitrification inhibitors to prevent potential N losses. These fertilizer products have been used extensively in horticultural applications, but limited information is available on their use for agronomic production (Motavalli, et al., 2006).

Challenges for widespread adoption of these fertilizers for agronomic uses include their higher cost compared to conventional fertilizers and lack of research-based recommendations on when they would be effective and how they should be managed under different environments and cropping systems. An alternative strategy is to focus on specific management practices, such as the timing and rate of fertilizer application. These management practices can also be combined with conservation practices such as buffer strips, continuous no-till, cover crops, and riparian buffers within intensively managed cropping systems to optimize yields and diminish nutrient losses (Fixen, 2006).

In recent years another important approach for NiUE has been developed. Microbial inoculants are promising components to reach a more environmentally-friendly, integrated nutrient management. Microbial inoculants include plant growth-promoting rhizobacteria (PGPR). PGPR application has shown effects on promoting plant growth and enhancing nutrient availability and uptake (Adesemoye, et al.,

2008). Specific effects on nutrient (N, P, K) assimilation have been reported due to the application of different microbial fertilizer in different crops like maize, cotton, palm seedlings, and rice (Amir, et al., 2005, Egamberdiyevaa and Hoflich, 2004, Wu, et al., 2005). It seems that microbial activity in the rhizosphere may affect the acquisition of mineral nutrients by roots either directly via effects on mobilization and/or immobilization or indirectly via effects on root morphology and/or physiology (Babalola, 2010).

To be adopted, NiUE must be cost effective and fit into the total farm management system. The main reasons why these enhancing tools are never adopted by farmers are because they are not cost effective for the cropping system and they do not fit into the total farm management system (Fixen, 2006). There is also a lack of research about the effectiveness of these tools on reducing some nutrient loss from the system. Achieving a successful NiUE can also have a positive effect on the economy. It was reported that with an improvement of 15-20% of N fertilizer use efficiency, the projected food production increase for 2030 can be obtained with 20 million tons less fertilizer N in total (Daberkow, et al., 1999). In the future, NiUE should be more focused on sound, environmentally-friendly options that also can improve the total soil system health. Production practices that have resulted in increased NiUE when compared to conventional or standard practices are those that will counter conditions, or environments, known to contribute to N loss from soil-plant systems.

IV. Plant nitrogen uptake

Nitrogen plays a central role in plant productivity because it is a major component of amino acids, proteins, nucleic acids, and chlorophyll. Organic N commonly constitutes 1.5 to 5% of the dry weight of plants. In both fertilized and unfertilized soils, ammonium and nitrate are the only major ionic forms of N actively absorbed by plants (Haynes, 1986, Pilbeam and Jan, 1999). Uptake of nutrient elements in ionic form by roots is an active physiological process. Absorption of nutrients from soil

solution is thus affected by many soil and environmental factors that can affect both the uptake process as well as the availability of nutrients within the soil. Because nutrients are captured by roots, an optimal root development is considered one important factor to uptake nutrients. The rate of root growth and the plasticity of root architecture along with development of the rhizosphere, through either root growth or extension of root hairs, are clearly important for effective exploration of soil and interception of nutrients by the plant (Richardson, et al., 2009).

Plants respond with one or more of three main strategies to optimize their acquisition of N. These strategies can be classified as (a) extending root length and branching or increasing root surface area via changes in root diameter or root hair morphology to explore a greater volume of soil; (b) using specific adaptive repsonse mechanisms in order to exploit spatial and temporal 'niches' such as N-rich patches due to the presence of particular forms of N (amino acids, NH_4^+ or NO_3^-); and (c) influencing plant available N in the rhizosphere through plant-microbial interactions (Richardson, et al., 2009).

Roots also respond to different levels of N in soil. The size and architecture of the root system is an important feature for ensuring adequate access to soil N, and root system size (relative to shoot growth) has generally been shown to increase when N is limiting. Roots can also indicate the availability of N in soil. It was demonstrated that the diameter of first and second order lateral roots were significantly thicker in cereals grown at high concentrations of NO_3^- (Pilbeam and Jan, 1999, Richardson, et al., 2009)
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Roots share an important relation with the rhizosphere. The rhizosphere can be defined as any volume of soil that is specifically influenced by plant roots and/or is associated with roots and hairs and plant-produced material (Mahaffee, 1997). Changes in the physical, chemical, and biological properties of rhizosphere soil have significant influence on the subsequent growth and health of plants (Glick, 1995). In terms of nutrient acquisition, both the structural and functional characteristics of roots have long been recognized as being important in determining the capacity for plants to access and mediate the availability of essential nutrients in soil (Haynes, 1986, Hinsinger, 1998, Yevdokimov and Blagodatsky, 1993).

Furthermore, roots interact with diverse populations of soil microorganisms that have significant implication for growth and nutrition (Brimecombe, et al., 1999).

V. Microbial inoculants and nitrogen uptake

Microbial associations with roots are complex in soil and can enhance the ability of plants to acquire nutrients from soil through a number of mechanisms. In this respect, plant growth-promoting rhizobacteria (PGPR) are of particular importance and have been studied most widely. PGPR can be described as bacteria having most of or all the following qualities: capacity to colonize plant roots, adherence to soil in the rhizosphere, and capacity to enter into root interior and establish endophytic populations that adapt to the niche and benefit the host plant (Compant, et al., 2005). They can stimulate plant growth through either a "biofertilizing" effect or a "biocontrol effect". The biofertilizer effect can also be considered a direct mechanism of plant growth promotion. Some mechanisms involved include production of metabolites or plant growth regulators/hormones (indole-3-acetic acid [IAA]), gibberellins, and cytokinins), which directly increase plant growth and lead to improved root growth with large surface area and increased number of root hairs (Bottini, et al., 2004, Khalid, et al., 2004, Ryu, Hu, et al., 2005, Tsavkelova, et al., 2006). Also, the capacity to enhance plant nutrient uptake (e.g., K, N, Fe, S) is considered a direct mechanism. The biocontrol effect is more related with an indirect mechanism of plant growth promotion. Some indirect mechanisms are antibiotic production, induced systemic resistance (ISR), parasitism, siderophore production, competition for binding sites on the roots, and cyanide production (Jacobsen, et al., 2004, Loper and Henkels, 1999, Zehnder, et al., 2001).

5.1. Overview of inoculants for enhancing fertilizer uptake

Biofertilizers are increasingly being reported as alternatives or suplements to fertilizers to stimulate improved uptake of nutrients as a possible solution to agro-environmental problems (Dibut, 2003, Martinez Viera and Dibut, 2006). Microbial inoculants are already on the market, and their popularity has increased substantially as extensive and systematic research has enhanced their effectiveness and consistency. Recent surveys of both conventional and organic growers indicate an interest in using microbial inoculants, suggesting that the market potential of these products will increase in coming years (Berg, 2009).

Many of the PGPR inoculants available have demonstrated enhanced nutrient uptake in the plants. In part, this effect is related to increased root surface area and root morphology. As a result, there is an indirect enhancement of nutrient uptake by the plant (Bákonyi, et al.). Improved nutrient uptake has been reported in different crops like rice, cotton, pea, tomato, maize, and peanut (Biari, et al., 2008, Dey, et al., 2004, Egamberdiyevaa and Hoflich, 2004, Kirankumar, et al., 2008, Meunchang, et al., 2006). These positive results have been observed in plants grown in the greenhouse and the field. Studies performed by Dibut and Martinez (2003) showed increments in yield in seven crops to which biofertilizers were applied. The increases ranged from 26% for tomatoes to 45% for cotton, with an average of 34%, while N fertilizer was reduced by 30%. The average weight of fruit increased by 36% with inoculation treatments. PGPR were also tested in organic based system, and the biofertilizer application resulted in significant increase in N uptake by leaves of raspberry (Orhan, et al., 2006).

PGPR strains isolatedfrom the rhizosphere can play an essential role in helping plants establish and grow in nutrient deficient conditions (Egamberdiyevaa and Hoflich, 2004). In addition to the indirect effect that affects root development, PGPR have been involved in a rhizosphere ”priming effect”. This effect suggests that a higher level of decomposition of native soil organic matter around roots by PGPR also increases availability of N near the root zone. A study conducted on peas inoculated with

Pseudomonas fluorescens showed a significant increase in uptake of N from ^{15}N enriched organic residues (Brimecombe, et al., 1999), which suggests that the enhanced N uptake by PGPR can be also be applied to organic systems involving organic amendments.

5.2. Brief comments on mycorrhizae

Arbuscular mycorrhiza fungi (AMF) are soil microorganisms that present symbiotic associations with plants' roots of different species. They are known for their capacity to facilitate nutrient transfer between the soil and the plant, thereby increasing plant nutrient and water uptake. Among the nutrients, AMF have the best capacity to enhance P-uptake of their associated plants in P-limited soils, due to their extamatrical fungi mycelium. The mycelium increases the soil volume explored by the plant (Ramirez, et al., 2009). Increases in nutrient uptake by mycorhiza has been reported in other nutrients such as potassium, magnesium, calcium (Liu, , et al., 2002), N (Vaast and Zasoski, 1992), copper (Li, et al., 1991), zinc (Faber, , et al., 1990), and iron (Caris, et al., 1998) .

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Microbial-Based Inoculants Impact N_2O Emissions from an Incubated Soil Medium Containing Urea Fertilizers

Abstract

There is currently much interest in developing crop management practices that will decrease nitrous oxide (N_2O) emissions from agricultural soils. Many different approaches are being investigated, but to date, no studies have been published on how microbial inoculants affect N_2O emissions. The current study was conducted to test the hypothesis that microbial-based inoculants known to promote root growth and nutrient uptake can reduce N_2O emissions in the presence of nitrogen fertilizers under controlled conditions. Carbon dioxide (CO_2) and methane (CH_4)fluxes were also measured to evaluate microbial respiration and determine the aerobic and anaerobic conditions of the incubated soil. The microbial-based treatments investigated were SoilBuilder™ (SB), a metabolite extract of SoilBuilder™ (SBF), and a mixture of four strains of plant growth-promoting *Bacillus* spp. (BM). Experiments included two different nitrogen fertilizer treatments, urea and urea ammonium nitrate 32% nitrogen (UAN), and an unfertilized control. Emissions of N_2O and carbon dioxide (CO_2) were determined from soil incubations and analyzed with gas chromatography. After 29 days of incubation (DAI), cumulative N_2O emissions were reduced 80% by SB and 44% by SBF in soils fertilized with UAN. Treatment with *Bacillus* spp. significantly reduced N_2O production on days 1 and 2 of the incubation in soils fertilized with UAN. In the unfertilized treatment, cumulative emissions of N_2O with SBF were significantly reduced 92%. Microbial-based treatments did not reduce N_2O emissions associated with urea application. Microbial-based treatments increased CO_2 emissions from soils fertilized with UAN, suggesting a possible increase in microbial activity. Overall, the results demonstrated that microbial-based inoculants can reduce N_2O emissions associated with nitrogen fertilizer application, and this response varies with the type of microbial-based inoculant and fertilizer.

I. Introduction

Recent concerns for increased accumulations of greenhouse gases in the atmosphere have stimulated interest to develop better crop management practices to decrease nitrous oxide (N_2O) emissions from agricultural soils. Agriculture is the single largest source of anthropogenic N_2O emissions (Bouwman, et al., 2005). Currently, agricultural N_2O emissions are more than 2 times that of pre-1940 management practices and about 6 times more than native vegetation (Del Grosso, et al., 2005). Nitrogen (N) fertilization is considered the major source of agricultural N_2O emissions, contributing 60 to 80% of the total emissions on a global scale (Dalal, et al., 2003, FAO, 2008). However, to meet growing food demands N fertilization is needed to optimize crop yield. Thus, considerable effort is being spent extensively studying fertilization practices to reduce N_2O emission.

Estimations of N_2O emissions from nitrogen fertilizers applied to agricultural crops vary widely, as N_2O fluxes depend on many factors such as type of nitrogen fertilizer and amount of nitrogen applied (Eichner, 1989). For instance, losses of N_2O are greater with ammonium nitrate than with urea (Harrison and Webb, 2001). Also, N_2O emission rates are 0.04% for nitrate, 0.15-0.19% for ammonium and urea, and 5% for anhydrous NH_3 (Breitenbeck, et al., 1980, Slemr and Seiler, 1984). However, the concentrations of ammonium (NH_4) and nitrate (NO_3) in soil have a greater effect on N_2O emissions than the specific fertilizer type applied (Harrison and Webb, 2001).

Microbial interactions in the soil are a very important aspect of N_2O emission from agricultural soils. Native soil microorganisms are responsible for the degradation and conversion of different forms of nitrogen in soil. The most important chemical reactions that take place in the nitrogen cycle are mineralization, immobilization, nitrification, denitrification, fixation, and volatilization. These chemical reactions are largely affected by environmental conditions such as temperature and soil moisture. Because environmental conditions are constantly changing, the interactions between all the chemical reactions are very dynamic. Harrison and Webb (2001) suggested that denitrification is the main process responsible

for N_2O emissions under anaerobic soil conditions while nitrification accounts for emissions under aerobic soil conditions.

Due to the great importance of the soil microbial community in N-cycling in the soil, alterations in community composition and abundance can change the rate of the nitrogen cycle processes(Cavigelli and Robertson, 2000). Hence, manipulating native soil microbial communities by chemical treatments or by inoculation with selected microorganisms can potentially alter N-cycling in soil. For example, adding nitrification inhibitors is a widely used method to reduce the rate of nitrification by inhibiting autotrophic ammonia oxidizing bacteria (Singh and Verma, 2007).

Over the past few decades, there has been increased interest in the use of beneficial microbial inoculations to improve plant and soil functions. Several microorganisms like plant growth-promoting rhizobacteria (PGPR) have been widely studied (Figueiredo, et al., 2010). PGPR stimulate plant growth through either a ”biofertilizing” effect or a biocontrol effect. There is currently much interest in PGPR and other microbial-based inoculants specifically as alternatives to or supplements with fertilizers to improve uptake of nutrients (Adesemoye, et al., 2009, Adesemoye, et al., 2010, Canbolat, et al., 2006, Idriss, et al., 2002). Among the PGPR microorganisms, *Bacillus* spp. are widely used mainly because they can survive as spores andcan potentially alter soil microbial composition.*Bacillus* spp. have a wide metabolic capability that allows them to play important roles in soil ecosystem functions and processes. Due to their heterotrophic nature, *Bacillus* spp. play an important role in the soil carbon cycle, soil nitrogen cycle, soil sulfur cycle, and transformation of other soil nutrients (Mandic-Mulec and Prosser, 2011). Furthermore, they work as biocontrol agents due to their wide range of antiviral, antibacterial, and antifungal compounds, which can control pathogens and have an effect on other soil microorganisms (Chaabouni, et al., 2012). Antibiotics are important metabolites that are produced by *Bacillus* spp. They not only can control pathogens but also confer a competitve advantage over other soil microorganisms (Stein, 2005).

Microbial-based inoculants are already on the market and, in recent years, the popularity of microbial-based inoculants has increased substantially due to extensive and systematic research to enhance their effectiveness and consistency. SoilBuilder™, manufactured by Advanced Microbial Solutions (Pilot Point, TX, USA), is an example of a microbial-based inoculant that is widely marketed.

Treatment of soils and plants with SoilBuilder™ has been shown to increase root growth and nutrient uptake (Yildirim, et al., 2006). In addition, a version of SoilBuilder™ (AgBlend) induced suppressiveness to root-knot nematodes and increased populations of aerobic spore-forming bacteria in the rhizosphere (Burkett-Cadena, et al., 2008). Given this demonstrated increase in bacterial populations, we were interested in determining if SoilBuilder™ can affect bacterial functions related to soil nitrogen transformations. Hence, SoilBuilder™ was selected as a model microbial-based inoculant in the current study and was compared to a mixture of *Bacillus* spp. PGPR as described below. In addition we were interested in testing the effects that the portion of SoilBuilder™ that contains metabolites may have directly or indirectly on soil nitrogen dynamics which could lead to changes in N_2O emissions from soils.

Although the use of microbial-based inoculants is increasing, currently there is a lack of information about how these products affect N_2O emissions from soils when nitrogen fertilizers are present. Thus, the objective of this article was to test the hypothesis that microbial-based inoculants, known to improve nutrient uptake, can reduce emissions of N_2O in the presence of nitrogen fertilizers (UAN and urea) under controlled conditions. This study is one of the first to evaluate the use of microbial-based inoculants (*Bacillus* PGPR mix, SoilBuilder™, and SoilBuilder™ filtered) for the purpose of reducing N_2O emissions from soil with common agricultural nitrogen fertilizers. Carbon dioxide and CH_4 were also evaluated in this study to determine the microbial-based inoculants impact on microbial respiration (CO_2) and if N_2O production was mainly an effect of aerobic and anaerobic conditions occurring under laboratory incubations.

II. Materials and methods

Soil Characterization

Initial soil analysis was performed by Auburn University Soil Testing Laboratory as described by Hue and Evans (1986). Briefly, total carbon and nitrogen was analyzed using an Elementarvario Macro C-N analyzer (Elementar Americas, Inc. Mt. Laurel, NJ, USA). Soil pH was determined on 1:1 soil/water suspensions with a glass electrode meter. Concentrations of P, K, Mg, and Ca were determined using Melich 1 (double acid extracting solution) (Olsen and Sommers, 1982) and measured using an ICAP 9000 (Thermo Jarrell Ash, Franklin, MA, USA). The Cation Exchange Capacity (CEC) was determined by base summation (Ca, Mg, K, and Na) according to procedures of Hue and Evans (1986).

Soil Microcosms

A soil:sand mixture was used as the soil medium for this study. Sand was mixed with the soil in order to improve water infiltration and minimize anaerobic conditions during the study. Briefly, a sandy loam soil with a texture of 72.8% sand, 10.4% clay, and 16.8% silt was mixed 3:1 (soil:sand; v:v) with white brick/Mason sand (particle size: 1/8 mm -1/4 mm). The mixture resulted in a soil medium with the texture of a loamy sand (78.8 % sand, 4.4% clay, and 16.8% silt). The soil:sand mixture had a pH of 6.14, CEC of 1.13 cmol kg^{-1}, total nitrogen concentration of 0.7 g kg^{-1}, organic matter concentration of 17 g kg^{-1}, total C concentration of 2.6 g kg^{-1}, NO_3 concentration of 10.53 μg g^{-1}, NH_4 concentration of 0.73 μg g^{-1}, Mg concentration of 236 μg g^{-1}, Ca concentration of 305 μg g^{-1}, P concentration of 4 μg g^{-1} , and K concentration of 51 μg g^{-1}.

Soils were incubated for flux measurements in 2L glass jars containing 400 g of the dry soil:sand mixture with a soil bulk density of 1.15 g cm^{-3}. The soil:sand mixture was then adjusted to 20% (gravimetric water content) moisture with the addition of the treatments. Treatments were organized in a complete randomized design (CRD) with a 4×3 factorial arrangement with three microbial-based

treatments and a water control (no microbial-based treatment) and two nitrogen fertilizer sources and an unfertilized control, each replicated four times. The nitrogen fertilizer treatments included (i) urea ammonium nitrate (UAN) -32% and (ii) urea.

Nitrogen fertilization was calculated based on 168 kg ha^{-1}. The amount of nitrogen applied was calculated based on one hectare furrow slice (topsoil 15 cm) which is equal to 1980000 kg of soil. Based on this calculation, each jar (400 g dry soil) received 0.03 g of nitrogen in the fertilizer treatments. The specific amount of fertilizer added was 0.0937 mL of UAN solution and 0.065 g of urea . The experiment was designed to provide the same amount of nitrogen regardless of nitrogen fertilizer source. Therefore, the quantity of nitrogen fertilizer added in each treatment was adjusted for each fertilizer type so that all treatments received the same amount of nitrogen.

Microbial Source Preparation

SoilBuilder™ is a commercially available microbial soil amendment manufactured by Advanced Microbial Solutions (AMS), Pilot Point, TX. SoilBuilder™ is prepared from a bioreactor system consisting of a continuously maintained microbial community (patent pending). The final product contains bacteria and bacterial metabolites derived from the bioreactor. Based on plate counts using tryptic-soy agar (TSA) (24 h at 25 °C incubation), the most commonly occurring bacteria within the final stabilized product are *Acidovoraxfacilis*; *Bacillus licheniformis*; *Bacillus subtilis*; *Bacillus oleronius*; *Bacillus marinus*; *Bacillus megaterium*; and *Rhodococcusrhodochrous* , each at $1x10^3$cfu/cc.

SoilBuilder™ filtered (SBF) consisted of SoilBuilder™ product without microbial cells and was prepared by filtering SB through a 0.45 µm filter and then through a 0.22 µm filter. SBF contains microbial metabolites derived from the bioreactor production system that in addition to other components includes organic acids, peptides, and enzymes.

The PGPR *Bacillus* mixture (referred to as BM) included four *Bacillus* strains: *Bacillus safensis*T4 (previously called *B. pumilus* T4), *Bacillus pumilus* INR7, *Bacillus subtilis* subsp. *subtilis*

IN937a (previously called *B. amyloliquefaciens*IN937a), and *Lysinibacillusxylanilyticus*SE56 (previously called *Bacillussphaericus* SE56). These strains were obtained from culture collections at the Department of Entomology and Plant Pathology, Auburn University (Auburn, AL, USA). These strains have been shown to have important plant growth-promoting effect (Enebak, et al., 1998, Jetiyanon, et al., 2003, Kokalis-Burelle, et al., 2003, Kokalis-Burelle, et al., 2002).

Microbial-based treatments were applied at a rate of 25 mL per jar. For the BM treatment, the bacterial mix was prepared by mixing each strain's spore suspension, which was previously quantified by plating the spore mix suspension on TSA and incubating for 48 h at 25 °C. The spore mix was then adjusted to a concentration of $l0^5$cfu mL^{-1}. The final concentration in each jar was of 6.2 l x 10^3cfu·g of dry soil. The SoilBuilder™ product (SB) solution was prepared according to the label instructions by mixing 16 mL of SoilBuilder™ in 1.0 L of distilled water immediately before setting up the experiment. SoilBuilder™ product contained 10^6cfu mL^{-1}, so the final concentration in each jar was 10^3 cfu·g of dry soil. The treatment of SoilBuilder™ filtered (SBF) was prepared the same way as SB but before applying the 25 mL to the incubated sample, the solution was filtered. Sterility of the filtrate was confirmed by plating onto TSA (48 h at 25 °C incubation) and observing no bacterial growth. Non-filtered SoilBuilder™ population concentrations were confirmed also by plate count on TSA after incubation for 48 h at 25 °C.

Incubation Methods

The fertilizer source corresponding to each nitrogen fertilizer treatment was added, followed by the appropriate microbial-based treatment. Four jars without soil, maintained the same way as jars with soil:sand mix, served as a blank. A 118 mL plastic container containing 10 mL of water was placed in each jar to maintain humidity. The soil moisture content of the incubation samples was maintained by weighing the experimental units on each sampling day and adding deionized water as necessary. Shortly following treatment application, incubation jars were sealed hermetically (jars remained sealed between

sampling intervals) with retrofitted lids containing butyl rubber stoppers to allow gas (CO_2, CH_4, and N_2O) sampling. Incubation jars were incubated in the dark at 25 °C for 29 days. At the same time, a separate set of jars with the same treatments was incubated simultaneously for destructive sampling in order to measure soil NH_4-N and NO_3-N content. On each sampling day, shortly following gas analysis, lids were removed for 5 min in order to prevent anaerobic conditions from occurring and allow gas to equilibrate with the ambient atmosphere.

Gas flux and soil NH_4-N and NO_3-N sampling

Gas samples were collected at 1, 2, 4, 8, 10, 15, 22, and 29 days after treatment. Soil NH_4-N and NO_3-N concentration was determined at 1, 4, 8, 15, 22, and 29 days after treatment (samples were taken from a second set of jars and not from the jar for gas sampling). Samples for gas analysis, collected by inserting a 23 gauge needle attached to gastight 10 mL polypropylene syringe through the rubber septum embedded in the lids of incubated jars, were injected into evacuated 6 mL glass vials fitted with butyl rubber stoppers. Samples were stored at 25 °C until analysis which was done within two weeks of collection. Gas samples were analyzed using a gas chromatography (GC) (Shimadzu GC-14B, Japan) equipped with an electron capture detector for N_2O and a flame ionization detector for CH_4 and CO_2. The gas chromatograph's detectors were calibrated by comparison to a standard curve using standards obtained from Scott Specialty Gases (Plumsteadville, PA, USA). Soil flux was determined by dividing the gas concentration (CO_2, CH_4, or N_2O) by the number of days incubated between sampling. Gas concentrations observed on each sampling day were added together to determine the total flux for the 29 day incubation.

Soil NH_4-N and NO_3-N concentration was determined by extracting 5 g of wet soil with 50 mL of 2M KCl for determination of inorganic nitrogen content as described by Keeney and Nelson (1982).

Soil extracts were measured colorimetrically for NH_4 and NO_2 + NO_3 using a Bran+Luebbe Auto Analyzer 3 (Bran+Luebbe, Norderstedt, Germany).

Statistical analysis

Analysis of variance (ANOVA), using a general linear model (GLM), was used to analyze each response variable for fertilizer type. Pearson correlations were also used to identify relations between variables (CO_2, N_2O, and CH_4). All statistical analyses were performed using SAS software version 9.2 (SAS Institute, 2010) and a significance level of α=0.05 set *a priori*. The least significant difference (LSD) test was used to identify significant differences between treatments (SB, SBF, BM, and control)

III. Results and discussion

Carbon dioxide and N_2O production rates differed significantly between fertilizer and microbial treatments (Table 1). In addition, the interaction of fertilizer treatment x microbial inoculant was significant (Table 1). These results indicate that emissions of N_2O and CO_2 depended on the interaction of both microbial and fertilizer treatments. This interaction was especially significant during the first eight days of the incubation.

Nitrous Oxide Emissions

Urea and UAN fertilized soils released 10 times more total N_2O (total N_2O after 29 DAI) than the unfertilized treatment (Table 2). These observations confirm reports that as more nitrogen cycles through the soil system, a greater quantity of nitrogen is converted into N_2O gas (Smith, et al., 1997). Previous reports from field studies have indicated that fertilizer-derived N_2O emissions from plots treated with nitrifiable forms of nitrogen fertilizer (ammonia or ammonium) are greater than those from plots receiving an equivalent application of nitrogen as NO_3-N (Breitenbeck and Bremner, 1986). In this case,

UAN had more NH_4-N (7.75 %) at the beginning of the experiment compared to urea (0% NH_4-N). When comparing the control treatment (no microbial-based treatment) of UAN and urea (Table 2), total N_2O emissions from UAN were 2 times more than the urea treatment.

Table 1. Analysis of variance for the effects of fertilizer and microbial-based treatments on CO_2, N_2O and CH_4 production.

Variable (µg trace gas kg soil$^{-1‡}$d^{-1})	Factors†	Days of incubation							
		1	2	4	8	10	15	22	29
	Fertilizer (F)	<0.0001	<0.0001	<0.0001	0.0006	0.6765	0.077	0.3013	0.0582
CO_2	Microorganism (M)	<0.0001	<0.0001	<0.0001	<0.0001	0.6289	0.0197	0.0034	0.0012
	M X F	0.0643	<0.0001	0.0120	0.0002	0.2450	0.0617	0.1698	0.3262
	Fertilizer (F)	0.0001	<0.0001	<0.0001	0.0003	0.1084	0.0051	0.0003	0.0685
N_2O	Microorganism (M)	0.0274	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
	M X F	0.2901	<0.0001	<0.0001	<0.0001	0.0041	0.0026	<0.0001	0.0202
	Fertilizer (F)	0.2497	0.5541	0.3897	0.2592	0.7267	0.2870	0.5172	0.6690
CH_4	Microorganism (M)	<0.0001	0.0073	<0.0001	<0.0001	0.4617	<0.0001	0.7200	<0.0001
	M X F	0.0007	0.1784	0.0188	0.0056	0.6101	0.0117	0.4638	0.9120

Analysis of variance P>F LSD (0.05).
† Fertilizer factor include the treatments UAN (urea ammonium nitrate), urea, and unfertilized. Microorganism factor includes SB (SoilBuilder™), SBF (SoilBuilder™ filtered), BM (*Bacillus* PGPR mix), and Control (no product applied).
‡Trace gases were calculated based on kg of dry soil.

Nitrous oxide emissions per sampling day peaked for all microbial-based treatments (SB, SBF, and BM) that received UAN and ureaon the 8th day of the incubation (DAI) (Fig. 2 and 3). High N_2O emissions measured on the first day after applying nitrogen fertilizer were also reported by Pathak et al. (2006) who suggested that higher emissions were due to N_2O formation during nitrification of NH_4 produced by hydrolysis of the applied urea. A peak was also observed in all treatments following the addition of nitrogen as urea followed by a decline(Bremner and Blackmer, 1978, Fujinuma, et al., 2011, Hou, et al., 2000). This peak was not observed in the unfertilized treatment (Fig.1) suggesting that nitrogen fertilizer was responsible for the N_2O peak.

Nitrous oxide production rates between microbial-based treatments varied among different days of the incubation (Fig. 1-3). In the unfertilized treatment (Fig.1), N_2O production rates were significantly lower for all three microbial-based treatments (SB, SBF, and BM), during the first eight DAI. After this time, N_2O production from the SB and BM treatments was significantly higher than the control (Fig 1), while with SBF, emissions stayed lower than the control throughout the experiment. This difference in emissions following treatments with SB or BM compared to SBF could be explained by the fact that both SB and BM contain living microorganisms, while SBF only has microbial metabolites. Accordingly, it is possible that the microorganisms in SB and BM did not survive past day 8 due to the low concentration of nitrogen in the soil. In support of this interpretation, West et al. (1985) reported that one of the main factors affecting survival of *Bacillus* species in soil is nutrient availability. In contrast with the SBF treatment, the total N_2O emission (Table 2) decreased 13 times compared to the control. It is possible that among the metabolites in SBF are phenolic compounds which are known to inhibit soil nitrifying bacteria communities (Bending and Lincoln, 2000). In support of this explanation, *Bacillus* spp. have been reported to produce phenolic compounds (Chaabouni, et al., 2012).

Table 2. Total N_2O and CO_2 production after 29 DAI.

Gas	Microbial Treatments	Fertilizer treatments†		
		urea	**UAN§**	**unfertilized**
N_2O (μg N kg soil^{-1}‡)	SB	1322.2 ab	376.7 c	194.9 a
	SBF	909.2 bc	1029.1 bc	14.1 b
	BM	1691.8 a	1628.8 ab	155.2 a
	Control	808.7 c	1639.2 a	181.8 a
CO_2 (μg C kg soil^{-1}‡)	SB	42319.6 b	35301.2 b	35786.5 ab
	SBF	57156.8 a	56785.6 a	45071.7 a
	BM	57814.3 ab	57011.6 a	40071.4 ab
	Control	52059.1 ab	48707.4 a	31393.8 b

†Means within a column followed by the same letter are not significantly different at the 0.05 level using LSD values.SB (SoilBuilder™), SBF (SoilBuilder™ filtered), BM (*Bacillus* PGPR mix), and Control (no product applied).
Trace gases were calculated based on kg of dry soil. Nitrogen (N) and Carbon (C).
§ Urea ammonium nitrate.

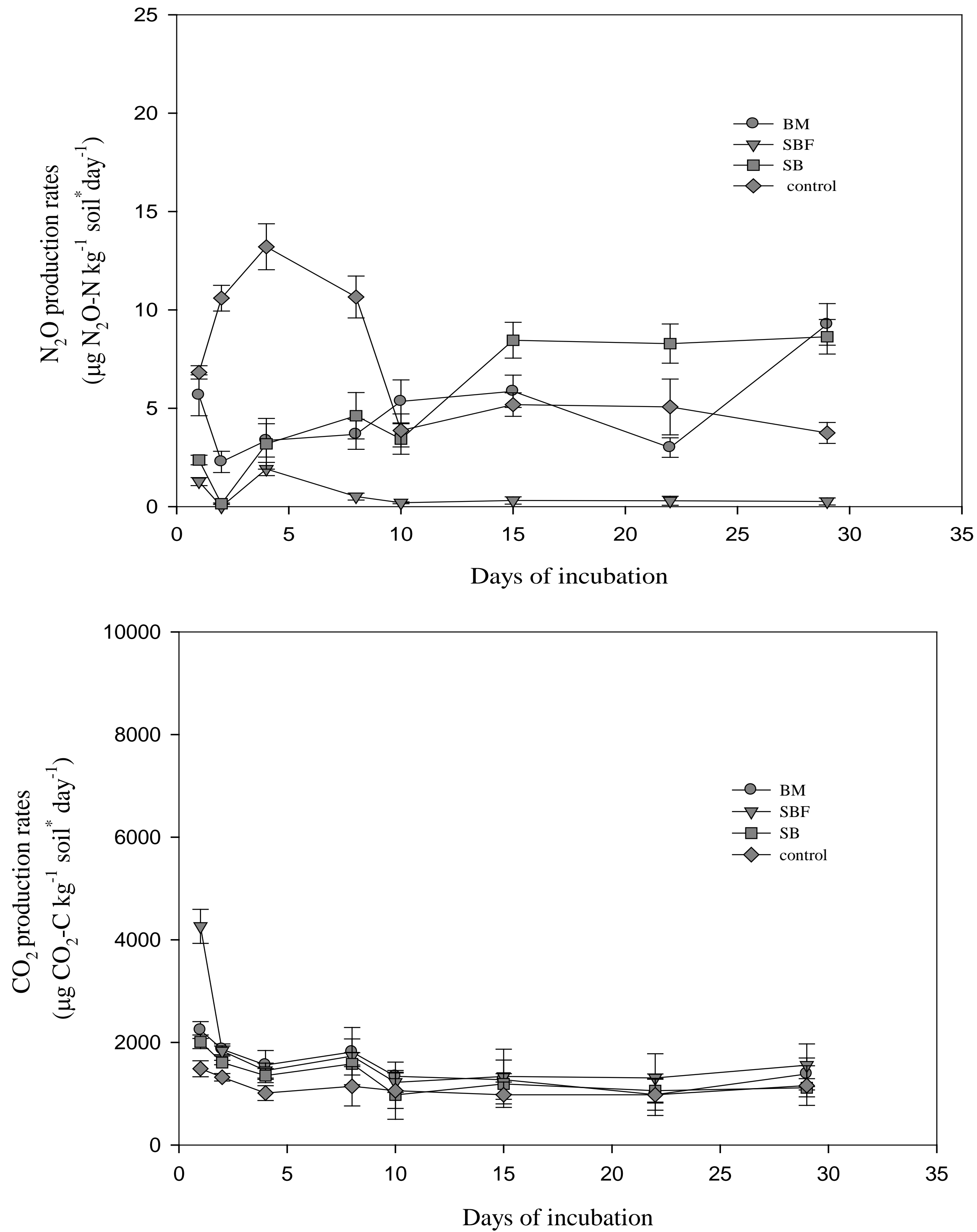


Figure. 1. Temporal changes in N_2O and CO_2 production rates with unfertilized treatment during 29 DAI (days of incubation). SB (SoilBuilder™), SBF (SoilBuilder™ filtered), BM (*Bacillus* PGPR mix), and Control (no product applied).

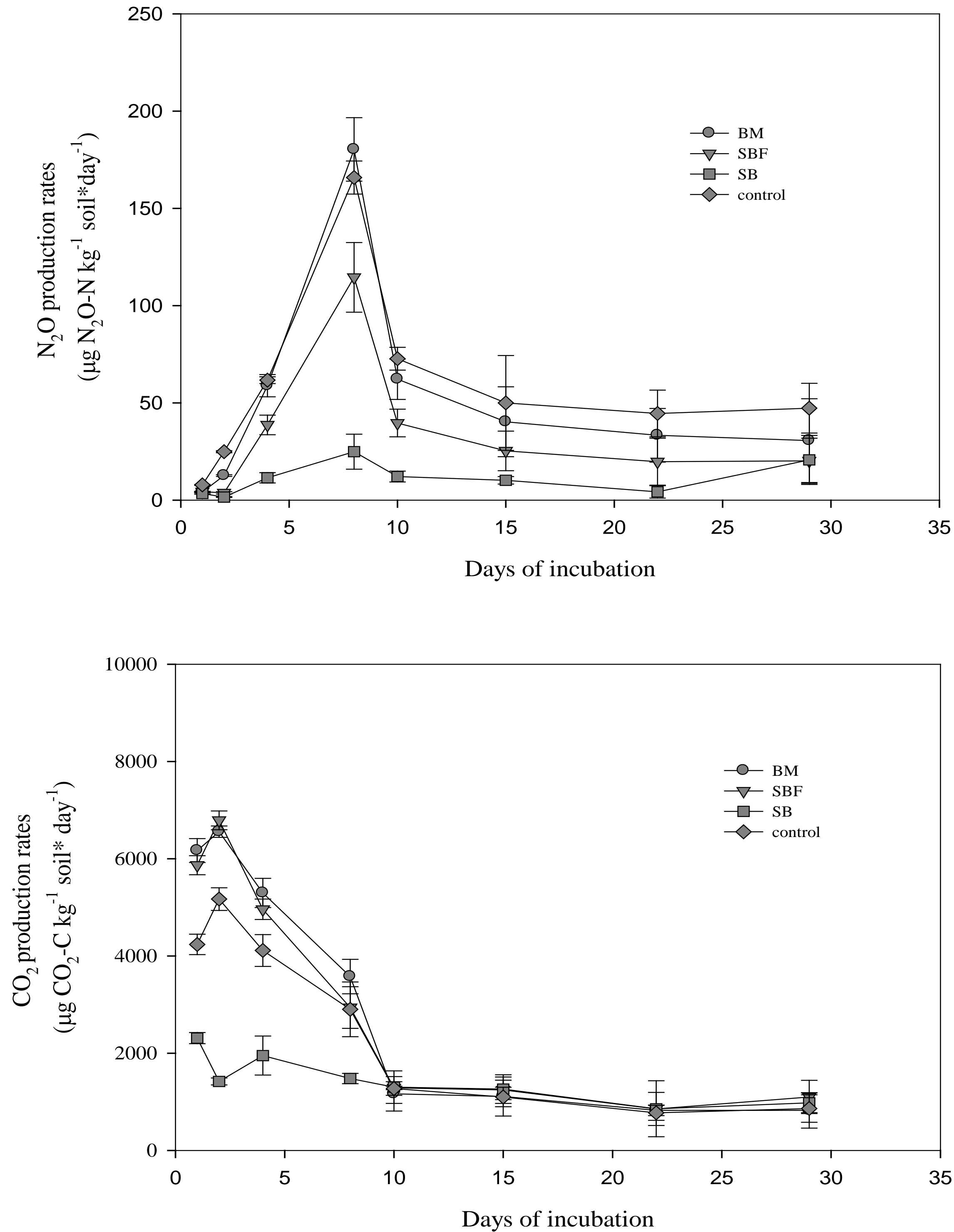
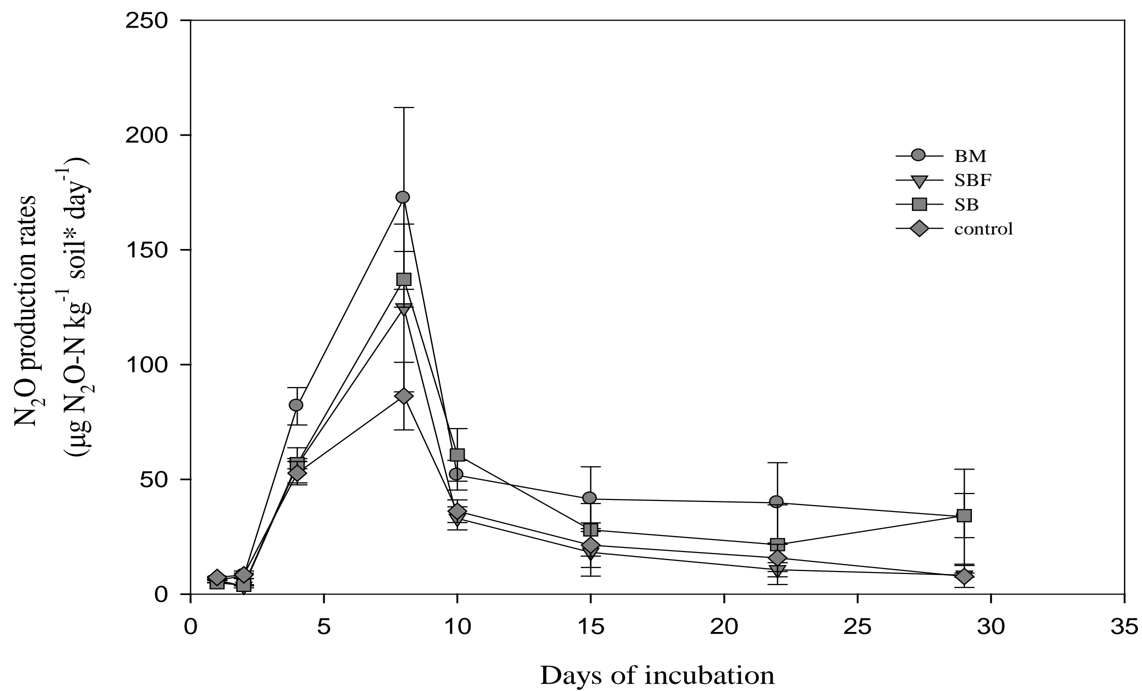


Figure. 2. Temporal changes in N_2O and CO_2 production rates with UAN treatment during 29 DAI (days of incubation). SB (SoilBuilder™), SBF (SoilBuilder™ filtered), BM (*Bacillus* PGPR mix), and Control (no produ applied).



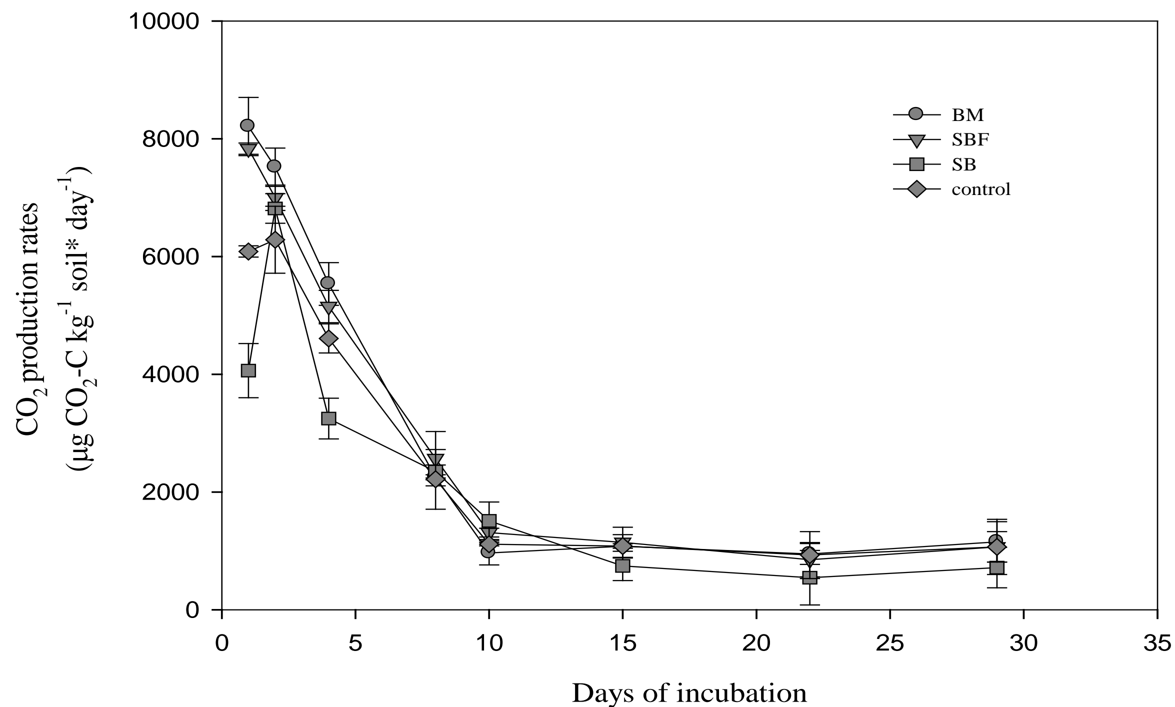


Figure. 3. Temporal changes in N_2O and CO_2 production rates with urea fertilizer treatment during 29 DAI (days of incubation). SB (SoilBuilder™), SBF (SoilBuilder™ filtered), BM (*Bacillus* PGPR mix), and Control (no product applied).

In UAN fertilized soils (Fig. 2), the BM treatment resulted in lower rates of N_2O during the first two DAI compared to the control (no microbial-based treatment). The SBF treatment also showed a similar pattern, but in this case, differences from the control lasted until day 4. The SB treatment produced significantly lower N_2O compared to the control during the first 22 DAI. The SB and SBF treatments significantly reduced the total N_2O production compared to the control treatment (no microbial-based treatment) (Table 2). With these two treatments, N_2O production was almost five times lower than that observed with the control. SB and SBF treatments also have in common the presence of microbial metabolites which could be responsible for the N_2O reduction. As mentioned above, it is possible that among the metabolites in SBF are phenolic compounds, which are known to inhibit soil nitrifying bacterial communities (Bending and Lincoln, 2000). The SB treatment, which contains both the living microorganisms + microbial metabolites, resulted in the greatest N_2O reduction. The presence of microorganisms most likely increased immobilization of the fertilizer nitrogen (Zak, et al., 1990), which could potentially reduce nitrification, and increased microbial competition for nutrients, which has been attributed before to microorganisms such *Bacillus* spp. (Stein, 2005, West, et al., 1985). SB and SBF could also alter soil chemistry. Kumar et al. (1988) observed that an increase in soluble salts could inhibit microbial processes such as nitrification. Also, decreases in pH could alter the nitrification and denitrification process (Bolan, et al., 2004, Broos, et al., 2007).

For urea-treated fertilized soils (Fig. 3), there were no significant differences in N_2O production among the control and SB, SBF, or BM for the first 10 days. After day 10, the BM treatment increased N_2Oproduction compared to the control. The total N_2O production after 29 days (Table 2) showed that the highest N_2O emissions were recorded with BM and SB treatments, which were significantly higher than the control treatment. The trends observed with urea fertilizer were different from those observed with UAN, even though both fertilizers contain urea. The amount of urea in UAN is only 16.5% ; thus, the mechanisms involved in N_2O production appear to be different.

The treatment effects observed with UAN are likely related more to the presence of NO_3-N and NH_4-N than to the presence of urea. Chapman and Leibig(1952) and Engel et al. (2009) found that localized application of urea can release significant amounts of NH_3, which could be toxic to many microorganisms. Hence, because the experiment was performed in a closed environment, the toxic effect of ammonia could have affected the added microorganisms in the BM and SB treatments. These two treatments also increased significantly the total N_2O emissions (Table 2) compared to a control (no microbial based treatment). This increase could be explained by the fact that dead bacterial cells become carbon and nitrogen sources that could indirectly increase populations of other microorganisms such as nitrifiers. In contrast, with SBF, the treatment that contained only the metabolites, total N_2O emissions did not differ significantly from the control. There were no reductions in N_2O with SBF in urea-soils. This could be explained by the fact that, unlike UAN, urea does not have a significant initial amount of NH_4-N. Bending and Lincoln (2000) showed that the inhibition of nitrifiers by phenolic compounds was recorded when a NH_4-N based fertilizer was applied to the soil. Hence in order for SBF to have an inhibitory effect on the population of nitrifiers, a significant initial amount of NH_4-N is needed, which was not the case when urea was applied.

Carbon Dioxide Emissions

The total CO_2 production was less from the unfertilized treatment than from the nitrogen fertilizer treatments (Figs. 1, 2, and 3). This finding differs from observations made by Kowalenkoet al.(1978)who found a consistent lowering of microbial activity due to nitrogen fertilization. However, Barabaszet al.(2002) suggested that nitrogen fertilization would increase microbial activity due to the addition of nutrients. This observation would be consistent with our results. The soil used in our experiment was nutrient poor, so when nitrogen fertilizer was applied it appears to have enhanced microbial activity.

None of the microbial-based treatments containing microorganisms (SB and BM) differed significantly from the control for CO_2 production after 29 DAI (Table 2) in unfertilized soils. The

metabolites based treatment (SBF) was the only one to significantly increase total CO_2 production. The SBF is the same treatment that had lower total N_2O production, so in this case greater CO_2 production was not related to greater N_2O production.

The SB treatment significantly reduced total CO_2 production in UAN and urea fertilized soils (Table 2). The SB treatment contains microbes plus metabolites, so the activity of SB could be more related to a decrease of microbial activity in the soil. Furthermore, SB also produced the least N_2O with UAN. So in the presence of UAN fertilizer, the decrease in microbial activity (represented by CO_2 production) most likely resulted in decreased total N_2O emissions. These results differ from results found by Ullah and Moore (2011) who observed that N_2O fluxes from well-drained soils correlated negatively and significantly with CO_2 emission rates.

On the other hand, the SB treatment in the urea-fertilized soil was more related to higher total N_2O production (Table 2). Therefore, these results clearly demonstrate that the dynamics of N_2O and CO_2 production are highly affected by fertilizer type. For instance, a treatment that could potentially decrease microbial activity in the presence of one fertilizer could show an opposite effect with another fertilizer. As mentioned before, a possible explanation could be the toxic effect of ammonia released from urea on the added microorganisms. However, when UAN was present and the toxic effect of NH_3 was lower, CO_2 total production also decreased in SB treatment. Nannipieri et al.(2003) pointed out an important concept about the link between microbial activity and microbial diversity. Assessing microbial activity by CO_2 production in the soil does not take into account the microbial species effectively involved in the measured process. In this case CO_2 is not reflecting the effect that the added microbial treatments (SB and BM) had. The reduction on N_2O total emission observed with SB and BM when UAN is present could be more related to a competition with nitrifiers, leading to a change in microbial diversity, more than to an increase in total microbial activity.

Methane Emissions

Methane emissions were low in all incubations, presumably because anaerobic conditions within the jars were negligible during the incubation (data not shown). Methane is formed in soils by the microbial breakdown of organic compounds in strictly anaerobic conditions (Smith, et al., 2003, Yu, et al., 2001). There were no significant differences in CH_4 emission between microbial-based treatments, leading to the conclusion that microbial-based products used for this study will not affect CH_4 emissions under aerobic conditions. These results suggest that soils were well aerated through the course of the incubations, which suggests N_2O fluxes reflect nitrification not denitrification.

Nitrate and Ammonium Concentration in Soil

As expected, nitrate and NH_4-N concentrations in soil extracts from the unfertilized treatment (Table 3) were much less than concentrations observed with the nitrogen fertilized treatments (Tables 4 and 5). In the unfertilized treatment there was significantly less soil NO_3-Non day 29 with the SB microbial-based treatment compared to the SBF and BM treatments, but not the control. On the other hand, no differences in NH_4-N concentration in soil were observed after 29 DAI.

Microbial-based treatments increased soil NO_3-N levels. Soil NO_3-N concentrations among treatments with UAN (Table 4) were significantly different on the 8th and 15th DAI. The NO_3-N concentration observed from the SB treatment was significantly higher than the control treatment. The SB treatment also produced lower N_2O emissions than the control. Furthermore, the highest N_2O emissions were recorded on the 8th and 15th DAI. The reduction in N_2O production was apparently related to a higher concentration of NO_3-N in soil on the 8th and 15 DAI.

Table 3. NO_3-N and NH_4-N concentration in the soil over time in µg g^{-1} soil. Unfertilized treatment.

Parameter	Treatments	Days of incubation†					
		1	4	8	15	22	29
	SB	7.91 a	8.96 a	7.76 a	8.27 a	7.94 a	6.42 b
NO_3-N	SBF	7.65 a	8.01 a	8.37 a	8.01 a	7.86 a	8.71 a
(µg g soil$^{-1‡}$)	BM	7.69 a	8.63 a	8.89 a	8.75 a	8.87 a	8.61 a
	Control	9.02 a	8.72 a	8.23 a	8.11 a	7.92 a	7.44 ab
	SB	1.09 a	1.18 a	1.28 b	0.98 b	1.08 bc	0.63 a
NH_4-N	SBF	1.08 a	1.08 a	1.23 b	1.28 a	1.03 c	0.65 a
(µg g soil$^{-1‡}$)	BM	0.81 a	1.14 a	2.67 a	0.74 c	1.29 ab	0.66 a
	Control	0.91 a	1.11 a	2.57 a	0.81bc	1.41 a	0.71 a

†Means within a column followed by the same letter are not significantly different at the 0.05 level using LSD values. SB (SoilBuilder™), SBF (SoilBuilder™ filtered), BM (*Bacillus* PGPR mix), and Control (no product applied).
‡NO_3^- -N and NH_4^+ -N were calculated based on kg of dry soil.

Microbial-based treatments also increased soil NH_4-N concentration. These differences were significantly higher at the end of the incubation (day 22 and 29) for SB and BM treatments. Thus, higher NH_4-N concentrations could be associated with a reduction in the nitrification process that converts NH_4-N to NO_3-N form. Less nitrification is also associated with less production of N_2O (Eichner, 1989).

Table 4. NO_3-N and NH_4-N concentration in the soil over time in µg g^{-1} soil.UAN (urea ammonium nitrate) treatment.

Parameter	Treatments	Days of incubation†					
		1	4	8	15	22	29
	SB	41.69 a	45.14 a	57.21 a	110.69 a	96.52 a	99.55 a
NO_3-N	SBF	33.97 a	43.73 a	50.93 ab	71.41 b	93.34 a	104.61 a
(µg g soil$^{-1‡}$)	BM	43.16 a	44.21 a	56.45 a	89.91 b	102.66 a	105.42 a
	Control	29.82 b	42.01 a	41.66 b	75.76 b	84.61 a	86.24 a
	SB	50.11 ab	96.29 a	99.77 ab	110.78 a	59.13 a	42.62 a
NH_4-N	SBF	38.66 ab	94.89 a	98.54 ab	86.65 a	53.35 ab	29.58 ab
(µg g soil$^{-1‡}$)	BM	57.66 a	98.63 a	110.63 a	96.31 a	61.25 a	43.86 a
	Control	32.21 b	80.26 a	67.22 b	55.45 a	25.41 b	16.371 b

†Means within a column followed by the same letter are not significantly different at the 0.05 level using LSD values. SB (SoilBuilder™), SBF (SoilBuilder™ filtered), BM (*Bacillus* PGPR mix), and Control (no product applied).
‡Trace gases were calculated based on kg of dry soil.

In the urea treatment, there were no significant differences in the NO_3-N or NH_4-N concentrations between microbial-based treatments during the first 8 DAI (Table 5). This indicates that the microbial-based treatments did not delay the release of NH_4-N from urea.   On the last day of incubation, significantly higher soil NH_4-N concentrations were observed with BM compared to the control. Nevertheless this higher NH_4-N concentration with BM treatment was not associated with less N_2O production.

Some *Bacillus* spp. were previously reported to have denitrification capacity that could potentially increase N_2O emissions (Verbaendert and DeVos, 2011). *Bacillus* spp. are present in SB and BM treatments. However neither of the results showed a consistent pattern regarding increaseof N_2O that could allow us to consider denitrification as an important issue in the present study. Furthermore, the conditions presented in this experiment were not oxygen-depleted, condition which is important for denitrification. PGPR *Bacillus* mix only increased N_2O with urea fertilizer but not with UAN or no fertilizer. Hence the increased on N_2O emissions could potentially be more related to the presence of urea than to denitrification.

Table 5. NO_3-N and NH_4-N concentration in the soil over time in µg g^{-1}soil. Urea treatment.

Parameter	Treatments	Days of incubation†					
		1	4	8	15	22	29
NO_3-N (µg g $soil^{-1‡}$)	SB	10.16 a	13.94 a	20.83 a	56.47 a	64.99 a	48.85 a
	SBF	9.96 a	14.22 a	23.21 a	53.48 a	55.11 a	57.67 a
	BM	9.51 a	13.21 a	21.67 a	38.55 a	54.26 a	49.24 a
	Control	9.71 a	15.91 a	22.23 a	52.95 a	59.38 a	51.98 a
NH_4-N (µg g $soil^{-1‡}$)	SB	16.83 a	56.27 a	52.03 a	52.91 a	7.71 a	1.57 b
	SBF	17.72 a	56.03 a	40.31 a	22.24 b	8.47 a	2.01 ab
	BM	16.93 a	55.51a	49.08 a	31.72 ab	8.15 a	3.21 a
	Control	19.09 a	53.66 a	42.78 a	42.76 ab	8.41 a	1.13 b

† Means within a column followed by the same letter are not significantly different at the 0.05 level using LSD values. SB (SoilBuilder™), SBF (SoilBuilder™ filtered), BM (*Bacillus* PGPR mix), and Control (no product applied).
‡Trace gases were calculated based on kg of dry soil.

Correlation analysis

Carbon dioxide and N_2O emissions were correlated in soils fertilized with UAN (r=0.611, p=<0.0001). Nitrous oxide emissions in the urea treatment were also significantly, but not highly correlated with CO_2 (r=0.315, p=0.0003). The relationship between N_2O and CO_2 is well documented for cultivated soils (Burford and Bremner, 1975) and also for tropical soils (Garcia-Montiel, et al., 2004). However to explain why N_2O emissions could be affected by microbial inoculations, one must consider that CO_2 could only give us information about the total microbial activity, not the microbial diversity in the soil (Nannipieri, et al., 2003). No significant correlation was found between CH_4 and CO_2 or CH_4 and N_2O.

IV. Conclusions

The results reported here partially support our hypothesis that microbial-based inoculants can reduce N_2O emissions from soils. The potential reduction of N_2O was affected by the type of fertilizer applied. The use of UAN fertilizer resulted in greater significant reductions in N_2O emissions with SB, and SBF. In the unfertilized control, significant reductions with SBF were observed. However, there was no significant reduction in N_2O emission when urea was applied to soil. A possible explanation for this is the toxicity effect that NH_3 could have on microbes, which would explain why the microbial treatments do not have an effect when urea is present. The results also showed that the microbes and microbial metabolites have effects on nitrogen dynamics and N_2O emissions from soil. Given the lack of an anaerobic environment, some possible mechanisms that could explain the reduction of N_2Oemissions include (i) production or presence of nitrification inhibitors; (ii) inhibition of nitrifying microorganisms; (iii) competition of applied microbial treatments with the native microbial nitrifiers; and (iv) immobilization of nitrogen fertilizer by microbes. Emissions of CO_2 did not show a clear pattern that could explain variations of N_2O.

The results presented in this article represent an important starting point for elucidating the effect of microbial and microbial metabolite treatments on production of N_2O from agricultural soils. Microbial-based treatments demonstrated potential to decrease N_2O emissions from agriculture soils. Further research is needed in order to better understand the processes involved in the dynamics between microbial-based treatments, the nitrogen cycle, and nitrogen fertilizers.

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Application of Microbial-Based Inoculants to Evaluate the N_2O Production from a Loamy Sand Soil Under Two Different Ammonium Nitrate-Based Nitrogen Fertilizers

Abstract

Considerable effort is being made among the scientific community to identify mechanisms to reduce nitrous oxide (N_2O) emissions from agriculture soils. Thus, the objective of this study was to test the hypothesis that microbial-based inoculants known to promote root growth and nutrient uptake will reduce N_2O emissions in the presence of ammonium nitrate-based nitrogen fertilizers under controlled conditions. The microbial-based treatments evaluated were SoilBuilder (SB), a metabolite extract of SoilBuilder (SBF), and a mixture of four strains of plant growth-promoting *Bacillus* spp. Experiments included an unfertilized control and two different nitrogen fertilizers: ammonium nitrate (AN-32) and calcium ammonium nitrate (CAN-17). Measurements of N_2O and carbon dioxide (CO_2) were determined from soil incubations and analyzed with gas chromatography. After 29 days of incubation, cumulative N_2O emissions were reduced 81%, 67%, and 50% for the SBF, SB, and BM treatments, respectively,in soils fertilized with CAN-17. In the unfertilized treatment, cumulative N_2O emissions with SBF were significantly reduced 92%, and emissions at 2, 4, and 8 days of incubation were significantly reduced by SB and *Bacillus.* Emissions from the AN-32 treatment were generally lower than those from CAN-17. Microbial-based treatments increased N_2O emissions when associated with AN-32 application, with SBF having the greatest flux. No differences in total CO_2 emissions were observed among treatments when AN-32 was applied. Microbial-based treatments increased CO_2 emissions from soils fertilized with CAN-17 and from the unfertilized control, indicating a possible increase in microbial activity. Overall, the results demonstrated that microbial-based inoculants can impact (reduce or increase) N_2O emissions from soil. This response is highly dependent on the fertilizer type and microbial-based product applied.

I. Introduction

Inefficient use of nitrogen (N) in agricultural systems tends to enhance nitrous oxide (N_2O) emissions. It is estimated that N use efficiency (NiUE) of crops in most agricultural ecosystems is approximately 30 -50% (Delgado, 2002). As a result, more than 50% of US cropland has a high N balance, leaving these soils highly susceptible N_2O loss (Millar et al., 2010). Thus, management practices are needed to reduce N_2O emissions, while at the same time increasing NiUE in agricultural production systems.

Currently, there is great interest in the development and implementation of agricultural greenhouse gas (GHG) reduction offset protocols that can be included in cap and trade markets (Millar et al., 2010). Direct strategies or technologies for N_2O reduction are limited. Research has been conducted on methodologies to utilize nitrification inhibitors and/or slow release fertilizers (Mosier et al., 1998, Singh and Verma, 2007). However, none of these strategies have included the application of microorganisms, which could play an important role in N_2O reduction by interacting with the native N-cycle microbes. Given the importance of the soil microbial community on N transformations, alterations in their community composition and abundance can change the rate of N cycle processes (Cavigelli and Robertson, 2000). Hence, manipulating native soil microbial communities by application of selected chemicals or inoculation via inoculation with specific microorganisms can potentially alter N_2O production from soil.

Applications of beneficial microbes such as plant growth-promoting rhizobacteria (PGPR) have increased in the past decades (Figueiredo et al., 2010). PGPR stimulate plant growth through either a “biofertilizing” effect or a biocontrol effect. There is currently much interest in PGPR and other microbial-based inoculants, specifically as alternatives to or supplements with fertilizers, to improve uptake of nutrients (Adesemoye et al., 2009, Adesemoye et al., 2010, Canbolat et al., 2006, Idriss et al., 2002). Among the PGPR microorganisms, *Bacillus* spp. are widely used. Due to their capacity to

survive in constantly changing environments like soil, *Bacillus* spp. have the potential to alter soil microbial composition. Characteristics like the multilayered cell wall, stress resistant endospore formation, and secretion of peptide antibiotics, peptide signal molecules, and extracellular enzymes contribute to their survivial (Kumar et al., 2011). Moreover, due to their heterotrophic nature, *Bacillus* spp. play important roles in the soil C cycle, soil N cycle, soil S cycle, and transformation of other soil nutrients (Mandic-Mulec and Prosser, 2011).

Microbial-based inoculants are already on the market, and, in recent years, the popularity of microbial-based inoculants has increased substantially (Babalola, 2010, Kennedy et al., 2004). SoilBuilder, manufactured by Agricen Sciences (Pilot Point, TX, USA), is an example of a microbial-based inoculant that is widely marketed. Treatment of soils and plants with SoilBuilder has been shown to increase root growth and nutrient uptake (Yildirim et al., 2006). In addition, a version of SoilBuilder (AgBlend) induced suppressiveness to root-knot nematodes and increased populations of aerobic spore-forming bacteria in the rhizosphere (Burkett-Cadena et al., 2008). Given this demonstrated increase in bacterial populations, we were interested in determining if SoilBuilder could affect bacterial functions related to soil N transformations. Hence, SoilBuilder was selected as a model microbial-based inoculant in the current study and was compared to a mixture of *Bacillus* spp. PGPR as described below.

Even though the use of microbial-based inoculants is increasing, currently there is a lack of information about how these inoculants affect N_2O, CO_2, and CH_4 emissions from soils when specific N fertilizers are present. In a previous study (Calvo et al., 2013), we observed that the use of the microbial inoculants PGPR, SoilBuilder, and the metabolite extract of SoilBuilder could reduce nitrous oxide emissions in the presence of urea-based fertilizers. Given that this was the first known study to evaluate the use of these microbial inoculants to reduce greenhouse gas emissions from soil, more research is needed to determine their effectiveness when used with other fertilizer sources. Thus, the objective of this study was to test the hypothesis that microbial-based inoculants, known to improve nutrient uptake, could reduce emissions of N_2O and indirectly alter the emissions of CO_2 and CH_4 gases in the presence of

ammonium nitrate-based fertilizers (calcium ammonium nitrate and ammonium nitrate) under laboratory incubations.

II. Materials and methods

Soil Characterization and Treatments

A soil:sand mixture was used as the soil medium for this incubation study. Briefly, a sandy loam soil with a texture of 72.8% sand, 10.4% clay, and 16.8% silt was mixed 3:1 (soil:sand; v:v) with white brick/Mason sand (particle size: 1/8 mm -1/4 mm). The mixture resulted in a soil medium with the texture of a loamy sand (85.2 % sand, 4.8% clay, and 10% silt).The soil medium had a pH of 6.14, a cation exchange capacity (CEC) of 1.13 cmol kg^{-1}, total N concentration of 0.7 g kg^{-1}, organic matter concentration of 4.5 g kg^{-1}, total C concentration of 2.6 g kg^{-1}, NO_3 concentration of 10.53 µg g^{-1}, NH_4 concentration of 0.73 µg g^{-1}, Mg concentration of 236 µg g^{-1}, Ca concentration of 305 µg g^{-1}, P concentration of 4 µg g^{-1}, and K concentration of 51 µg g^{-1}.

Background analysis for the soil medium was performed by Auburn University Soil Testing Laboratory as described by Hue and Evans (1986). Briefly, total C and N was analyzed using an Elementarvario Macro C-N analyzer (Elementar Americas, Inc. Mt. Laurel, NJ). Soil pH was determined on 1:1 soil/water suspensions with a glass electrode meter. Concentrations of soil P, K, Mg, and Ca were determined using a Melich 1 double acid extracting solution (Olsen and Sommers, 1982) and measured using an ICAP 9000 (Thermo Jarrell Ash, Franklin, MA). The CEC was determined by base summation (Ca, Mg, K, and Na) according to procedures of Hue and Evans (1986).

Treatments were organized in a complete randomized design with a 4×3 factorial arrangement with four microbial-based treatments and three N fertilizer sources, each replicated four times. The four microbial treatments consisted of non-microbial control, SoilBuilder™, SoilBuilder™ filtered, and a

PGPR and *Bacillus* spp.mixture. The N fertilizer treatments included: (i) unfertilized control, (ii) ammonium nitrate (AN-32) (99 % pure Sigma), and (iii) calcium ammonium nitrate -17 (CAN-17).

Microbial and Nitrogen Treatment Preparations

SoilBuilder™ is a commercially available microbial soil amendment manufactured by Agricen Sciences (Pilot Point, TX, USA). SoilBuilder™ is prepared from a bioreactor system consisting of a continuously maintained microbial community (patent pending). The final product contains bacteria and bacterial by-products derived from the bioreactor. Based on plate counts using tryptic-soy agar (TSA) (48h at 25˚C incubation), the most commonly occurring bacteria within the final stabilized product are *Acidovoraxfacilis* $1x10^3$cfu/cc, *Bacillus licheniformis* $1x10^3$ cfu/cc, *Bacillus subtilis* $1x10^3$cfu/cc, *Bacillus oleronius*$1x10^3$cfu/cc, *Bacillus marinus*$1x10^3$cfu/cc, *Bacillus megaterium* $1x10^3$cfu/cc, and *Rhodococcusrhodochrous* $1x10^3$ cfu/cc.

SoilBuilder™ filtered (SBF) consisted of SoilBuilder™ product without microbial cells and was prepared by filtering SB through a 0.45µm sterile filter and then through a 0.22µm filter. The SBF contains microbial metabolites derived from the bioreactor production system that among other components include organic acids, peptides, and enzymes.

The PGPR *Bacillus* mixture (referred to as BM) included four *Bacillus* spp. strains: *Bacillus safensis* T4 (previously called *B. pumilus* T4), *Bacillus pumilus* INR7, *Bacillus subtilis*subsp. *subtilis*IN937a (previously called *B. amyloliquefaciens*IN937a), and *Lysinibacillus xylanilyticus* SE56 (previously called *Bacillus sphaericus* SE56). These strains were obtained from culture collections at the Department of Entomology and Plant Pathology, Auburn University (Auburn, AL, USA). These strains have been shown to have important plant growth promoting effects (Enebak et al., 1998, Jetiyanon et al., 2003, Kokalis-Burelle et al., 2003, Kokalis-Burelle et al., 2002).

Microbial-based treatments were applied at a rate of 25 mL per jar. For the BM treatment, the bacterial mix was prepared by mixing each strain's spore suspension, which was previously quantified by

plating the spore mix suspension on TSA and incubating for 48h at 25˚C. The spore mix was then adjusted to a spore concentration of $l0^5$cfu mL^{-1}. The final concentration in each jar was 6.2 l x 10 ^{3}cfu· g of dry soil $^{-1}$. The SoilBuilder™ product (SB) solution was prepared according to the label instructions by mixing 16 mL of SoilBuilder™ in 1.0 L of water immediately before setting up the experiment. SoilBuilder™ product contained 10^6cfu mL^{-1}, so the final concentration in each jar was 10^3 cfu· g of dry soil $^{-1}$. The treatment of SoilBuilder™ filtered (SBF) was prepared the same way as SB, but before applying the 25 mL to the incubated sample, the solution was filtered. Sterility of the filtrate was confirmed by plating onto tryptic soy agar (24h at 25˚C incubation) and observing no bacterial growth.

Nitrogen fertilizer was applied at a rate of 168 kg ha^{-1}. The N applied was calculated based on one hectare furrow slice (topsoil 15 cm), which is equal to 1,980,000 kg of soil. Based on this, 0.03 g of N was added to 400 g of incubated soil (dry wt. basis). The specific amount of each fertilizer added was CAN-17 solution =0.1875 ml and AN-32=0.085 g. The experiment was designed to provide the same amount of nitrogen regardless of nitrogen fertilizer source. To facilitate uniform addition, the N fertilizers were dissolved with deionized water and added to samples using a micropipette.

Incubation Methods

Four hundred grams of soil medium compacted to a bulk density of 1.15 g cm^{-3} were incubated for flux measurements in 2L glass jars. The soil medium was adjusted to 20% moisture (gravimetric water content) with the addition of treatments. First, the fertilizer source corresponding to each N treatment was added, followed by the appropriate microbial-based treatment. Four jars without soil were maintained in the same way as jars with the soil medium to serve as blanks. A 118-mL plastic container containing 10 mL of water was placed in each jar to maintain humidity. Soil moisture content of the incubation samples was maintained by weighing experimental units on each sampling day and adding deionized water as necessary. Shortly following treatment application, incubation jars were sealed hermetically (jars remained sealed between sampling intervals) with retrofitted lids containing butyl rubber stoppers to

allow gas (CO_2, CH_4, and N_2O) sampling and incubated in the dark at 25˚C for 29 days. At the same time, a separate set of jars with the same treatments was incubated for destructive sampling in order to measure soil NH_4-N and NO_3-N content. On each sampling day, shortly following gas analysis, lids were removed for 5 min in order to prevent anaerobic conditions from occurring and allow gas to equilibrate with the ambient atmosphere.

Gas flux and soil NH_4-N and NO_3-N sampling

Gas samples were collected at 1, 2, 4, 8, 10, 15, 22, and 29 days after treatment application. Soil NH_4-N and NO_3-N concentration was determined at 1, 4, 8, 15, 22, and 29 days after treatment application (samples were taken from a second set of jars and not from the jar for gas sampling). Samples for gas analysis, collected by inserting a 23 gauge needle attached to gastight 10 mL polypropylene syringe through the rubber septum embedded in the lids of incubated jar, were injected into evacuated 6 mL glass vials fitted with butyl rubber stoppers. Samples were stored at 25˚C until analysis. Gas samples were analyzed using a gas chromatograph (GC) (Shimadzu GC-14B, Japan) equipped with an electron capture detector for N_2O and a flame ionization detector for CH_4 and CO_2. The gas chromatograph's detectors were calibrated by comparison to a standard curve using standards obtained from Scott Specialty Gases (Plumsteadville, PA, USA). Soil flux was determined by dividing the gas concentration (CO_2, CH_4, or N_2O) by the number of days incubated between sampling. Gas concentrations observed on each sampling day were added together to determine the total flux for the 29 day incubation.

Soil NH_4-N and NO_3-N concentration was determined by extracting 5 g of wet soil with 50 mL of 2M KCl for determination of inorganic N content as described by Keeney and Nelson (1982). Soil extracts were measured colorimetrically for NH_4 and NO_2 + NO_3 using a Bran+Luebbe Auto Analyzer 3 (Bran+Luebbe, Norderstedt, Germany).

Statistical Analysis

Analysis of variance (ANOVA), using a general linear model (GLM), was used to analyze each response variable for fertilizer type. Pearson correlations were also used to identify relations between variables (CO_2, N_2O, NO_3-N, and NH_4-N). All statistical analyses were performed using SAS software version 9.2 (SAS Institute, 2010) with a significance level of α=0.05 set *a priori*. The least significant difference (LSD) test was used to identify significant differences among treatments (SB, SBF, BM and the control).

III. Results and discussion

After 48h of incubation, N_2O and CO_2 production was significantly affected by the fertilizer type, the microorganism treatment, and the interaction of both factors (Table 1), with the greatest impact occurring with N_2O emissions. Methane emissions were only affected by the fertilizer type. An effect of fertilizer type on N_2O emissions has been reported by Qin et al. (2011). These results suggested that N_2O emissions, as affected by soil microbial-based treatments, are dependent on the type of fertilizer present.

Carbon Dioxide production

Total CO_2 production (sum of all the microbial- based treatments: SB, SBF, BM, and control) significantly differed among fertilizer treatments. The unfertilized treatment produced significantly more CO_2 (18361.3 µg CO_2 kg dry soil^{-1}) compared to the AN-32 treatment (16181.4 µg CO_2-C kg dry soil^{-1}, P=0.025) and to the CAN-17 treatment (12405.9 µg CO_2-C kg dry soil^{-1}, P<0.0001). The CAN-17 treatment produced less CO_2 than AN-32 (P=0.0002). These findings are in agreement with others who have observed decreases in soil CO_2 emissions with N fertilization (Al-Kaisi et al., 2003, Kowalenko et al., 1978, Ma et al., 1999).

Table 1. Analysis of variance results of trace gas production rates: CO_2, N_2O, and CH_4 per each sampling day for main factors and interactions.

Trace gas production rates (µg trace gas kg^{-1} soil d^{-1})	Sources of variation	df	*F-value*							
			Days of incubation							
			1	2	4	8	10	15	22	29
CO_2	Fertilizer (F)	2	12.87**	35.52**	33.90**	36.64**	9.65**	27.18**	13.05**	16.97**
	microorganism (M)	3	9.68**	12.49**	3.65*	6.11**	0.29	3.36*	0.94	1.87
	M X F	6	6.54**	2.17	2.06	2.41*	1.11	2.65*	1.71	2.19
N_2O	Fertilizer (F)	2	0.81	58.84 **	140.51**	345.51**	189.86**	48.63**	165.61**	142.43**
	microorganism (M)	3	1.88	95.55**	38.08**	55.91**	11.95**	1.84	9.21**	12.93**
	M X F	6	1.54	44.85**	63.11**	111.06**	53.02**	16.81**	40.67**	40.81**
CH_4	Fertilizer (F)	2	17.95**	1.72	27.18**	26.07**	1.04	9.39**	0.26	15.45**
	microorganism (M)	3	2.69	0.16	2.66	0.9	0.34	2.11	0.92	0.51
	M X F	6	0.87	0.79	3.17*	0.13	0.88	1.05	0.91	0.24

* Significant at 0.05 probability level, **Significant at the 0.01 probability level,

When comparing the microbial-based treatments within each fertilizer we found different trends depending on the fertilizer type. None of the microbial-based treatments impacted CO_2 production when applied with AN-32 (Fig.1). These results showed indirectly that total microbial activity was not enhanced nor decreased in the presence of microbial-based treatments.On the other hand, when CAN-17 was present, all the microbial-based treatments significantly increased total CO_2 production (Fig. 1) by an average of 28% compared to the control (no microbial-based treatment). In the unfertilized treatment only, the SBF treatment significantly increased total CO_2 production 30% compared to the control (no microbial-based treatment).

Carbon dioxide production rates provide important information about the dynamics of CO_2 production over time (Fig.2). For all three fertilizer treatments, the highest CO_2 peak was observed after 24h of incubation. After the first peak in CO_2, rates decreased until day 10 and then were constant until

the last day of incubation. Carbon dioxide production response curves from all three microbial-based treatments and the control were similar.

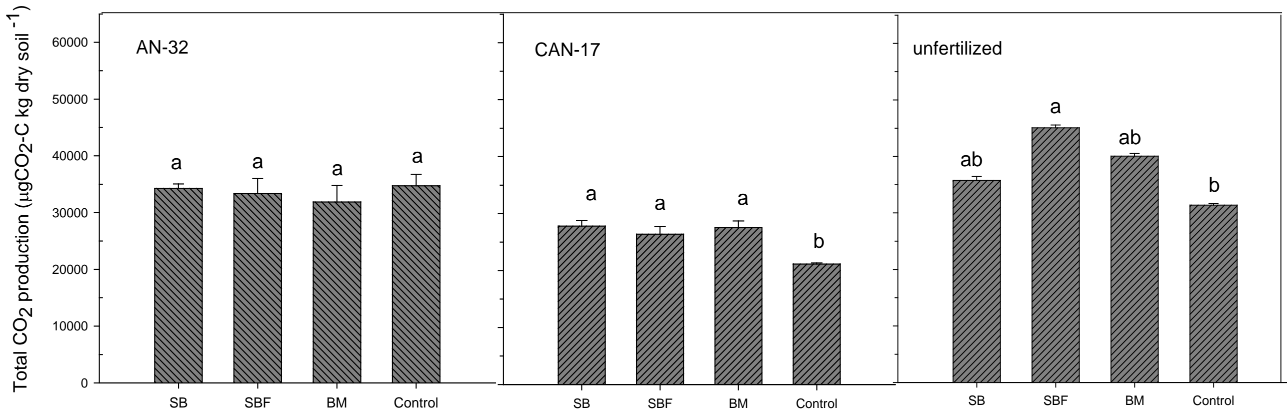

Figure. 1. Total CO_2 production after 29 days of incubation under 3 different fertilizer treatments and three microbial-based products:SB (SoilBuilder), SBF (SoilBuilder filtered), BM (PGPR*Bacillus* mix), control (no product applied).Columns labeled with the same letter are not significantly different based on LSD test (α =0.05). Error bars indicate the standard error of the mean (n=4).

When CAN-17 was present, the CO_2 production rate of the control (no microbial-based treatment) was slightly lower during the whole incubation experiment compared to the microbial-based product treatments. In the unfertilized treatment, a different behavior of the CO_2 rates was observed, especially at the beginning of incubation. After 24 hours of incubation, a significant peak in CO_2 was observed when the SBF treatment was present. This increase in CO_2 could be related to enhance microbial activity, which could be explained by the presence of microbial metabolites in the SBF treatment.

Garcia-Montiel et al. (2004) proposed that higher CO_2 emissions indicate more microbial decomposition, which consumes oxygen and creates low-oxygen or anaerobic conditions needed for N_2O production. In our study, the addition of microbes and metabolites could have indirectly affected O_2 availability due to increased microbial metabolism (Gillam et al., 2008). This phenomenon was observed with CAN-17 and the unfertilized control, where the microbial-based treatments significantly increased

(Fig. 1) total CO_2 emission. Under these conditions denitrification could be occurring due to increased microbial metabolism that reduced O_2 availability.

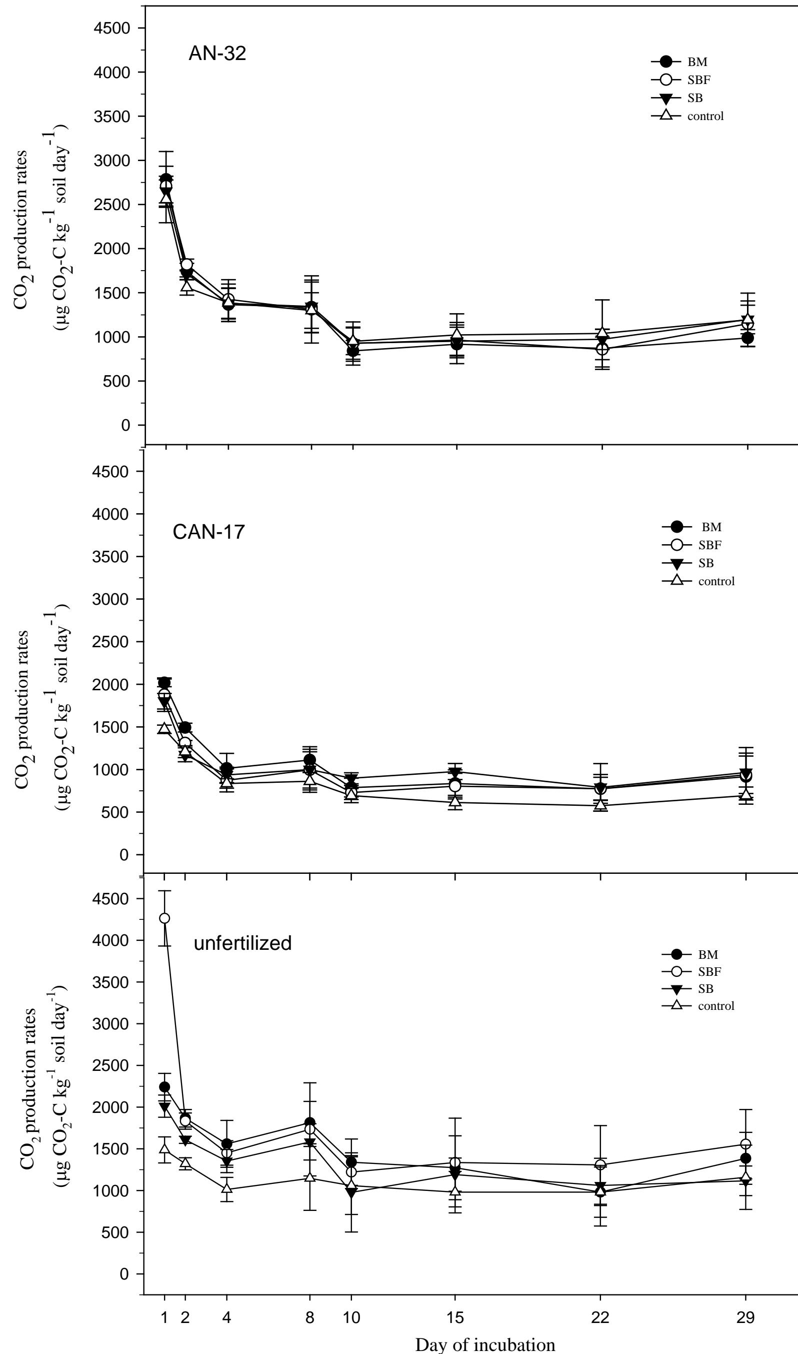

Figure. 2. CO_2 production rates as a function of days of incubation (1 -29 days) of three different fertilizer treatments and three microbial-based products: SB (SoilBuilder), SBF (SoilBuilder filtered), BM (*Bacillus* mix), control (no product applied). Error bars indicate the standard error of the mean (n=4).

Meanwhile, with the AN-32 treatment there was no difference in CO_2 emissions, indicating that microbial activity was not significantly enhanced by the microbial-based treatment. This lack of effect on microbial activity could be related to chemical properties in soil affected by the N fertilizer treatments. In this case more nitrification than denitrification could be occurring for two reasons. One, O_2 availability at the microsite level is higher because microbial metabolism is not enhanced. Two, ammonium content of AN-32 is higher and contributes more to the nitrification process. Even though no differences were observed between treatments for total CO_2 emissions when AN-32 was present, an effect on microbial composition instead of microbial activity by the microbial-based treatments could not be rejected as an explanation. For instance, Peacock et al. (2001) reported no differences in microbial biomass between the fertilized and unfertilized treatments. However, a significant effect on microbial composition was found between treatments.

Methane Emissions

Methane emissions were low for all treatments and fertilizer type throughout the incubation (data not shown). This was expected due to the aerobic conditions that were maintained during the experiment. Methane is a product of anaerobic decomposition of soil organic matter by methanogenic micro-organisms (Lelieveld et al., 1993). There was no effect from the microbial treatments on CH_4 emissions (Table 1), leading to the conclusion that microbial-based treatments used for this study do not affect CH_4 emissions under aerobic conditions.

Nitrous oxide production

Total N_2O production was affected by fertilizer type, and decreased in the order of CAN-17 $\geq$ AN-32 $\geq$ unfertilized control (Fig. 3). The unfertilized control showed the lowest total average production; this was expected due to the soil medium's low N concentration. The more N that is being cycled through the system, the greater the quantity converted into N_2O and released to the atmosphere

(Firestone and Davidson, 1989). It has been reported that N_2O emission is affected by the fertilizer type applied (Millar et al., 2010). Between the two fertilized treatments, CAN-17 produced higher total N_2O. This result agrees with the previous observations of De Klein et al. (2001) who reported that CAN-17 fertilizer had a higher N_2O emission factor (%N applied) compared to other nitrogen fertilizers. On the other hand, our results differ from findings of Galbally(1985) who reported that N fertilizers containing more ammonium resulted in higher N_2O production. In this study, ammonium content of AN-32 (22.5%) was higher than CAN-17 (8.1%); however, the total N_2O production was higher with CAN-17. Minami and Fukushi(1983) found that additions of other nutrients such as Ca in N- containing fertilizers could increase N_2O emissions under anaerobic conditions. However, Millar et al. (2010) emphasized that comparing the effects of different fertilizers with other studies could be inaccurate mainly due to other sources of variations such as environmental conditions and methodology.

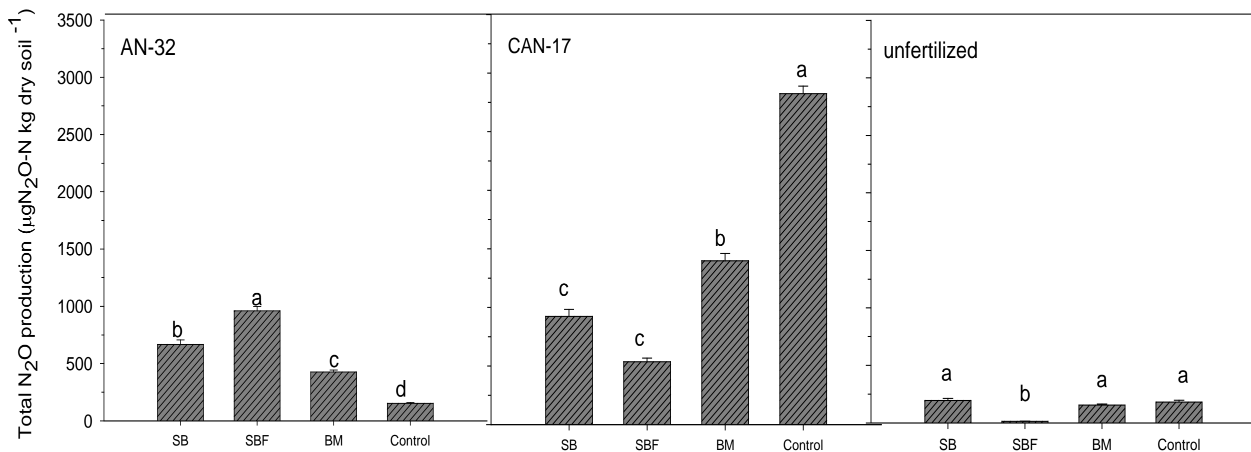

Figure 3. Total N_2O production after 29 days of incubation under 3 different fertilizer treatments and three microbial-based products:SB (SoilBuilder), SBF (SoilBuilder filtered), BM (PGPR *Bacillus* mix), control (no product applied).Columns labeled with the same letter are not significantly different based on LSD test (α =0.05). Error bars indicate the standard error of the mean (n=4).

Within each fertilizer type there were also treatment differences. The three microbial treatments (SB, SBF, and BM) showed significantly higher total N_2O production (Fig. 3) compared to the control (no microbial-based product applied) when AN-32 was present. On the other hand, microbial treatments (SB,

SBF, and BM) significantly reduced the total N_2O production in the presence of CAN-17 (Fig. 3), resulting in a reduction of 81% , 67% , and 50% for the SBF, SB, and BM treatments, respectively. There were no significant differences observed between SBF and SB, suggesting that the effect of N_2O reduction could be related more to the microbial metabolites in the product than to the microbes in SB. However, the treatment with only PGPR *Bacillus* mix (BM) also reduced total N_2O, which indicates that the mechanisms involved in this reduction are different from those observed in the SBF and SB treatments. In the unfertilized control treatment, although N fertilizer was not added, a significant reduction in total N_2O production was observed with the SBF treatment (92%) compared to the control (no microbial-based product applied). These results agree with results obtained with the CAN-17 fertilizer treatment where the SBF also had a higher reduction. In addition, it was observed that the SBF treatment had higher CO_2 emissions in both CAN-17 and unfertilized treatments. In summary, in the presence of CAN-17 fertilizer and the unfertilized treatment an increase in total CO_2 emissions was related to a decrease in total N_2O emissions.

Nitrous oxide production rates varied widely through incubation days and between fertilizer treatments (Fig. 4). In the unfertilized treatment, N_2O production rates were significantly lower for all three microbial-based treatments (SB, SBF, and BM). There was no clear N_2O emission peak that was consistent for all treatments. The SBF and control (no microbial-based treatment) peaked on the 4th day of incubation, while the SB and BM treatments (both treatments that contained living microorganisms) peaked on the 29th day of incubation. This difference in emissions following treatments with SB or BM compared to SBF could be explained by the fact that both SB and BM have living microorganisms, while SBF (SB filtered) only has microbial metabolites. Under the low N content and low organic matter in the soil used for this study, the microorganisms applied struggled to survive. West et al. (1985) also reported that one of the main factors affecting survival of *Bacillus* species in soil is nutrient availability. In contrast, with the SBF treatment, N_2O emissions remained very low during the whole incubation. As a result, effects of the SBF treatment could be due to the presence of metabolites that could indirectly affect

the nitrification or denitrification process occurring in soil. It is possible that among the metabolites in SBF are phenolic compounds, which are known to inhibit soil nitrifying bacteria communities (Bending and Lincoln, 2000). This explanation is supported by the fact that *Bacillus* spp., which are present in the SB treatment, have been reported to produce phenolic compounds (Chaabouni et al., 2012).

When CAN-17 fertilizer was applied, BM, SB, and the control (no microbial-based treatment) treatments peaked on the 8th day of incubation (Fig. 4). The control treatment reached its highest peak at 133 ± 2.31 μg N_2O-N kg soil^{-1} d^{-1}. The peak behavior observed is the same as previous observations made by Qin et al. (2011) who reported a peak in N_2O emissions during the second week after CAN-17 application. After the peak, N_2O production decreased and remained constant after the 10th day. However, N_2O emissions when SBF was applied remained low during the entire incubation, except for a slight peak on day 22.

On the other hand, N_2O emissions when AN-32 was present showed a different behavior, with the highest N_2O production rate being different for each microbial-based treatment. Nitrous oxide production rates reached the highest peak with SBF treatment (49.07 ± 10.38) on the 15th day of incubation. Meanwhile, the control treatment (no microbial-based product applied) reached its highest peak at 5.90 ± 0.11 μg N_2O-N kg soil^{-1} d^{-1} on the 1st day of incubation. Even though a delay in the N_2O peak was observed with the application of SB, this is not related to a reduction in total N_2O emissions. The peaks observed in this study differ from previous observations made by Tenuta and Beauchamp (2003) who observed a very distinct peak when AN-32 was applied to soil after 3 days of incubation.

Our results showed contradictory results. The application of microbial-based treatments reduced N_2O emissions in the presence of the CAN-17 fertilizer and unfertilized treatments but increased emissions when AN-32 was present. One possible explanation is that AN-32 could affect the soil native microbial population and the microbial-based treatments due its capacity to reduce the soil pH (Peacock et al., 2001). Barak et al. (1997) also found an acidification effect with the use of ammonium nitrate after long-term applications. Even though we did not evaluate the long-term effect of AN application in this

study, the fact that the test system was in an enclosed environment could have resulted in the acidification effect being magnified.

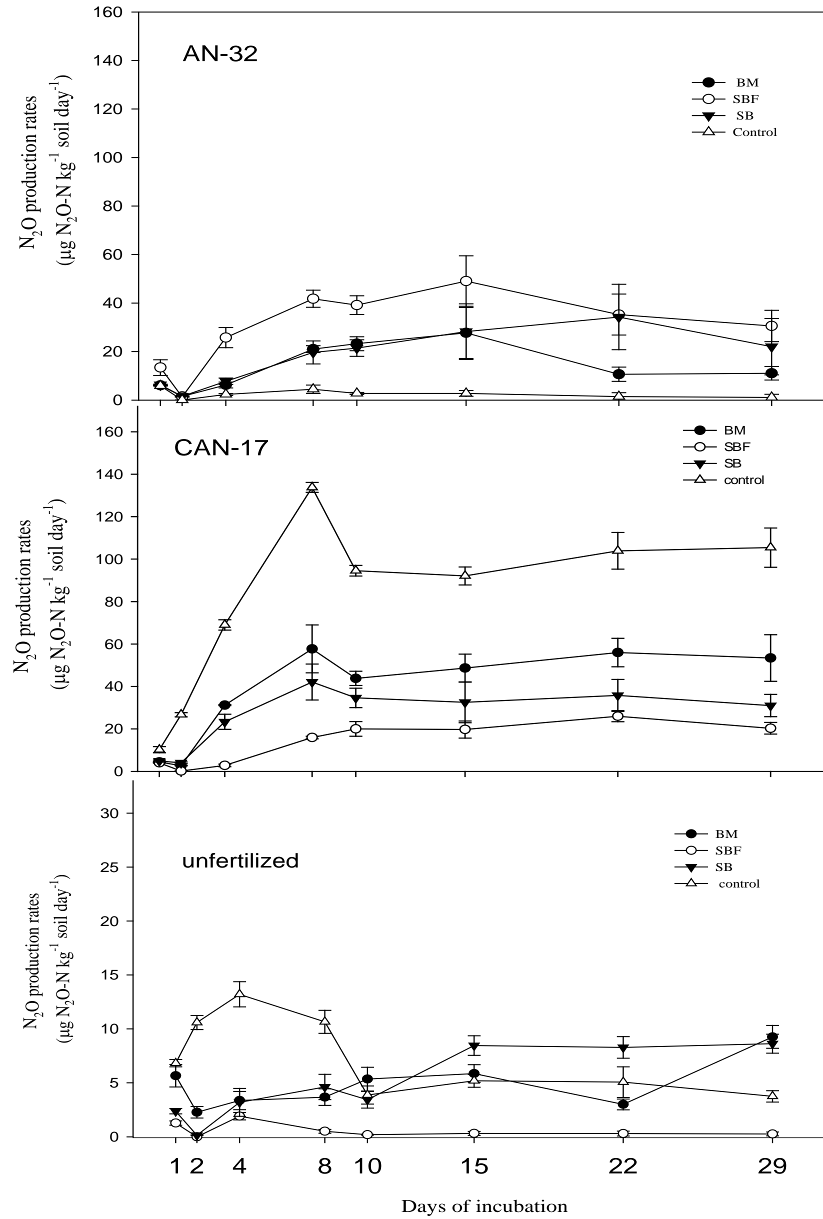

Figure 4. N_2O production rates as a function of days of incubation (1 -29 days) of three different fertilizer treatments and three microbial-based products: SB (SoilBuilder), SBF (SoilBuilder filtered), BM (*Bacillus* mix), control (no product applied). Error bars indicate the standard error of the mean (n=4).

Another effect of acidification is a reduction in the nitrification and denitrification process (Bramley and White, 1990), which could explain the overall low N_2O emissions from the AN fertilizer. Unlike AN-32, CAN-17 fertilizers tend to have a neutral effect on soil pH (Pakarab, 2012). This effect could explain the overall higher N_2O emission compared to AN-32. The different effects on soil properties from each N fertilizer play an important role not only in N_2O emissions but also in the potential effects of the microbial-based treatments. Looking at total N_2O emissions, it is possible to identify that the SBF treatment, one of the treatments that reduced N_2O emissions with CAN-17 fertilizer and unfertilized treatment, is also the treatment that increased N_2O emissions the most with AN-32. The SBF treatment contains several microbial metabolites, such as organic acid and enzymes, that could alter the native microbial composition and potentially interact with the nitrification pathway. Subbarao et al. (2006) demonstrated that plant metabolites released from roots of *Brachiariahumidicola* have the capacity to reduce nitrification. Moreover, iso-thiocyanates (ITCs) produced by plants have been found to inhibit nitrification by either reducing the abundance of nitrifying bacteria or lowering nitrification rates (Bending and Lincoln, 2000). Therefore, the idea that metabolites produced by bacteria can affect nitrification should be considered. Microorganisms present in SB and BM treatments also reduced N_2O with CAN-17. In this case, the mechanisms involved could be related to a direct competition with autotrophic nitrifiers for N in soil. Philippot et al. (2009) supported the idea that heterotrophic microbes are more competitive compared to autotrophic nitrifiers in the soil environment,. This idea could explain and support the use of microorganisms to help control populations of nitrifiers in soil, especially when N fertilizer is added to the system.

Nonetheless, even though aerobic conditions were predominant in this experiment, it is important to consider that denitrification conditions still could have been present. Skiba et al. (1993) found that there is a thin line between the nitrification and denitrification processes in soil. Furthermore, Davidson et al. (2000) showed that a soil with 60% water field pore space (WFPS) and good aeration can contain anaerobic microsites (Parton et al., 1996). With regard to the CO_2 emissions discussed above, it was

observed that microbial activity was enhanced when CAN-17 was applied, indicating that lower O_2 availability was likely present. As a result, the presence of anaerobic microsites would be increased; leading to the possibility that denitrification was involved in N_2O emissions. The N_2O fluxes from the CAN-17 fertilizer (Fig. 4) showed a very distinct peak during the incubation. Maag and Vinther(1996) found that denitrification processes contribute more to spikes in N_2O fluxes than do nitrification processes, which supports the conclusion that dentrification could be involved in the observed N_2O fluxes with the CAN-17 fertilizer treatments. Determining which process (nitrification or denitrification) is predominant in the current experiment is not possible. The effects of the microbial-treatment application depend on the type of N fertilizer present, which also somewhat affects the soil pH and structure of the microbial population.

Another process that could be related to the observed N_2O reduction is immobilization. The amount of available N at any specific moment during the incubation will determine the rate of microbial activity. Nitrous oxide emitting pathways compete for N with assimilatory N immobilization by microbes. Only when N applied to soil exceeds microbial immobilization will N_2O emissions increase (Davidson et al., 2000). When microbial-based treatments were applied and CAN-17 was present, total CO_2 emissions were enhanced by microbial-based treatments due to increased microbial activity. Hence, immobilization processes that reduce the amount of N available and potentially reduce the loss of N_2O could be occurring. However, this same process seems not to be happening when AN-32 was present, as evidenced by lack of difference in total CO_2 emissions between treatments.

Dynamics of soil NO_3-N and NH_4-N

Concentrations of NO_3-N and NH_4-N observed on all days during the incubation were higher in the CAN-17 treatment than in AN-32. Although the same amount of N was applied, 24h after initiation of incubation the concentrations of NO_3-N and NH_4-N were lower in AN-32 treated soils compared to

those treated with CAN-17. As expected, the concentrations of NO_3-N and NH_4-N in the unfertilized treatment were much lower than concentrations observed with the N-fertilized treatments (Fig. 5).

Concentrations of NO_3-N in soils treated with AN-32 were similar among the microbial-based treatments during most of the incubation. The NO_3-N dynamics in all microbial-based treatments and the control (no microbial-based product) followed the same behavior, tending to increase and peak at the 15th day of incubation and then decrease until the end of incubation. On the last day of incubation, there was a significant difference in soil NO_3-N, with the BM treatment having a significantly higher concentration compared to the control. The soil dynamics of NH_4-N concentrations followed the same trend for all treatments, with higher initial values reducing over time. Among the microbial-based treatments and control, there were no significant differences at the beginning or the end of incubation. However, between day 4 and day15 of incubation, NH_4-N concentrations for the control (no microbial-based product) were lower. The highest N_2O emissions observed in this study could be associated with periods of increased NO_3-N content in soil. This association would be consistent with the nitrification process, which increases N_2O emissions. For instance, NH_4-N availability is the factor that most frequently limits the overall rate of nitrification (Haynes, 1986). In this case, the control that produced less N_2O also had a faster decrease in NH_4-N compared to the microbial-based treatments. Hence, there is no relation between faster decrease of NH_4-N (control) and higher N_2O emissions (microbial-based treatments).

Nitrate concentrations with the CAN-17 fertilizer treatment were variable between the microbial treatments. The NO_3-N concentrations tended to initially increase, peaking on day 15, then decreasing until day 22, and finally increasing again until the end of the incubation. When compared with the other treatments, SBF showed a different dynamic. It maintained high values during the incubation and also had a higher significant final NO_3-N value compared to the control (Fig. 5). Temporal patterns of NO_3-N concentration are typical of nitrification systems (Venterea and Rolston, 2000). However, in this case, NO_3-N concentrations did not follow a specific pattern. This observation supports the idea that nitrification may not be the only process responsible for N_2O emissions. The continuous increase and

decrease of NO_3-N concentration could indicate that nitrification and denitrification processes could be occurring at the same time. The control (no microbial-based treatment) in general had lower NO_3-N in soil compared to the other microbial treatments and also tended to decrease even more by the end of incubation. On the other hand, SB and BM treatments containing microbes also decreased N_2O emissions, but the effect on NO_3-N was different from SBF.

Soil concentrations of NH_4-N slightly increased until day 4 and then decreased rapidly until the end of incubation. The concentration dynamics were very similar among all the treatments. Nevertheless, the control showed a lower concentration between day 1 and day 22 compared to the other 3 treatments. There was a clear delay of the NH_4-N disappearance by the microbial-based treatments compared to the control, especially by SBF (Fig. 5). In summary, the presence of SBF on the soil delayed the disappearance of NH_4-N and also maintained the soil concentration of NO_3-N at higher levels. This delaying effect on the N fertilizer transformation could be associated with a reduction in the nitrification process that converts NH_4-N to NO_3-N and also produces N_2O (Eichner, 1989, Synder, 2009).

Completely different dynamics of NO_3-N and NH_4-N concentrations were observed in the unfertilized treatment. The dynamics differed depending on the treatment, and there were no clear peaks. The control (no microbial-based product) and SB treatments showed significantly lower NO_3-N concentration at the end of incubation. In contrast, the BM and SBF treatment showed significantly higher NO_3-N concentrations at the end of incubation. Increases in NO_3-N concentration have been related to an increase in nitrification and denitrification that resulted in increased N_2O emissions (Davidson et al., 2000). However, it was recently reported by Müller et al. (2004) that high NO_3-N concentrations in soil are not a prerequisite for N_2O production. This observation agrees with our results, which showed that SBF reduced N_2O emissions the most and also had higher NO_3-N concentration at the end. Meanwhile, NH_4-N concentration remained very similar during all of the incubation. The control and the BM treatments were the only treatments that resulted in a peak at day 8 of incubation.

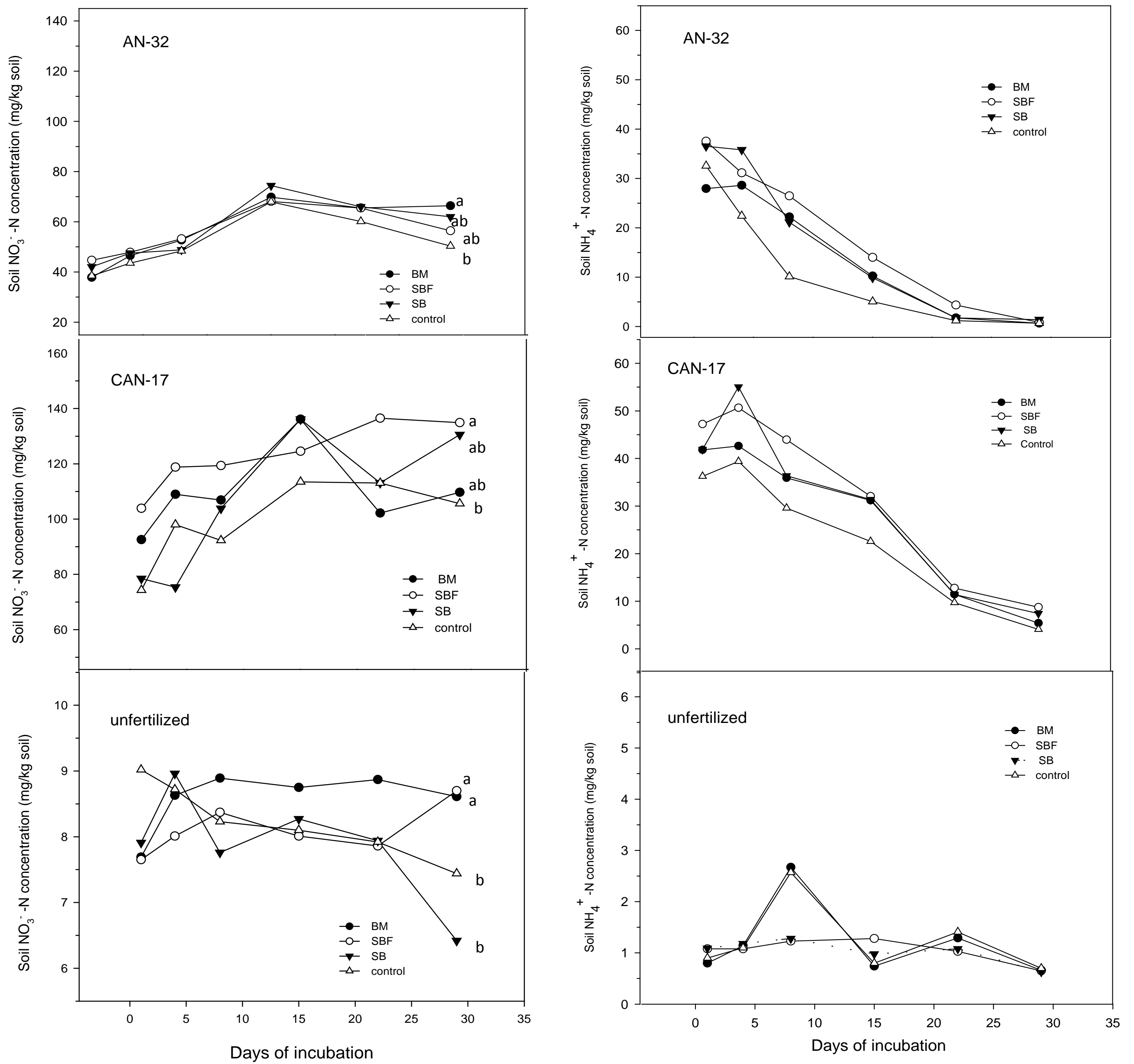

Figure 5. Nitrate (NO_3-N) and Ammonium (NH_4-N) soil concentrations as a function of days of incubation (1 -29 days) of three different fertilizer treatments and three microbial-based products: SB (SoilBuilder), SBF (SoilBuilder filtered), BM (Bacillus mix), control (no product applied). Points labeled with the same letter are not significantly different based on LSD test (α =0.05).

Correlation Analysis

Significant negative correlations between CO_2 and N_2O were found only with CAN-17 fertilizers treatment (Table 2). This result agrees with observations of Ullah and Moore (2011), who also reported that N_2O fluxes from well-drained soils correlated negatively with CO_2 emission rates. This also agrees with the previous observations that linked the treatments that reduced N_2O (SBF, SB, and BM) with the same treatments that produced less CO_2. No significant correlation was found for AN-32 fertilizer and the unfertilized treatment.

Table 2. Pearson correlations values per fertilizer type : CAN (calcium ammonium nitrate), AN (ammonium nitrate), and control (no fertilizer) between nitrous oxide (N_2O), carbon dioxide (CO_2), soil nitrate concentration (NO_3-N), and soil ammonium concentration (NH_4-N) (n =96) (α=0.05).

Fertilizer		**Parameter**		
		N_2O	**CO_2**	**NO_3-N**
CAN	N_2O			
	CO_2	-0.7088		
	NO_3-N	ns	0.4243	
	NH_4-N	-0.4893	-0.3985	-0.2597
AN	N_2O			
	CO_2	ns		
	NO_3-N	0.4313	0.4191	
	NH_4-N	-0.4579	-0.3822	-0.5876
Control	N_2O			
	CO_2	ns		
	NO_3-N	ns	ns	
	NH_4-N	ns	-0.3231	ns

†ns= not significant

Negative correlations between the two inorganic forms of N in the soil (NO_3-N and NH_4-N) were only significant for CAN-17 and AN-32 fertilizer treatments, with the AN-32 treatment having more

negative correlations than CAN-17 (Table 2). This finding agrees with reports by Davidson et al. (2000) who also found that NH_4-N was negatively correlated with NO_3-N. Furthermore, when AN-32 was applied, a significant positive correlation was also found between N_2O emissions and NO_3-N concentrations, and a significant negative correlation was observed between N_2O emissions and NH_4-N concentration. Meanwhile, when CAN-17 fertilizer was applied, a significant negative correlation was observed only for the N_2O emissions with the NH_4-N concentration. The fact that N_2O and NH_4 –N concentrations were negatively correlated in both fertilizers indicates that nitrification was present at some point, and the effect of the microbial-based treatment may be related somehow to this process.

IV. Conclusions

The results of this study showed that microbial-based inoculants reduced N_2O emissions from unfertilized and CAN-17 fertilized soils but increased emissions when AN-32 was applied. All three microbial-based treatments significantly reduced N_2O emission with CAN-17. In the unfertilized control, significant reductions were observed only with SBF. Conversely, the SBF treatment increased emissions the most when AN-32 was applied. This contradictory result could be attributed to changes in soil chemical properties and microbial dynamics following the AN-32 application. This idea is supported by the finding that CO_2 was not affected when AN-32 was present, indicating that treatments applied did not enhance or reduce soil microbial activity. However, with CAN-17 and the unfertilized treatments, CO_2 emissions were significantly increased by the three microbial-based treatments. With CAN-17, the relation between NO_2 and CO_2 was confirmed with correlation analysis that showed a negative significant correlation between these two variables. Although this incubation study was done under aerobic conditions, the presence of anaerobic microsites should not be discarded. Some possible mechanisms that could explain the reduction of N_2O emissions include (i) production or presence of nitrification and/or denitrification inhibitors; (ii) inhibition of nitrifying and/or denitrifying microorganisms; (iii) competition

of applied microbial treatments with the native microbial nitrifiers and/or denitrifiers; and (iv) immobilization of nitrogen fertilizer by microbes.

The results presented here complement results presented by Calvo et.al (2013) and confirm that application of microbial-based inoculants somehow interacts with the nitrogen cycle by reducing or increasing N_2O emissions under fertilized and unfertilized conditions. Further research is needed in order to understand the specific conditions and mechanisms that are involved in reducing N_2O emissions by microbes and by microbial metabolites. These findings represent a starting point to elucidate the potential of inoculants to reduce nitrogen losses from agricultural systems.

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Effect of Microbial-Based Inoculants on N_2O Emissions from Greenhouse Corn (*Zea mays* L.) under Different Nitrogen Fertilizer Regimens

Abstract

Emissions of nitrous oxide (N_2O) are increasing due to several factors, including increased use of nitrogen (N) fertilizers. New management tools are needed to reduce N_2O emissions from production agriculture. One potential tool is the use of microbial inoculants. In a previous soil incubation study, we found that application of microbial-based inoculants reduced N_2O emissions when N fertilizers were present. The current study compared emissions of N_2O and CO_2 following applications of microbial based inoculants to corn planted in field soil in a greenhouse test. Treatments consisted of SoilBuilder (SB) from Agricen Sciences, a metabolite extract of SoilBuilder (SBF), and a mixture of four strains of plant growth-promoting *Bacillus* spp. (BM). Experiments included an unfertilized control and three nitrogen fertilizers: urea, urea ammonium nitrate 32% N (UAN), and calcium ammonium nitrate 17% N (CAN). Cumulative fluxes of N_2O from pots at 41 DAP showed significant reductions of 15% (SB), 41% (BM), and 28% (SBF) with CAN fertilizer. When UAN was used, reductions of 34% (SB), 35% (SBF), and 49% (BM) were obtained. However, no reduction of N_2O occurred with urea fertilizer. Microbial-based treatments do not affect total CO_2 emissions from any of the fertilized treatments and the unfertilized control. Nitrogen uptake was increased by microbial-based treatments on an average of 56% over the control (no microbial based treatments). Significant increases in plant height, SPAD reading, and fresh and dry shoot weight were also observed when microbial-based treatments were applied. Overall, the results demonstrate that microbial inoculants can reduce emissions of N_2O resulting from N fertilizer application depending on the type of N fertilizer and enhance N uptake and plant growth. Future studies are planned to determine the mechanisms of N_2O reduction by microbial inoculants.

I. Introduction

Nitrous oxide (N_2O) emissions from agriculture soil management is the largest source of emissions in the United States, accounting for about 69% of total 2011 N_2O emissions in the United States (EPA, 2013). Nitrous oxide is an atmospheric trace gas that contributes to global warming and the depletion of stratospheric ozone (IPCC, 2007). Emissions of N_2O in agriculture are predominantly from soils amended with N-rich amendments (fertilizers, manure, and compost), which release inorganic N in the soil. Soil inorganic N is converted to N_2O by soil bacteria. On average, 1 to 2% of the N applied from fertilizers and organic amendments (manure, compost, and other organic fertilizers) will be lost as N_2O (IPCC, 2007). In general, N_2O emissions are directly related to the type, quantity, and method of application of fertilizer, but other factors such as soil type and weather patterns also influence emissions (EPA, 2013). Nitrous oxide is a side product of the aerobic nitrification process and an obligate intermediate in the denitrification pathway. Therefore, it can be emitted by both nitrifiers and denitrifiers (Conrad, 1996). Sustainable agronomic practices need to be designed in order to decrease N_2O emissions and improve the system's efficiency.

Microbes play an important role in soil N cycling. Soil microorganisms are responsible for processes like mineralization, immobilization, nitrification, and denitrification. When aerobic conditions are predominant in the soil, nitrification is the main process responsible for N_2O emissions. Meanwhile, if anaerobic conditions are present, denitrification is the one that produces more N_2O. These two processes are not exclusive, and in some cases, both processes could occur at the same time in the soil (Harrison and Webb, 2001). Alteration of the composition and abundance of microbial communities in the soil has affected the N cycle process (Cavigelli and Robertson, 2000). Application of chemical treatments such as nitrification inhibitors which inhibit autotrophic ammonia oxidizing bacteria, has shown a reduction in nitrification rates (Singh and Verma, 2007). Furthermore, arecent soil incubation study indicated that

application of microbial-based inoculants could reduce N_2O emission when some types of N fertilizer are present (Calvo, et al., 2013).

The importance of microbial-based inoculants in agriculture is increasing in the last few years. Besides their recently reported potential as tools to reduce N_2O emissions from soils, they also play an important role in promoting plant growth (Bloemberg and Lugtenberg, 2001). It has been extensively reported that microbial-based inoculants such as plant growth-promoting rhizobacteria (PGPR) have the capacity to promote plant growth by increasing plant nutrient uptake (Adesemoye, et al., 2008, Canbolat, et al., 2006, Figueiredo, et al., 2010). Microbial based-inoculants can consist of one or more than one specie or strain of microorganisms. Among the PGPR microorganisms, *Bacillus* spp. are widely used, mainly because they can survive as spores and can potentially alter soil microbial composition. *Bacillus* spp. have a wide metabolic capability that allows them to play important roles in soil ecosystem functions and processes. Due to their heterotrophic nature, *Bacillus* spp. play an important role in the soil carbon cycle, soil N cycle, soil sulfur cycle, and transformation of other soil nutrients (Mandic-Mulec and Prosser, 2011). In the last few years a microbial-based inoculants that consist of microorganisms plus their fermentation metabolites have been commercialized. One example of such a product that is already on the market is SoilBuilder™, manufactured by Agricen Sciences (Pilot Point, TX, USA). This product has been shown to increase root growth and nutrient uptake (Yildirim, et al., 2006).

Nitrogen-use efficiency (NiUE) practices in agriculture have been proposed as effective methods for reducing N_2O emissions and promoting a more efficient use of N by plants. Nitrogen-use efficiency relies on the application of precise amounts of Nous fertilizer or manure to crops based on N estimates from soil and plant tissues tests (Synder, 2009). Even though this method is increasingly used, there is a lack of sustainable tools that could help accomplish nitrogen-use efficiency practices. In this context, microbial-based inoculants could potentially be included as tools to increased NiUE. Microbial-based inoculants could help accomplish NiUE in two ways: by decreasing N_2O emission from N-fertilized soils

(Calvo, et al., 2013) and/or increasing directly or indirectly the plant N uptake which also will reduced N losses from the system (Adesemoye, et al., 2010, Bashan, 1998, Biari, et al., 2008) .

Given the previously demonstrated potential of microbial-based inoculants to decrease N_2O emissions in an incubation study using only soil, we were interested in testing the gas emissions (N_2O, CO_2, and CH_4) from corn planted in field soil in a greenhouse test treated with the same microbial-based inoculants. The objective of this study was to test the hypothesis that microbial-based inoculants (*Bacillus* PGPR mix, SoilBuilder™, and SoilBuilder™ filtered) can reduce emissions of N_2O in the presence of N fertilizers (UAN, urea, and CAN) in a greenhouse study using corn plants . This study also aimed to confirm the results obtained in a previous study by Calvo et al. (2013). Carbon dioxide and CH_4 were also evaluated to determine the microbial-based inoculants' impact on microbial respiration (CO_2) and if N_2O production was mainly an effect of aerobic and anaerobic conditions. In addition, we tested the hypothesis that microbial-based inoculants could increase plant growth and N uptake of corn plants evaluated 43 days after planting.

II. Materials and methods

Soil Characterization

Initial soil analysis was performed by Auburn University Soil Testing Laboratory as described by Hue and Evans (1986). Briefly, total carbon and N were analyzed using an Elementarvario Macro C-N analyzer (Elementar Americas, Inc. Mt. Laurel, NJ, USA). Soil pH was determined on 1:1 soil/water suspensions with a glass electrode meter. Concentrations of P, K, Mg, and Ca were determined using Melich 1 (double acid extracting solution) (Olsen and Sommers, 1982) and measured using an ICAP 9000 (Thermo Jarrell Ash, Franklin, MA, USA). The Cation Exchange Capacity (CEC) was determined by base summation (Ca, Mg, K, and Na) according to procedures of Hue and Evans (1986).

Soil Microcosms

A soil:sand mixture was used as the soil medium for this study. Sand was mixed with the soil in order to improve water infiltration and minimize anaerobic conditions during the study. Briefly, a sandy loam soil with a texture of 72.8% sand, 10.4% clay, and 16.8% silt was mixed 3:1 (soil:sand; v:v) with white brick/Mason sand (particle size: 1/8 mm -1/4 mm). The mixture resulted in a soil medium with the texture of a loamy sand(85.2 % sand, 4.8% clay, and 10% silt) The soil:sand mixture had a pH of 6.14, CEC of 1.13 cmol kg^{-1}, total N concentration of 0.7 g kg^{-1}, organic matter concentration of 4.5 g kg^{-1}, total C concentration of 2.6 g kg^{-1}, NO_3 concentration of 10.53 µg g^{-1}, NH_4 concentration of 0.73 µg g^{-1}, Mg concentration of 236 µg g^{-1}, Ca concentration of 305 µg g^{-1}, P concentration of 4 µg g^{-1}, and K concentration of 51 µg g^{-1}.

The experiment was performed in the greenhouse at the USDA National Soil Dynamics Laboratory, Auburn, Alabama. Each pot was filled with 6 kg of dry soil: sand mix and adjusted to 60 % WHC. Treatments were organized in a complete randomized block design (CRBD) with a 4×4 factorial arrangement with three microbial treatments and a control (no microbial treatment) and three N fertilizer regimens , each with five replications. The N fertilizers sources included (i) unfertilized control, (ii) urea ammonium nitrate (UAN) -32%, (iii) urea, and (iv) calcium ammonium nitrate (CAN)-17%. Nitrogen fertilization was calculated based on 168 kg ha^{-1}. The amount of N applied was calculated based on one hectare furrow slice (topsoil 15 cm), which is equal to 1 980 000 kg of soil. Based on this calculation, each pot (6 L, C600 Classic from Nursery supplies, Kissimmee, FL) received the same amount of N regardless of N fertilizer source in the fertilizerregimes. Calcium fertilization was applied as $CaCl_2$ at a rate of 6.6 g/pot (UAN, urea, and unfertilized control) and 5.9 g/pot (CAN). Phosphorus and potassium fertilization was applied as K_2HPO_4 at a rate of 1.3 g/pot.

Microbial Source Preparation

SoilBuilder™ is a commercially available microbial biofertilizer manufactured by Agricen Sciences, Pilot Point, TX. SoilBuilder™ is prepared from a bioreactor system consisting of a continuously maintained microbial community (patent pending). The final product contains bacteria and bacterial metabolites derived from the bioreactor. Based on plate counts using tryptic-soy agar (TSA) (24 h at 25 °C incubation), the most commonly occurring bacteria within the final stabilized product are *Acidovorax facilis*, *Bacillus licheniformis*, *Bacillus subtilis*, *Bacillus oleronius*, *Bacillus marinus*, *Bacillus megaterium*, and *Rhodococcus rhodochrous*, each at $1x10^3$cfu/cc.

SoilBuilder™ filtered (SBF) consisted of SoilBuilder™ product without microbial cells and was prepared by filtering SB through a 0.45 µm filter and then through a 0.22 µm filter. SBF contains microbial metabolites derived from the bioreactor production system that, in addition to other components, include organic acids, peptides, and enzymes.

The PGPR *Bacillus* mixture (referred to as BM) included four *Bacillus* strains: *Bacillus safensis* T4 (previously called *B. pumilus* T4), *Bacillus pumilus* INR7, *Bacillus subtilis* subsp. *subtilis* IN937a (previously called *B. amyloliquefaciens*IN937a), and *Lysinibacillus xylanilyticus* SE56 (previously called *Bacillus sphaericus* SE56). These strains were obtained from culture collections in the Department of Entomology and Plant Pathology, Auburn University (Auburn, AL, USA). These strains have been shown to have important plant growth-promoting effects (Enebak, et al., 1998, Jetiyanon, et al., 2003, Kokalis-Burelle, et al., 2003, Kokalis-Burelle, et al., 2002).

Microbial treatments were applied at a rate of 375 ml per pot. For the BM treatment, the bacterial mix was prepared by mixing each strain's spore suspension, which was previously quantified by plating the spore mix suspension on TSA and incubating for 48 h at 25 °C. The spore mix was then adjusted to a concentration of 10^5cfu mL^{-1}. The final concentration in each pot was of 6.25×10^3cfu·g of dry soil. The SoilBuilder™ product (SB) solution was prepared according to the label instructions by mixing 16 mL of

SoilBuilder™ in 1.0 L of distilled water immediately before setting up the experiment. SoilBuilder™ product contained 10^6cfu mL^{-1}, so the final concentration in each jar was 10^3 cfu·g of dry soil. The treatment of SoilBuilder™ filtered (SBF) was prepared the same way as SB, but before applying the 375 mL to each pot, the solution was filtered. Sterility of the filtrate was confirmed by plating onto TSA (48 h at 25 °C incubation) and observing no bacterial growth. Non-filtered SoilBuilder™ population concentrations were confirmed also by plate count on TSA after incubation for 48 h at 25 °C.

Experimental Setup

The fertilizer source corresponding to each treatment was added in liquid form, followed by the appropriate microbial treatment, and two seeds of corn hybrid DKC61-73 (Dekalb Seed Company, Cedar, Iowa) per pot. Four days after seed germination, plants were thinned to one plant per pot. Plants were watered each day, assuring that the soil moisture was maintained at 20% in each pot. Soil moisture was controlled by using a WaterScout SM 100 Soil moisture sensor (Spectrum technology, Inc.). Trace gases emitted from the pots were sampled using the static closed chamber method (Hutchinson and Davidson, 1993; Hutchinson and Mosier, 1981). Custom-made gas flux chambers were designed and constructed based upon criteria described in the GRACEnet protocol (Baker, et al., 2003, Parkin and Kaspar, 2006) to accommodate nursery containers. A structural base consisting of polyvinyl chloride (PVC) cylinders [25.4 cm (10 in) inside diameter by 38.4 cm (15.1 in) tall] was sealed at the bottom. During gas measurement, the entire plant-pot system was placed inside the base cylinder and a vented flux chamber [25.4 cm (10 in) diameter x 11.4 cm (4.5 in) height] was placed on top of the base cylinder (Fig 1). The top flux chambers were constructed of PVC, covered with reflective tape, and contained a center sampling port.

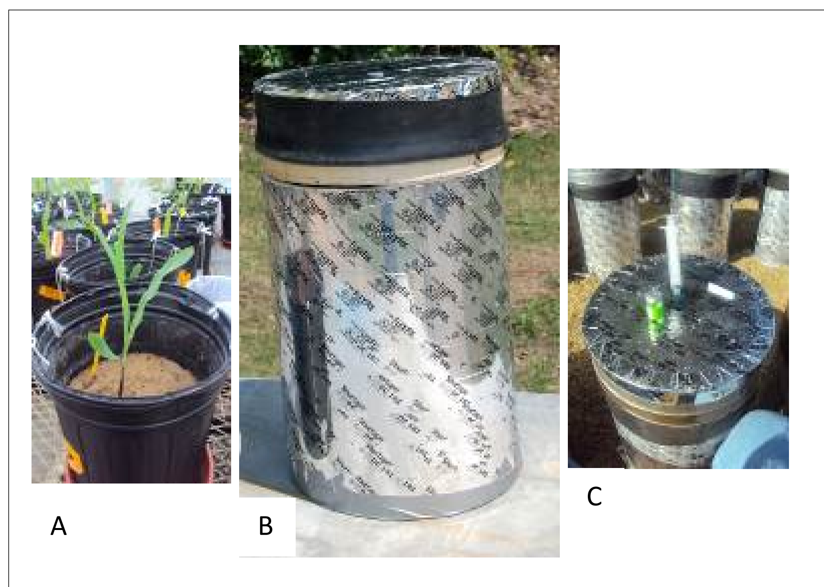

Figure 1. Photos of the gas-sampling system. A. Corn plant to be evaluated. B. The pot containing the corn plant was placed inside the custom-made gas flux chambers. C. Gas samples were taken at: 0, 20, and 40 minutes after closing the chamber using a syringe through a rubber septum.

Gas Flux and Soil NH_4-N and NO_3-N Sampling

Gas sampling was performed at 1, 3, 6, 10, 15, 21, 27, 34, and 41 days after treatment at the same time of the day (10:00-12:00 pm). Gas samples for CO_2, CH_4, and N_2O were taken at 0, 20, and 40 minute intervals following chamber closure. At each time interval, gas samples were collected by inserting a 23 gauge needle attached to gastight 10 mL polypropylene syringe through the rubber septum embedded in the lids of the chambers. They were then injected into evacuated 6 mL glass vials fitted with butyl rubber stoppers as described by Parkin and Kaspar (2006) an samples were stored at 25 °C until analysis, which was done within two weeks of collection. Gas samples were analyzed using a gas chromatograph (GC) (Shimadzu GC-14B, Japan) equipped with an electron capture detector for N_2O and a flame ionization detector for CH_4 and CO_2. The gas chromatograph's detectors were calibrated by comparison to a standard curve using standards obtained from Scott Specialty Gases (Plumsteadville, PA, USA). Gas fluxes were calculated from the rate of change of the concentration of trace gas (CO_2, N_2O, or CH_4) in the

chamber headspace during the time intervals while chambers were closed (0, 20, and 40 minutes) as described by Parkin and Venterea (2010). Calculations in this study were used to express data as mg (CO_2-C and CH_4-C) and ug (N_2O-N) trace gas per kg of dry soil (per day). Total trace gas efflux was calculated by extrapolating daily averages over the course of 41 days.

Soil nitrate and ammonium concentrations were determined at 3, 6, 10, 15, 21, 27, 34, and 41 days after treatment. Soil NH_4-N and NO_3-N concentrations were determined by extracting 5 g of wet soil with 50 mL of 2M KCl for determination of inorganic N content as described by Keeney and Nelson (1982). Soil extracts were measured colorimetrically for NH_4 and NO_2 + NO_3 using a Bran+Luebbe Auto Analyzer 3 (Bran+Luebbe, Norderstedt, Germany).

<u>Plant Evaluation</u>

Plants from each treatment were harvested and evaluated at the end of the experiment when plants reached the V6 stage (43 DAP). Plant parameters measured included fresh shoot and root weight, dry shoot and root weight determination (70 °C oven dry), chlorophyll content (SPAD 502 meter), and plant height were measured. Plant nutrient analysis was performed on the dried shoot tissue. Ground tissue from each plant shoot was analyzed for N using the combustion method (LECO FP-528 N Analyzer). Nitrogen uptake by the plant shoot was calculated using the N concentration and the dry shoot weight of the shoot.

<u>Statistical Analysis</u>

Analysis of variance (ANOVA), using a general linear model (GLM), was used fisrt to analyze the effect of microbial-based treatment, fertilizer and the interaction of both on each parameter (N_2O,CO_2,CH_4, NO_3^-, and NH_4^+). Then the response of eachvariable per fertilizer type was analyzed using the least significant difference (LSD) test was used to identify significant differences between treatments (SB, SBF, BM, and control). All statistical analyses were performed using SAS 9.2 (SAS Institute, 2010) and a significance level of α=0.05 set *a priori*.

III. Results and discussion

Nitrous oxide fluxes were significantly affected by the fertilizer type, microorganisms and the interaction of both factors (Table 1). Greater significant differences were found during the first 15 days of evaluation. Meanwhile CO_2 and CH_4 fluxes were not affected to the same degree by the fertilizer, microorganisms, and the interaction. Between these two, CH_4 was almost not affected at all. This result was not a surprise due to lack of anaerobic conditions or animal manure, which are factors responsible for CH_4 production from agricultural sources (Yu, et al., 2001). The type of fertilizer affected CO_2 only during the first 6 days of evaluation. The application of microbial-based treatments only affected the CO_2 fluxes during the first day of evaluation. There were no effects from the interaction of fertilizer and microbial-based treatment on CO_2 fluxes.

Table 1. Analysis of variance for the effects of fertilizer and microbial-based treatments on CO_2, N_2O, and CH_4 fluxes during 41 days after planting (DAP).

Variable (μg trace gas kg soil$^{-1‡}$d^{-1})	Factors†	ANOVA P > F LSD (0.05)								
		1d	3d	6d	10d	15d	21d	27d	34d	41d
CO_2	Fertilizer (F)	0.0048	<0.0001	0.0012	0.6856	0.7914	0.6585	0.7791	0.9040	0.1807
	Microbial –based treat. (M)	0.0006	0.9467	0.3286	0.0668	0.2134	0.9723	0.3209	0.6407	0.0839
	M X F	0.3621	0.7730	0.6980	0.7823	0.7944	0.3986	0.1350	0.8560	0.7611
N_2O	Fertilizer (F)	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0085	<0.0001	0.0147
	Microbial –based treat. (M)	<0.0001	<0.0001	0.0023	0.0005	0.2807	0.0003	0.1924	0.0113	0.0206
	M X F	<0.0001	<0.0001	0.0023	0.0251	<0.0001	0.0489	0.4658	0.1105	0.0057
CH_4	Fertilizer (F)	0.8012	0.2136	0.2041	0.6154	0.3657	0.3958	0.1970	0.8696	0.6512
	Microbial –based treat. (M)	0.0420	0.4272	0.9740	0.0725	0.4554	0.0438	0.2138	0.4055	0.7308
	M X F	0.6272	0.3288	0.4617	0.0975	0.8235	0.0129	0.4523	0.8733	0.7134

Analysis of variance LSD; P>0.05.
† Fertilizer factor include UAN-32 (urea ammonium nitrate), urea, CAN-17 (calcium ammonium nitrate), and unfertilized. Microbial based treatments factor includes SB (SoilBuilder™), SBF (SoilBuilder™ filtered), BM (*Bacillus* PGPR mix), and Control (no product applied).
‡Trace gases were calculated based on kg of dry soil.

Nitrous Oxide Emissions

Total N_2O production was affected by fertilizer type and decreased in the order of UAN-32 ≥ CAN-17 ≥ Urea ≥ unfertilized control (Fig. 2). UAN-32 and CAN-17 fertilized soils released on average 4.5 times more total N_2O (total N_2O after 41 DAP) than the unfertilized treatment (Fig 2). The more N that is being cycled through the system, the greater the quantity converted into N_2O and released to the atmosphere (Firestone and Davidson, 1989). The effect of N fertilizer on N_2O emissions is well known (Smith, et al., 1997) and explains why the three N fertilizer produced significantly more total N_2O than the unfertilized control. Breitenbeck et al. (1986) reported that plots treated with nitrifiable forms of N fertilizer (ammonia or ammonium) produced more N_2O than plots that received an equivalent application of N as NO_3^--N. In this case, CAN-17 and UAN had more NH_4-N (8.1% and 7.75%, respectively) at the beginning of the experiment compared to urea (0% NH_4-N). This explains the differences found between the total N_2O production of urea and the two other fertilizers.

Microbial based treatments (SB, SBF, and BM) significantly reduced total N_2O emissions when CAN-17,UAN-32, and unfertilized control were present (Fig.2). With UAN-32 total N_2O emissions were reduced by 15%, 28%, and 41% when SB,SBF, and BM were applied, respectively. With CAN-17 the reductions were 34%, 35%, and 49% when SB, SBF, and BM were applied, respectively. Finally, in unfertilized control, significant reduction also were observed: SB (26%), SBF (26%), and BM (28%). It is possible to conclude that, among the three treatments, BM was the one that consistently reduced N_2O total emissions at the greatest level. However, this reduction was only significantly lower than SB and SBF when CAN-17 was applied. These results agree in part with previously reported soil incubation studies performed with the same microbial-based treatments (Calvo, et al., 2013). In that study the three microbial-based treatments reduced total N_2O emissions, and SBF and SB significantly reduced emissions the most when CAN-17 was applied. In the present study, BM was the treatment that reduced N_2O total emissions the most with the same fertilizer. This enhanced effect of BM could be also related to

the fact that in the present study living plants were added to the system, while the previous study evaluated effect in soil without plants.

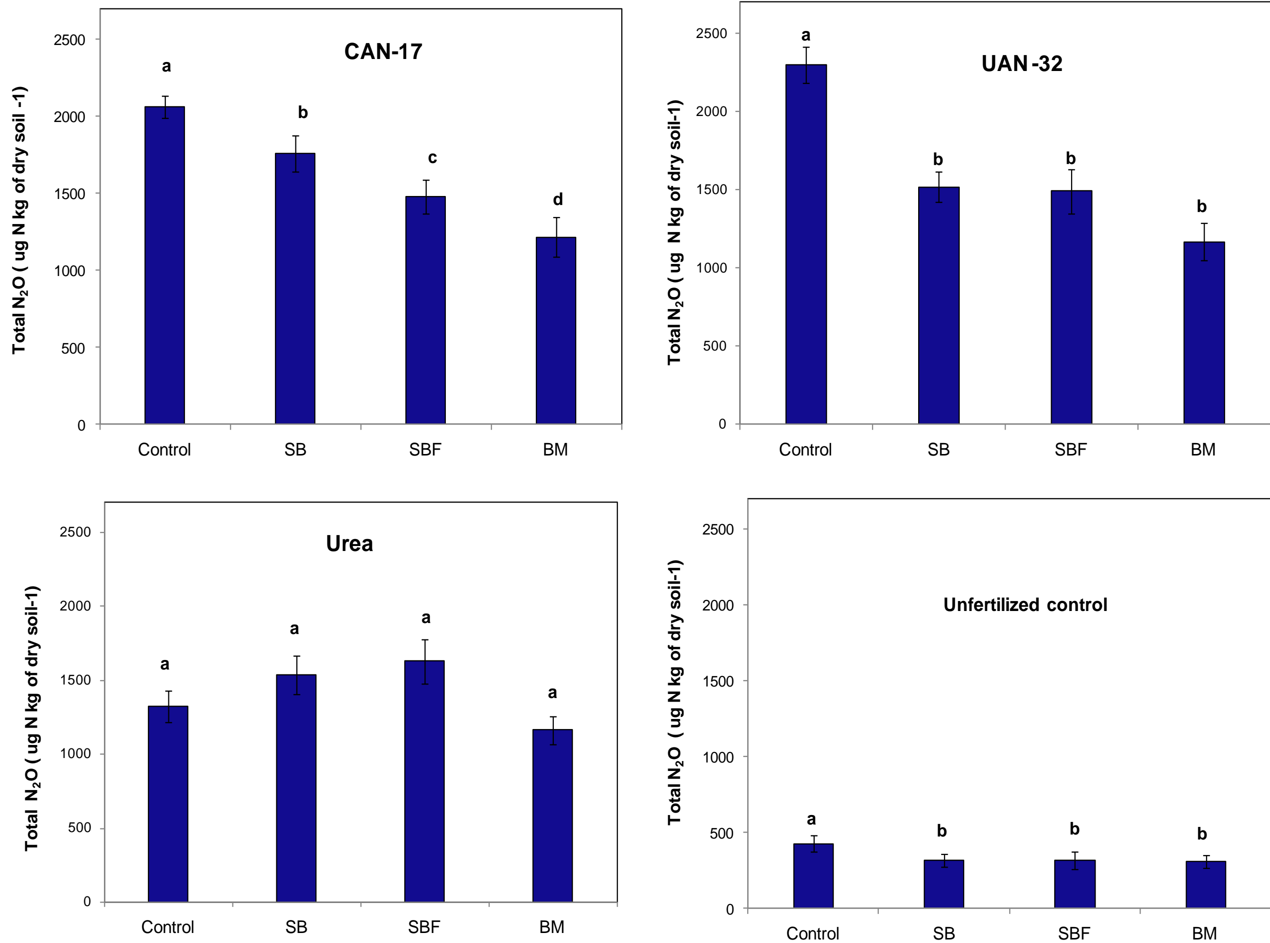

Figure 2.Total N_2O production from soil planted with corn after 41 days of evaluation per type of N fertilization regimen. Microbial- based treatment: SB (SoilBuilder™), SBF (SoilBuilder™ filtered), BM (*Bacillus* PGPR mix), and Control (no product applied).Bars followed by the same letter are not significantly different at the 0.05 level using LSD values.

The interaction among soil-roots-bacteria-N is more complex than only bacteria-soil-N. Additional mechanisms like root colonization and plant growth promotion by BM could indirectly allow the plant to take more N from the soil, which indirectly would be translated in less N_2O lost from the system. Regarding the difference between SB and SBF, no significant difference between them was observed with UAN-32 and unfertilized control (Fig 2). However, with CAN-17, SBF significantly

reduced N_2O emissions more than SB. This observation suggests that in the specific case of CAN-17 the application of only microbial metabolites (SBF) could have a greater effect than the application of microbial metabolites + microorganisms (SB). It was previously reported that phenolic compounds produced by some microorganisms could have an inhibitory effect on nitrifiers' bacteria, which could potentially reduce N_2O emissions (Bending and Lincoln, 2000). No significant reduction was observed when urea was applied. This observation supports results previously published by Calvo et al. (2013), where the same microbial-based treatments had less effect when urea was present in a soil incubation experiment. The lack of effect of microbial-based treatments when urea was applied could be due to the release of significant amounts of NH_3, which could be toxic to many microorganisms (Chapman and Leibig, 1952, Engel, et al., 2009).

Nitrous oxide fluxes per sampling day or days after planting (DAP) peaked for the three fertilizers (UAN-32, CAN-17, and urea) between the 1 and 6 DAP. At the same time, the unfertilized control showed a different trend, and the N_2O flux of all microbial-based treatments peaked at the 1 DAP (Fig. 3). After N_2O fluxes peaked, an almost constant flux decline was observed in the three fertilizer treatments and the unfertilized control. The N_2O emissions peak observed were like those previously observed by Bouwman (1996) and Mosier et al. (1994), who found that fertilizer application resulted in short-term increased N_2O emissions that lasted between several days and a few weeks . Furthermore, Pathak et al. (2006) suggested that higher emissions after application of chemical N fertilizers were due to N_2O formation during nitrification of NH_4 .

Nitrous oxide fluxes between microbial-based treatments varied among different days of the incubation (Fig. 3). In the unfertilized treatment, N_2O production fluxes were lower for all three microbial-based treatments (SB, SBF, and BM) throughout the experiment compared to the control (no microbial-based treatment). This last treatment also showed a very distinct and high peak at the 1 DAP.

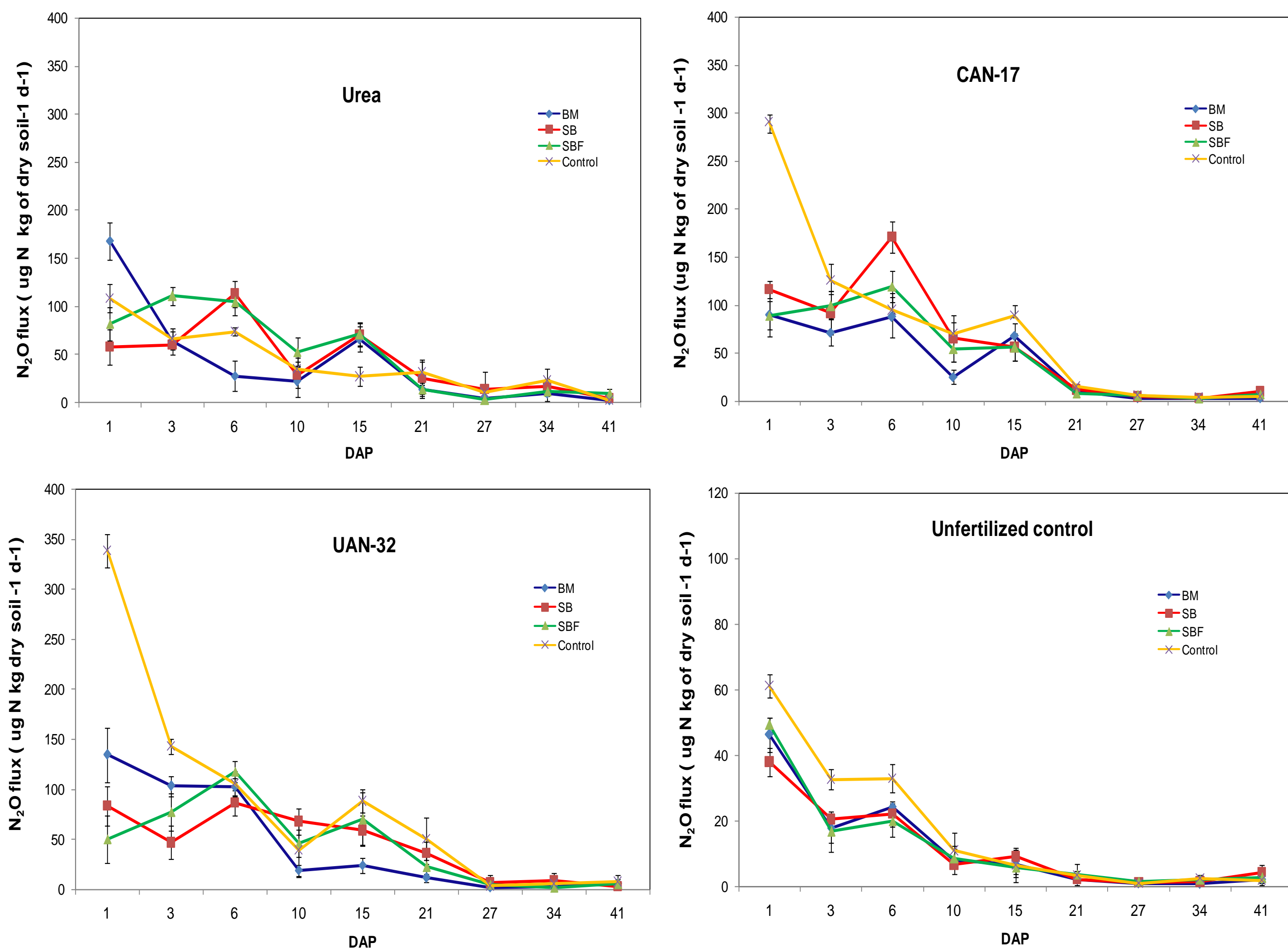

Figure 3.N_2O fluxes during 41 days of evaluation per type of N fertilization regimen: calcium ammonium nitrate (CAN-17), urea ammonium nitrate (UAN-32), urea, and unfertilized control. Microbial-based treatment: SB (SoilBuilder™), SBF (SoilBuilder™ filtered), BM (*Bacillus* PGPR mix), and Control (no product applied).

In CAN-17 fertilized soils, the control (no microbial-based treatment) was the one that showed the highest peak at the 1 DAP. All the microbial-based treatments (SB, SBF, and BM) peaked on the 6 DAP, with SB having the higher peak when CAN-17 was presented. In a similar way, N_2O fluxes of plants that received UAN-32 also showed the highest peak with the control (no microbial-based treatment) on the 1 DAP , and the three microbial-based treatments also peaked on the 6 DAP. However,

in this case none of the three microbial-based treatments produced a distinctly higher peak. In contrast, adifferent results were observed when urea was present. Unlike the other two fertilizers and the unfertilized control, the control (no microbial-based treatment) did not show the higher peak. In this case BM treatment showed the higher peak at 1 DAP; SB and SBF peaked at 6 DAP.

In order to explain the effect of microbial-based treatments on N_2O emissions it is important to consider that N_2O is regulated by oxygen partial pressure and nitrification, which is also controlled by the concentration of ammonia and pH in the soil (Philippot, et al., 2009). The N_2O emission mechanisms involved when chemical N fertilizers are applied to a soil system are different from the mechanisms involved when plant roots are present. It is important to take into account that plants affect local conditions in the rhizosphere soil in many ways that influence microbial activity, abundance, and community composition (Sørensen, 1997). Several of these factors have a direct impact on microbial communities emitting greenhouse gases (GHG), which are of major concern for global climate change (Molina and Rovira, 1964). The oxygen partial pressure can be altered in the rhizosphere because of respiration by roots and root-associated microorganisms, root consumption of water, and root penetration into the soil, which decreases soil compaction and creates channels for gas transfer (Philippot, et al., 2009). The complex interaction among plant roots, soil, N, and microorganisms creates difficulty in explaining the capacity of the microbial-based treatments to reduce N_2O by only one mechanism. Enwall et al. (2007) observed that plants could stimulate nitrification due to increased organic matter that in turn enhances N turnover in the soil, in combination with increased aeration. On the other hand, Herman et al. (2006) reported that in some cases the activity of nitrifiers in the rhizosphere was decreased due to the lack of ammonium taken up by the plant or used by other microbes that compete with autotrophic nitrifiers in this carbon rich environment. In this case, microbial-based treatments that contain living microorganisms like BM and SB could be associated with plants roots and compete with autotrophic nitrifiers for the carbon source and the ammonium. Therefore, in this way, the populations of nitrifiers decrease, and less N is available to be converted into N_2O.

Denitrification is the predominant N_2O-producing process soils with more than 70–80% water-filled pore space (Davidson, 1991). In the present study, WFPS was maintained at no more than 60%. However, denitrification is possible in soil microsites, where this process may increase due to the presence of carbon sources. When plants are present in the system, the production of exudates could potentially increase N_2O production via denitrification. However, this potential increase on denitrifiers' activity in the rhizosphere has not been confirmed (Philippot, et al., 2009). It is still important to take into account that the capacity of microbial-based treatments to reduce N_2O emissions could be related to an effect on the denitrification process.

Carbon Dioxide Emissions

Carbon dioxide flux curves showed a very similar pattern in all three fertilizers and the unfertilized control (Fig 4). Fluxes follow a quadratic pattern. They begin high then continuously . It is possible to observe that the CO_2 fluxes for the unfertilized control were in general significantly lower than the ones from the three fertilizers. This observation agrees with that of Barabasz et al. (2002), who suggested that N fertilization could increase microbial activity due to the addition of nutrients. The soil used in the present study was nutrient poor, so when N fertilizer was applied it appears to have enhanced microbial activity.

Carbon dioxide fluxes among microbial-based treatments in each fertilizer type were very similar. Fluxes among microbial-based treatments showed significant differences only with urea on 1 DAP (Fig 4). At this day, the control (no microbial-based treatments) and BM CO_2 fluxes were not significantly different. However, BM and SB (P=0.0252) and BM and SBF (P=0.0465) were significantly different from each other at α=0.05. Carbon dioxide fluxes from the control (no microbial-based treatments) were also significant different from SB (P= 0.0255) and from SBF (P=0.0471) at α=0.05.

Table 2.Total CO_2 production per type of fertilization regimen after 41 days of evaluation.

Treatment	**CO_2 total production (mg C kg of dry soil $^{-1}$)†**			
	Fertilizer			
	CAN-17‡	UAN-32§	Urea	Control
BM	158.71a	195.27 a	227.88 a	195.66 a
SB	210.48 a	246.84 a	239.8 a	193.83 a
SBF	227.34 a	212.3 a	201.15 a	203.23 a
Control	217.74 a	230.69 a	200.88 a	205.57 a

†Means within a column followed by the same letter are not significantly different at the 0.05 level using LSD values.SB (SoilBuilder™), SBF (SoilBuilder™ filtered), BM (*Bacillus* PGPR mix), and Control (no product applied).
‡ Calcium ammonium nitrate, § Urea ammonium nitrate

Total CO_2 emissions after 41days of evaluation presented no significant difference among any microbial-based treatment and the control (no microbial-based treatment) in any of the three fertilizer treatments and the unfertilized control (Table 2). These results differ from the ones observed in a previous study by Calvo et.al (2013) where the same microbial-based treatments did affect the CO_2 emissions in a soil incubation study. However, the present study includes a plant, which affects the CO_2 emissions. Regarding the CO_2 fluxes during the 41 days of the experiment, it is important to notice that in the first 15 DAP (Fig 4) the flux constantly decreased, which agrees with the previous observation in the soil incubation study (Calvo, et al., 2013). After the first 15 DAP, CO_2 fluxes began to increase. This increase coincides with the time in which the seedling has already germinated. It was reported before that when a plant is present in the chamber, CO_2 fluxes are the result of dark chamber respiration that includes plant, root, and soil microbial respiration (Artz, et al., 2013, Zou, et al., 2005). Therefore, at 15 DAP it is difficult to know what specific process accounts for the increase of CO_2.

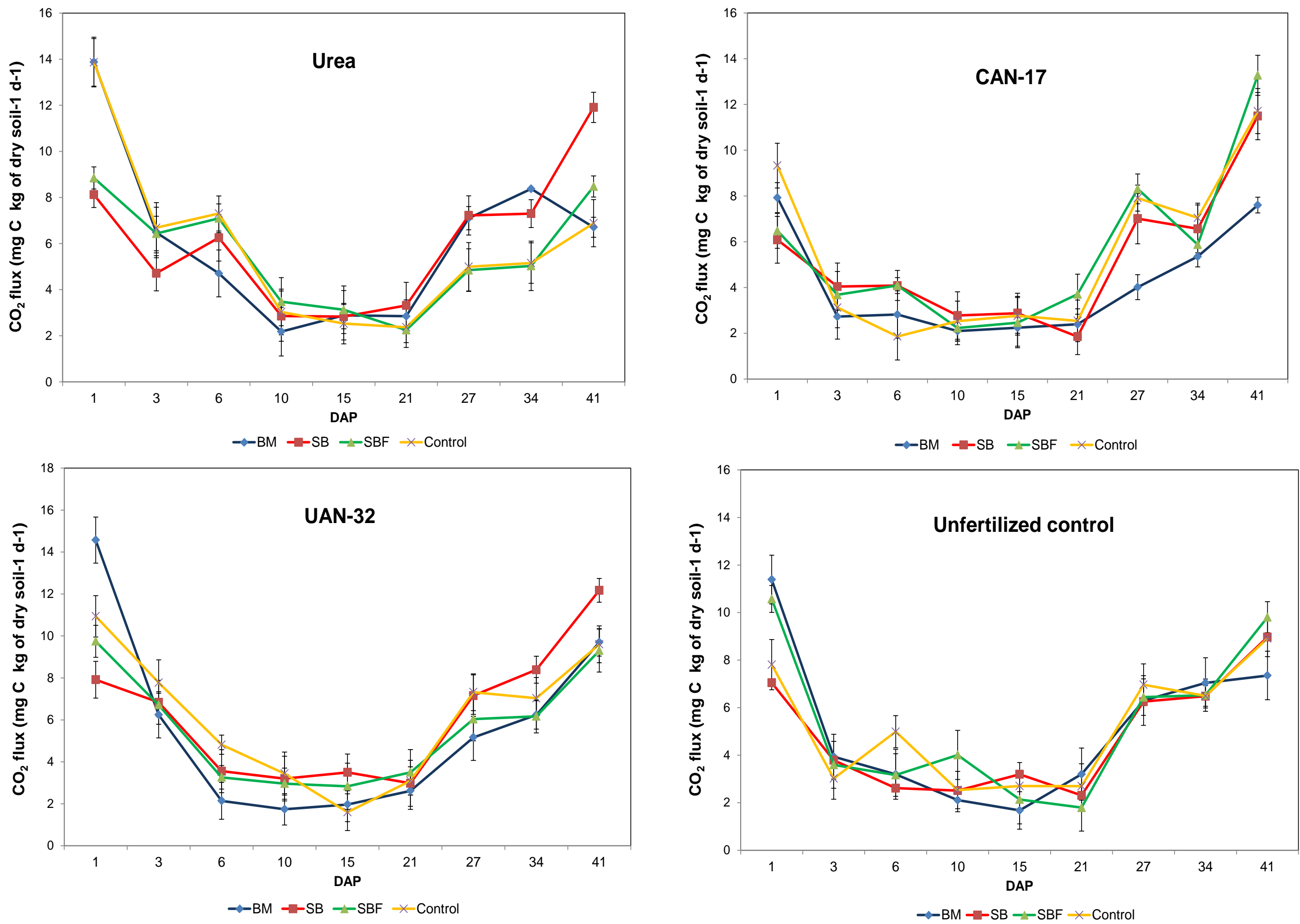

Figure 4. CO_2 fluxes during 41 days of evaluation per type of N fertilization regimen: calcium ammonium nitrate (CAN-17), urea ammonium nitrate (UAN-32), urea, and unfertilized control. Microbial- based treatment: SB (SoilBuilder™), SBF (SoilBuilder™ filtered), BM (*Bacillus* PGPR mix), and Control (no product applied).

Nitrate and Ammonium Concentrations in Soil

As expected, the concentrations of NO_3^--N and NH_4^+-N in soil extracts from the unfertilized treatment (Fig.5 and Fig.6) were much less than concentrations observed with the N-fertilized treatments.

UAN-32 was the fertilizer that showed a higher amount of NH_4^+-N, which helps explain why the greater amounts of N_2O emissions were produced by this fertilizer. As mentioned above, fertilizers with higher amounts of NH_4^+-N could potentially loose N as N_2O due to the nitrification process.

There is not a specific pattern for the ammonium concentration in all fertilizer types and the unfertilized control (Fig 5). For urea and the unfertilized control, NH_4^+-N levels began high and then diminished progressively until reaching small values at 41 DAP. Meanwhile, for CAN-17 and UAN-32, values show a diminishing trend. After one day of planting with CAN-17, SBF treatment had a significantly higher NH_4^+-N value compared to the other treatments (Table 3). Having significantly more NH_4^+-N in the system could mean that less NH_4^+-N was converted to N_2O trough nitrification, and this could help to explain why SBF reduced N_2O at 3 DAP (Fig 3). However, the other two microbial-based treatments also reduced N_2O at 3 DAP, and they did not show significantly more NH_4^+-N at 3 DAP. The fact that SBF affected NH_4^+-N at 3 DAP is an indication that it has little or no direct connection with the reduction of N_2O. On 15 DAP, the control (no microbial-based treatment) showed a spike on NH_4^+-N. This value is significantly higher compared to the other treatments. This spike on NH_4^+-N could be the result of many different processes going on at the same time. It is important to remember that more NH_4^+-N could have been added to the system due to mineralization process. Also, at 15 DAP plants are already germinated and absorbing nutrients from the soil, so the presence of more NH_4^+-N could mean that plants are not absorbing this nutrient too quickly. Furthermore, Nguyen (2003) observed that plants also release readily available organic compounds in soil solution through rhizodeposition, of which root exudation is the largest component . So increases of NH_4^+-N at 15 DAP could be explained by root exudates that mineralize and convert to NH_4^+-N. Another important consideration is that the application of microbes could indicate that immobilization of N occurred, leaving less immediately available NH_4^+-N present in the soil . Immobilization could be also one of the processes that could explain how bacteria might reduce N_2O emissions. Less NH_4^+-N is available in the soil so less nitrification could be happening (Eichner, 1989).

Table 3. Ammonium (NH_4^+-N) soil content per day after planting (DAP).Microbial-based treatment: Bacillus mix (BM), Soil Builder (SB), Soil Builder filtrated (SBF), and control (no microbial-based treatment).

Fertilizer Treatments	**Microbial-based Treatments**	**NH_4^+-N (mg/kg soil) ‡**							
		Days after planting (DAP)†							
		3	**6**	**10**	**15**	**21**	**27**	**34**	41
CAN-17ǂ	BM	46.46 b	36.96 a	40.04 a	29.73 bc	17.38 b	17.75 a	7.77 a	4.48 a
	SB	36.08 b	46.79 a	27.29 b	31.76 b	11.3 bc	14.61 a	1.6 b	1.38 b
	SBF	82.7 7 a	37.77 a	29.32 b	21.38 c	26.55 a	3.44 b	1.01 b	2.76 ab
	Control	39.31 b	35.82 a	27.43 b	66.01 a	10.44 c	14.06 a	1.62 b	2.65 ab
UAN-32ǂ	BM	90.53 bc	71.89 a	69.11 ab	55.69 b	31.24 a	25.91 a	9.73 a	1.73 bc
	SB	71.51 c	52.91 b	50.57 c	62.29 b	22.42 ab	23.81 a	2.98 b	5.44 a
	SBF	111.4 ab	45.62 b	78.17 a	50.32 b	29.02 a	28.37 a	8.54 a	3.21 b
	Control	118.14 a	71.82 a	57.98 bc	115.37 a	16.71 b	4.31 b	1.01 b	0.84 c
Urea	BM	26.06 c	37.49 a	27.49 b	46.23 a	25.12 a	11.43 a	0.92 a	1.518 b
	SB	45.88 b	47.76 a	46.15 a	35.34 b	24.28 a	4.33 bc	1.36 bc	4.594 a
	SBF	51.23 b	32.09 a	28.16 a	29.78 bc	26.36 a	7.64 b	2.17 b	2.48 ab
	Control	69.05 a	43.24 a	37.32 ab	24.93 c	23.4 a	2.98 c	2.76 c	1.49 b
Unfertilized control	BM	5.21 a	2.06 a	0.75 a	0.49 a	0.11 b	0.58 a	0.73 a	0.76 a
	SB	3.58 a	2.04 a	1.34 a	0.02 b	0.73 a	0.32 a	0.26 ab	0.75 a
	SBF	3.96 a	2.31 a	2.07 a	0.23 ab	0.36 c	0.16 a	0.47 ab	0.48 a
	Control	3.94 a	2.17 a	0.71 a	0.26 ab	0.05 b	0.2 a	0.21 b	0.07 b

†Means within a column followed by the same letter are not significantly different at the 0.05 level using LSD values. SB (SoilBuilder™), SBF (SoilBuilder™ filtered), BM (*Bacillus* PGPR mix), and Control (no product applied).

ǂ Calcium ammonium nitrate (CAN-17) ,and urea ammonium nitrate (UAN-32).

‡ NH_4^+ -N values were calculated based on kg of dry soil.

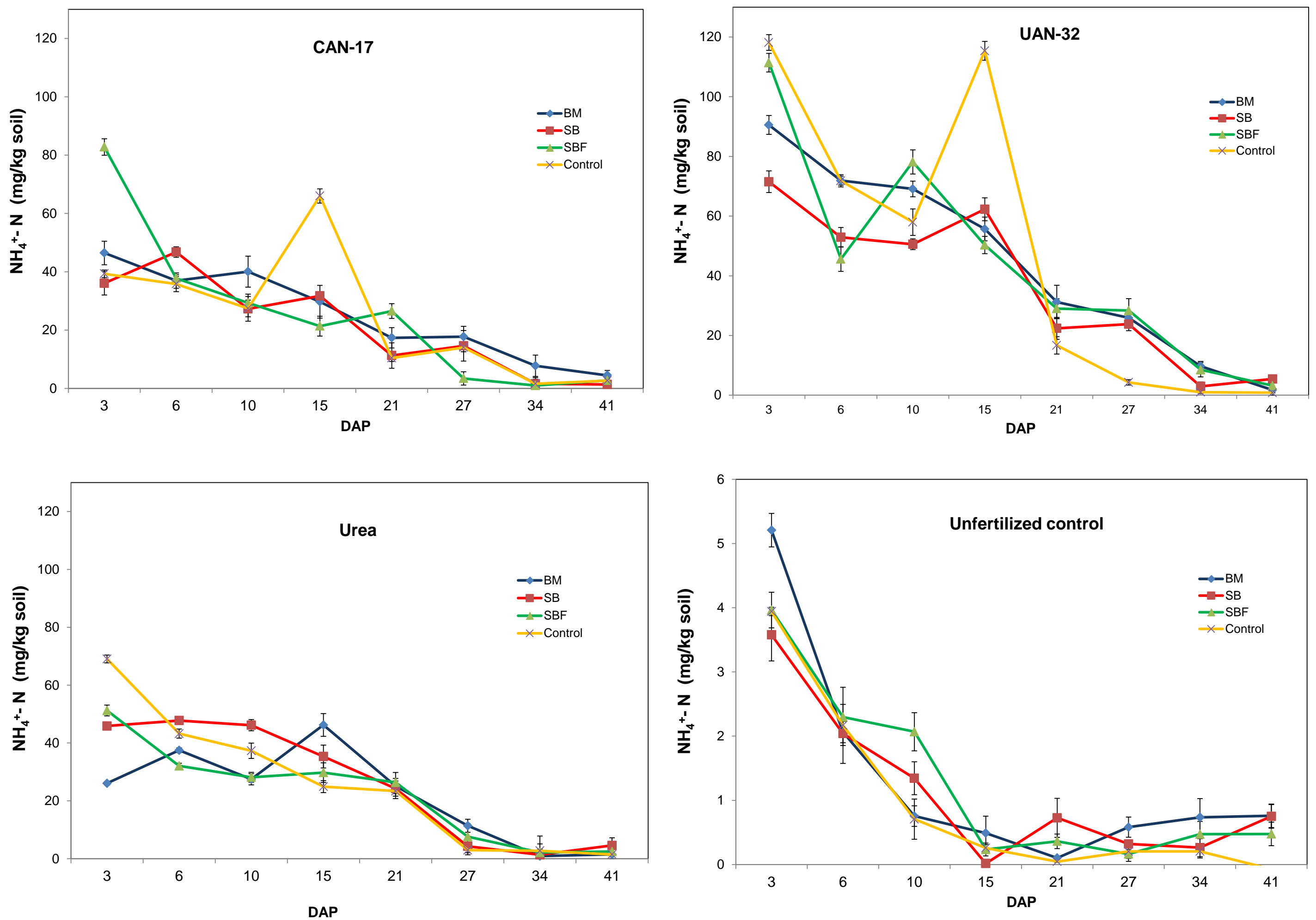

Figure 5.Ammonium (NH_4^+-N) soil concentration during 41 days of evaluation per type of N fertilization regimen: calcium ammonium nitrate (CAN-17), urea ammonium nitrate (UAN-32), urea, and unfertilized control. Microbial-based treatment: SB (SoilBuilder™), SBF (SoilBuilder™ filtered), BM (*Bacillus* PGPR mix), and Control (no product applied).

In contrast with CAN-17, when UAN-32 was present in the system, the control (no microbial-based treatment) and SBF are the treatments that had significantly higher amounts of NH_4^+-N at 3 DAP compare to the other two treatments. Interestly, the control (no microbial-based treatment) also had a significantly higher peak at 15 DAP that is similar to the one observed with CAN-17. However, with UAN-32 soil, NH_4^+-N soil dynamics are different than with CAN-17. UAN-32 contains 16.5 % of urea in its composition. This urea becomes NH_4^+-N by the action of urease enzymes. Therefore, the peak at 15

DAP observed could be due to the conversion of urea to NH_4^+-N. Nevertheless, it is important to note that in both fertilizer regimens (CAN-17, UAN-17) in which microbial-based treatments have a significant effect on the reduction of N_2O emissions, a peak in NH_4^+-N was observed. Even though these two fertilizers have different forms of N content and their soil dynamics are different, there could be a common soil nutrient cycle process affected by the microbial-based treatments that is responsible for the reduction in N_2O emissions.

Table 4. Nitrate (NO_3^--N) soil content per day after planting (DAP).Microbial- based treatment: Bacillus mix (BM), Soil Builder (SB), Soil Builder filtrated (SBF), and control (no microbial-based treatment).

Fertilizer Treatments	**Microbial-based Treatments**	**NO_3^--N (mg/kg soil) ‡**							
		Days after planting (DAP)†							
		3	**6**	**10**	**15**	**21**	**27**	**34**	41
	BM	66.75 b	44.74 a	71.99 a	38.99 c	55.68 a	48.82 ab	68.84 a	92.36 a
CAN-17ǂ	SB	68.75 b	67.75 a	33.26 b	50.13 b	39.87 ab	57.45 a	37.19 b	53.71 b
	SBF	111.03 a	43.09 a	44.45 b	39.63 bc	35.37 b	36.65 b	64.3 a	55.03 b
	Control	41.32 b	74.68 a	38.99 b	133.04 a	35.84 b	55.50 a	53.91 ab	43.75 b
	BM	29.47 ab	19.21 a	35.11 ab	59.39 ab	32.43 a	51.07 a	53.15 a	31.06 a
UAN-32ǂ	SB	19.57 b	22.42 a	32.68 b	47.11 b	32.63 a	49.05 a	70.92 a	28.82 a
	SBF	38.75 a	14.09 a	48.76 a	50.93 b	31.62 a	50.11 a	62.71 a	37.88 a
	Control	45.85 a	22.84 a	35.33 ab	76.58 a	38.09 a	49.12 a	56.17 a	26.97 a
	BM	1.72 a	3.92 a	9.25 c	35.37 a	39.42 b	23.47 b	57.57 a	39.61 a
Urea	SB	4.81 a	5.06 a	16.63 a	23.72 a	29.71 bc	26.62 b	59.52 a	26.48 b
	SBF	4.79 a	4.15 a	13.47 ab	30.66 a	51.85 a	31.91 b	41.52 a	29.91 ab
	Control	3.09 a	4.54 a	11.22 bc	38.15 a	23.43 c	54.66 a	41.03 a	23.82 b
	BM	2.84 a	2.95 b	6.19 a	5.49 a	5.19 a	6.16 a	1.78 ab	3.23 a
Unfertilized control	SB	2.22 a	2.29 b	5.55 a	6.78 a	5.91 a	6.53 a	0.89 b	3.46 a
	SBF	1.69 a	3.07 b	5.15 a	3.04 a	6.69 a	6.29 a	1.66 ab	2.31 ab
	Control	3.51 a	4.05 a	5.47 a	4.99 a	5.94 a	4.14 a	4.31 a	0.87 b

†Means within a column followed by the same letter are not significantly different at the 0.05 level using LSD values. SB (SoilBuilder™), SBF (SoilBuilder™ filtered), BM (*Bacillus* PGPR mix), and Control (no product applied).
ǂ Calcium ammonium nitrate (CAN-17) ,and urea ammonium nitrate (UAN-32).
‡NO_3^- -N values were calculated based on kg of dry soil.

With urea, NH_4^+-N values started higher at 3 DAP but were lower than the other two fertilizers. This was expected due to the lack of NH_4^+-N in the fertilizer. As mentioned above, urea needs to go through a process to be converted to NH_4^+-N. No distinct peak was observed during the 41 days of the experiment, and NH_4^+-N content throughout the experiment was similar among the microbial-based treatments and the control (no microbial-based treatments). It seems that none of the microbial-based treatments had a greater effect on NH_4^+-N in this case. Finally, when no N fertilizer was applied, microbial-based treatment presented a significantly higher amount of NH_4^+-N at days 21, 34, and 41 (Table 3) compared to the other treatments. The mechanisms involved in the reduction of N_2O emissions may be different when no N fertilizer is present in the system.

Nitrate (NO_3^--N) content in the three fertilizer treatments and the unfertilized control did not follow a specific trend (Fig 6). They increased and decreased constantly throughout the experiment. With CAN-17, significantly more nitrate was found at 3 DAP when SBF was applied (Table 3). Then, at 15 DAP, the control (no microbial-based treatment) showed a higher and significant peak of NO_3^--N. Ae significant event may be occurring at 15 DAP in the control (no microbial-based treatment) that is not only affecting NO_3^--N in the soil but NH_4^+-N content as well. By the final day of the incubation, a significant increase in NO_3^--N was observed by BM treatment.

With UAN-32 a significantly higher amount of NO_3^--N was produced by SBF and the control (no microbial-based treatment) at 3 DAP. These results are very similar with ones observed with NH_4^+-N content, where the same two treatments presented significantly higher NH_4^+-N contents. At 15 DAP, once again the control (no microbial-based treatment) presented significantly higher amounts of NO_3^--N. Nevertheless, this higher content was not as high as the one observed with CAN-17.

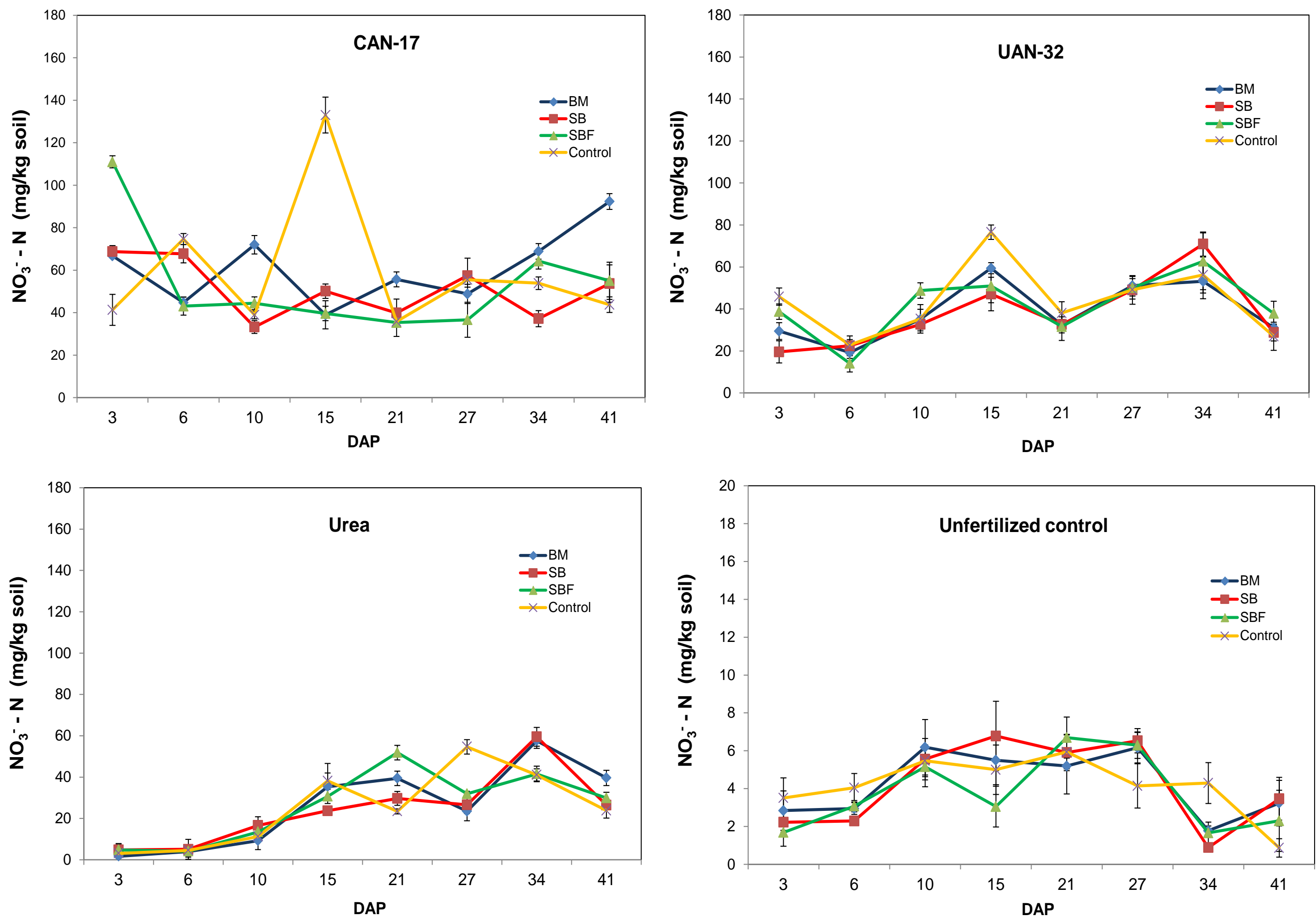

Figure 6. Nitrate (NO_3^-N) soil concentration during 41 days of evaluation per type of N fertilization regimen: calcium ammonium nitrate (CAN-17), urea ammonium nitrate (UAN-32), urea, and unfertilized control. Microbial-based treatment: SB (SoilBuilder™), SBF (SoilBuilder™ filtered), BM (*Bacillus* PGPR mix), and Control (no product applied).

Nitrate values in urea-treated soils were considerably lower at the beginning of the experiment compared to the other two fertilizer treatments. This difference was due to the lack of a nitrate source in the fertilizer. Nitrate levels tended to increase, and all treatments presented very similar curves. At the end of the incubation, BM treatment showed a significantly higher amount of NO_3^--N. Similar to urea, the unfertilized control showed very low values of NO_3^--N due to the lack of N fertilizer. The only difference among NO_3^--N values was observed at the final day of the experiment when BM and SB had significantly higher amount of NO_3^--N compared to the control (no microbial-based treatments).

Ammonium and nitrate soil contents in this experiment give important information about the N soil dynamics and could give some clues about the effect of microbial-based treatments on the N content of the soil. In many cases the variations in NO_3^--N and NH_4^+-N contents in the soil are related to N_2O emissions. This relationship can help in understanding the processes that could be responsible for any increases or decreases of N_2O emissions. This approach works better when a plant is not included in the system. In the present study the plant is also affecting on the N content in the soil. Nitrogen content in the soil becomes a very complex dynamic with the plant included, adding to the variables of fertilizer type and the microbial-based treatment. Because the microbial-based treatment is also affecting the plant growth and its nutrient uptake, it is very difficult to find a direct link only between N content in the soil and N_2O emissions. Nevertheless, some trends and differences on N content among the microbial-treatments and the control are present. These differences show that microbial-based treatments also affect the N soil dynamics that could be indirectly related to the N_2O reduction effect. Furthermore, with urea there was a negligible effect of microbial -based treatments on NO_3^--N and NH_4^+-N. Coincidentally urea was the only fertilizer in which microbial-based treatments had no effect on reducing N_2O emissions.

Plant Growth Promotion and Nitrogen Uptake

Microbial treatments had an effect on all the parameters evaluated, and the effect was dependent on the type of fertilizer applied. When CAN-17 was applied, differences in fresh and dry shoot weights and N uptake were observed according to microbial-based treatments, with SBF enhancing those parameters the most (Fig 7). SBF was able to increase plant N uptake by 100% over the control. No effects of microbial-based treatments on root parameters, SPAD, and plant height were observed (Fig 8).

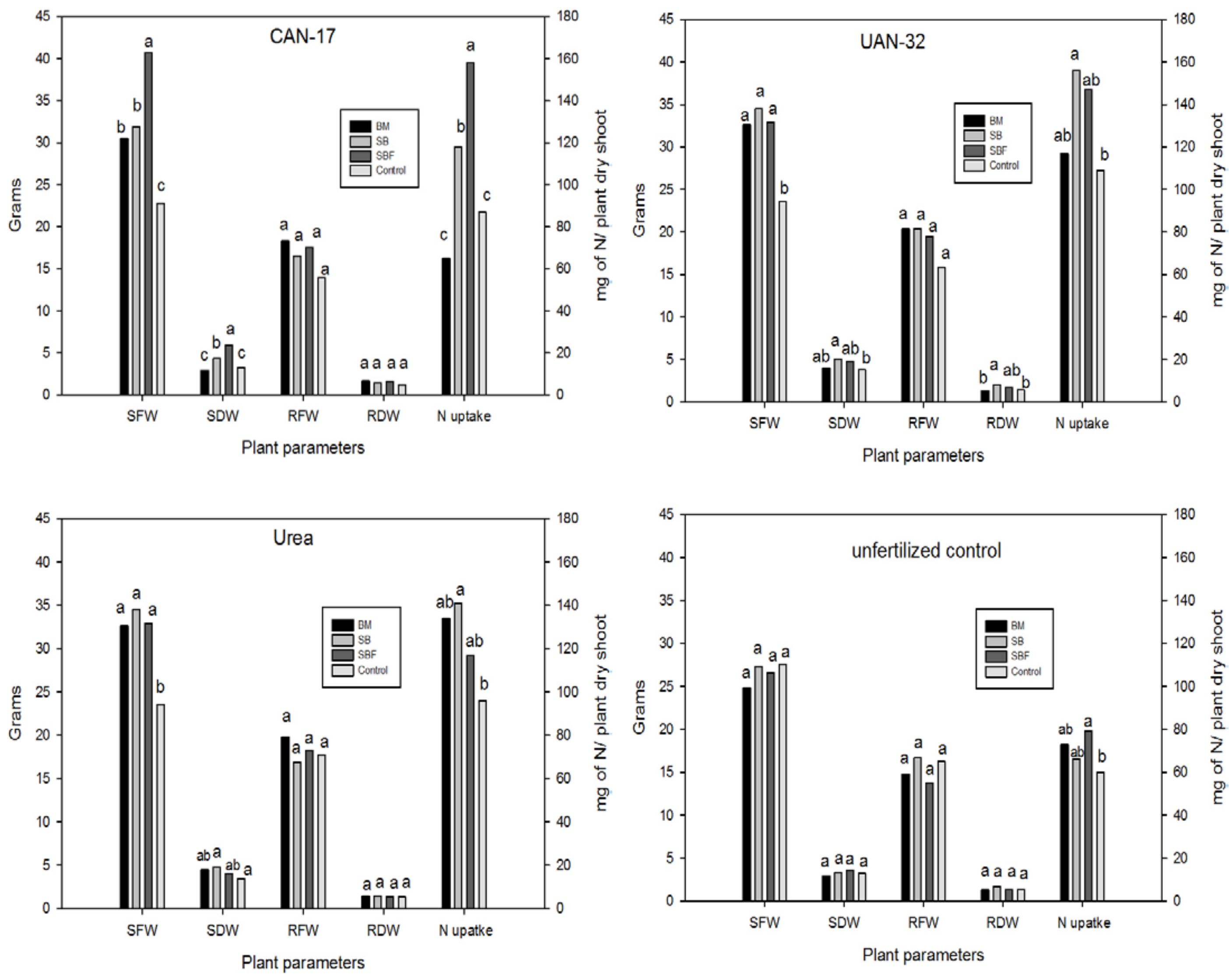

Figure 7. Plant parameters evaluated at V6 stage (43 DAP). Shoot fresh weight (SFW), shoot dry weight (SDW), root fresh weight (RFW), root dry weight (RDW), N uptake measured as total N content in a plant shoot (N uptake) . Microbial- based treatment: SB (SoilBuilder™), SBF (SoilBuilder™ filtered), BM (*Bacillus* PGPR mix), and Control (no product applied). Bars within the same plant parameter followed by the same letter are not significantly different at the 0.05 level using LSD values.

When UAN-32 was applied, plant height, SPAD shoot and root dry weight, and N uptake were the parameters in which microbial treatments showed significant differences compared to the control (no microbial treatments) (Fig 8),. From the three microbial-based treatments, SB resulted in more consistent increases in the four parameters. The increased N uptake was 43% greater than the control (no microbial-based treatment). Significant increases were also observed when urea fertilizer was applied. In this case microbial-based treatments showed a significant effect on SPAD (BM), fresh and dry shoot weights, and N uptake. SB treatment again showed more consistent increases in the last three parameters. Nitrogen uptake was the parameter that was enhanced the most by SB. It increased 47% compared to the control. When no N fertilizer was applied, microbial treatments also increased SPAD, plant height, and N uptake (Fig 8). This last parameter was increased by 32% over the control when SBF was applied.

When looking at all fertilizers, none of the microbial-based treatments showed any effect on fresh and dry root weights in CAN-17, urea, and the unfertilized control. Significant differences on root dry weight were only observed when SB was applied in the presence of UAN-32. There was not a single microbial-based treatment that consistently performed best in all fertilizer regimes. Hence, there is an effect of the N fertilizer on the ability of the microbial-based treatment to enhance plant growth. It was previously reported that the capacity of microbial-based treatment such as PGPR to enhance nutrient uptake was due to an increase in root growth, which allowed the plant to absorb more nutrients (Hayat, et al., 2010). However, in the present study we found that microbial treatments resulted insignificant differences in the plant N uptake, but an increase in root biomass was not reported except with UAN-32. This last observation agrees with a previous study by Bertrand et al. (2000) who reported that inoculation with the PGPR *Achromobacter* spp. increased N uptake rate without increasing root biomass. In light of these results, one hypothesis is that N uptake may involve other mechanisms that are not related to root growth or root morphology.

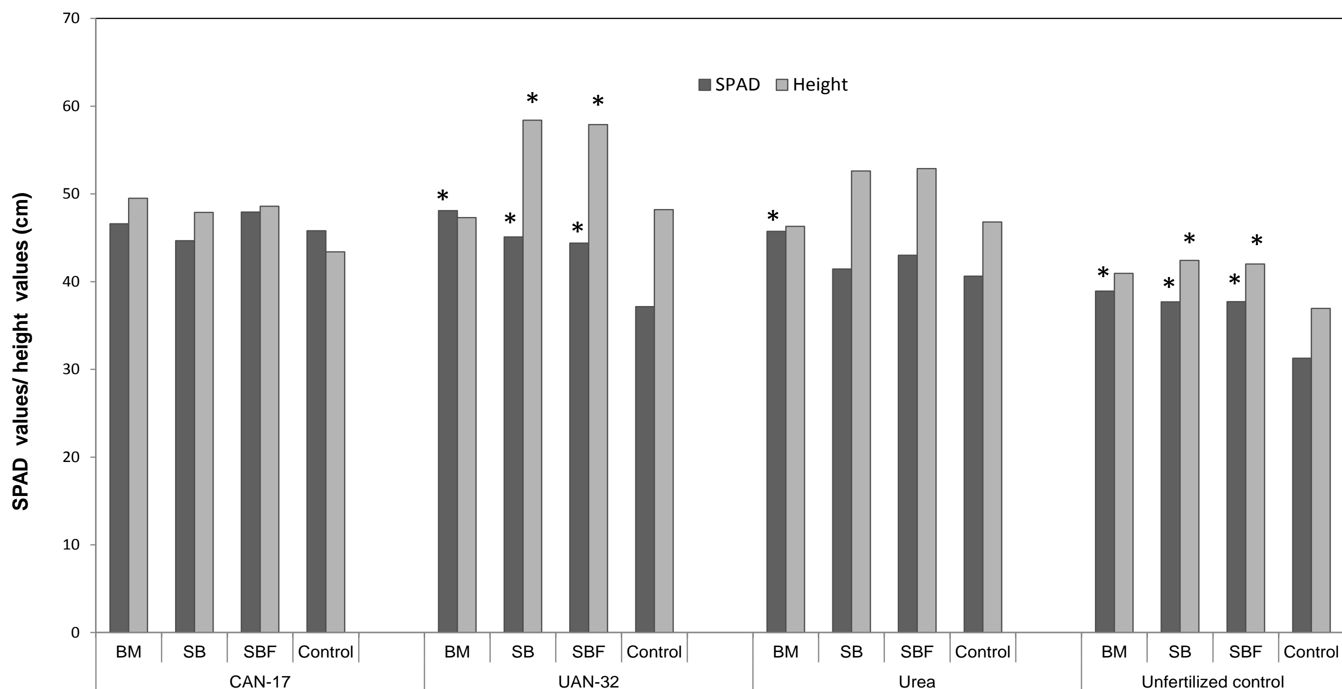

Figure 8.Values of SPAD and plant height (cm) evaluated at V6 stage (43 DAP) per type of N fertilizer. Microbial-based treatment: SB (SoilBuilder™), SBF (SoilBuilder™ filtered), BM (*Bacillus* PGPR mix), and Control (no product applied). Bars within the same plant parameter and within the same fertilizer type followed by an asterisk are significantly different from the control (no microbial-based treatment) at the 0.05 level using LSD values.

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IV. Conclusions

The results reported here support our hypothesis that microbial-based inoculants can reduce N_2O emissions from a soil-plant system. The results obtained also support the previous results reported by (Calvo, et al., 2013) in a soil incubation study using the same microbial-based treatments and the same fertilizers. Similar to the results obtained in the soil incubation study, microbial-based treatments were able to reduce N_2O emissions in the unfertilized control and with all fertilizers except urea. Nitrous oxide emissions were reduced the most when CAN-17 fertilizer was applied. Higher reductions of N_2O emissions were observed by BM treatment. This microbial treatment reduced N_2O emissions by 41%, 49%, and 28% with UAN-32, CAN-17, and unfertilized control, respectively. No effect on total CO_2 emissions by any of the microbial-based treatments in any of the fertilizers was observed.

Microbial-based treatments increased plant growth promotion and plant N uptake. SB treatment increased plant parameters and N uptake the most with urea and UAN-32, while SBF was the treatment that enhanced plant parameters and N uptake the most with CAN-17 and unfertilized control. Even though root parameters (fresh and dry weight) were almost never increased by microbial-based treatments, N uptake was significantly increased. The highest increase of N uptake was 100% over the control when SBF was applied with CAN-17.

How the microbial-based treatments were able to reduce N_2O emissions cannot be explained by only one mechanism. The presence of plants roots in the system added one more variable and increased the complexity of the interactions. Some possible mechanisms involved in this reduction effect include (i) production or presence of nitrification inhibitors; (ii) inhibition of nitrifying and/or denitrifying microorganisms; (iii) competition of applied microbial treatments with the native microbial nitrifiers and/denitrifiers; and (iv) immobilization of N fertilizer and root exudates by microbes.

The results presented in this section complement and confirm the results obtained in a previous soil incubation study. Microbial-based treatments demonstrated that they have the potential to decrease N_2O emissions from agriculture soils when a plant is present in the system. Further research is needed in order to know if these results will be also observed in a field environment. Additional research will be needed to better understand the processes involved in the dynamics between microbial-based treatments, the N cycle, plants, and N fertilizers.

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Effect of Microbial-Based Inoculants on Nutrient Uptake and Early Root Morphology of Corn (*Zea mays* L.)

Abstract

Microbial-based inoculants have been reported to stimulate plant growth and nutrient uptake. However, their effect may vary depending on the crop stage evaluated and on the chemical fertilizer applied. Thus, the objective of this study was to test the hypothesis that microbial-based inoculants known to promote root growth and nutrient uptake would promote plant growth, enhance early root development and increase nutrient uptake of corn plants evaluated at 4 different growth stages and in the presence of three different nitrogen fertilizers. The microbial-based treatments evaluated were SoilBuilder (SB), a metabolite extract of SoilBuilder (SBF), and a mixture of four strains of plant growth-promoting *Bacillus* spp. Experiments included an unfertilized control and three different nitrogen fertilizers: urea, urea ammonium nitrate (UAN-32), and calcium ammonium nitrate (CAN-17). Corn plants were evaluated at V2, V4, V6, and VT stages. Plant growth parameters such plant fresh and dry biomass, plant height, and SPAD were enhanced by the three microbial-based treatments. A more significant effect of microbial-based treatments was observed when plants were evaluated at V6 and VT stages. Early root development parameters such as total root length (TRL), root surface area (RSA), and length of fine roots were enhanced when microbial-based treatments were applied. Finally, nutrient uptake of N, P, and K was increased by microbial-based treatments compared to the non-inoculated control. Increases in plant N uptake among microbial-based treatments were on average 72 % with CAN-17, 61% with UAN-32, 72% with urea, and 54% in the unfertilized treatment. Phosphorus uptake was increased the most (138%) when BM was applied with CAN-17. In the same way, when CAN-17 was present, K uptake was increased the most by BM (95%) and on average 65% when SB and SBF were applied. Overall, the results demonstrated that microbial-based inoculants can positively impact corn growth and nutrient uptake,

especially at late vegetative stages. The enhanced nutrient uptake may be related to the capacity of microbial-based treatments to affect the root morphology at early stages of corn growth.

I. Introduction

Modern agricultural practices rely on high inputs of mineral fertilizers to achieve optimum yield; however, a majority of the nutrients applied are often not utilized, leaving the excess susceptible to loss. It has been estimated that nutrient use efficiency (NUE) is on average 50% for nitrogen (N) (Chien, et al., 2009, Roberts, 2008), between 15- 30% for phosphorus (P) (Syers, et al., 2008), and about 40% for potassium (K) (Zou and Lu, 2010). These levels demonstrate the importance of improving crop nutrient uptake. Thus, improvements in NUE may decrease fertilizer input costs and have widespread implications in reducing the environmental impacts of N and P losses, which have been linked to surface and ground water contamination and greenhouse gas emissions. Moreover, as the human population continues to grow, the demand for more food with less input will likely increase. Thus, considerable effort is needed from the scientific community to identify practices that could improve plant production without increasing nutrient inputs.

Plant nutrient acquisition from soil occurs via the root system. Improving soil\root interactions may increase crop production while minimizing the demand for fertilizer inputs. Since soil bordering the root surface is a zone of intense microbial activity that can influence plant nutrient uptake, manipulating the rhizophere processes may be the most effective approach to increase yields and nutrient use. For example, it has been reported that a symbiotic relationship occurs between plants and root-associated bacteria in most rhizosphere environments, but not all (Bashan, Y., and G. Holguin, 1998; Compant et al., 2005). Therefore, dynamically changing the microbial populations in the rhizosphere may influence nutrient transformations, availability, and uptake by plants (Shen et al., 2012). Modulating the root zone with additions of microbial inoculant could promote root growth, increase access to soil moisture, and improve nutrient uptake, thereby potentially reducing the overreliance on fertilizer inputs.

Among the microbial-based inoculants, plant growth-promoting rhizobacteria (PGPR) have received considerable attention over the past two decades with respect to plant growth promotion and root growth (Adesemoye, et al., 2010, Canbolat, et al., 2006, Idriss, et al., 2002). Plant growth-promoting rhizobacteria, usually referred to as beneficial free living bacteria (Kloepper et al., 1989), include bacterial species and strains belonging to the genus *Azotobacter*, *Azospirillum*, *Pseudomonas*, *Acetobacter*, *Herbaspirilum*, *Burkholderia*, and *Bacillus* (Glick 1995, Probanza et al., 1996), with *Bacillus* spp.being among the most commonly reported PGPR (Calvo, et al., 2010, Compant, et al., 2005). These rhizobacteria can influence crop growth and development by physiologically changing the status (Glick and Bashan, 1997;Volpin and Phillips, 1998) and morphological characteristics of inoculated roots (Yanni et al., 1997) to favor improved nutrient uptake (Okon and Kapulnik, 1986; Biswas, et al., 2000).

The plant growth-promoting effects of rhizobacteria may include production of phytohormones (Tien et al., 1979; Hussain et al., 1987; Chabot et al., 1996), N_2 fixation (Urquiaga et al, 1992), more efficient use of N sources (Yanni et al., 1997) and other nutrients (chabot et al., 1996), and production and secretion of siderophores (Neilands and Leong, 1986). Although several studies have evaluated the impact of PGPR inoculants on agricultural crops, most of this research had been conducted on the use of PGPR as biological control agents for plant diseases (Compant et al., 2005; Haas and Défago, 2005; Someya and Akutsu, 2006). There are still many questions regarding their impact on plant growth promotion through nutrient uptake. Enhanced rooting, leading to a greater surface area and increased root hairs (Mahaffee and Kloepper, 1994; Mantelin and Touraine, 2004), has been reported with the use of PGPR. Increased soil P and K solubilization, improved plant N uptake, and other soil nutrients (de Freitas et al., 1997; Rodriguez and Graga 1999; Joo et al., 2004; Sheng and He 2006; Glick et al., 2007) have been enhanced with PGPR inoculations. Thus, integrating microbial-based inoculant use into crop management systems to improve plant growth promotion and nutrient uptake could be advantageous to the future of agricultural production.

Microbial-based inoculants have become commercially available in recent years. SoilBuilder (Agricen Sciences) is an example of a microbial-based inoculant that is being widely marketed. However, there is a lack of published scientific data on the effects of SoilBuilder on plant growth promotion and nutrient uptake. Hence, SoilBuilder was selected as a microbial-based inoculant for the current study and evaluated with a mix of four *Bacillus* spp. strains. We were also interested in testing the effects that the metabolite portion (without microbes) of SoilBuilder may have on growth promotion and nutrient uptake. In addition, it is important to know how different chemical fertilizer sources could affect the microbial-based inoculants. Thus, information is needed about how these products affect nutrient uptake at different stages of plant growth and if there is an effect on root morphology that could be related to plant growth and nutrient uptake using different fertilizer sources. The objective of this study was to test the hypotheses that (1) microbial-based inoculants could increase plant growth and nutrient uptake of corn evaluated at different growth stages (V2, V4, V6, and VT) with different N fertilizer sources (UAN, urea, and CAN) under greenhouse conditions; and (2) microbial-based inoculants could affect early root growth and morphology of corn, which is related with nutrient uptake.

II. Materials and methods

Study Site and Soil Characteristics

A greenhouse pot experiment was performed at the USDA National Soil Dynamics Laboratory in Auburn, Alabama. The soil medium used for this study consisted of a soil:sand mixture. Sand was mixed with soil in order to improve water infiltration and minimize anaerobic conditions during the study. Briefly, a sandy loam soil with a texture of 72.8% sand, 10.4% clay, and 16.8% silt was mixed 3:1 (soil: sand; v:v) with white brick/Mason sand (particle size: 1/8 mm -1/4 mm). The mixture resulted in a soil medium with the texture of loamy sand sand (85.2 % sand, 4.8% clay, and 10% silt). The soil:sand

mixture had a pH of 6.2, CEC of 1.13 cmol kg^{-1}, total N concentration of 0.7 g kg^{-1}, organic matter concentration of 5.3 g kg^{-1}, total C concentration of 2.6 g kg^{-1}, NO_3 concentration of 17.47 µg g-1, NH4 concentration of 0.73 µg g^{-1}, Mg concentration of 125 µg g^{-1}, Ca concentration of 460 µg g^{-1}, P concentration of 7 µg g-1, and K concentration of 41 µg $g-^{1}$. Initial soil analysis was performed by Auburn University Soil Testing Laboratory as described by Hue and Evans (1986). Briefly, total carbon and N were analyzed using an Elementar vario Macro C-N analyzer (Elementar Americas, Inc. Mt. Laurel, NJ, USA). Soil pH was determined on 1:1 soil/water suspensions with a glass electrode meter. Concentrations of P, K, Mg, and Ca were determined using Melich 1 (double acid extracting solution) (Olsen and Sommers, 1982) and measured using an ICAP 9000 (Thermo Jarrell Ash, Franklin, MA, USA). The Cation Exchange Capacity (CEC) was determined by base summation (Ca, Mg, K, and Na), according to procedures of Hue and Evans (1986).

<u>*Microbial Source Preparation*</u>

SoilBuilder™ is a commercially available microbial soil amendment manufactured by Agricen Sciences (Pilot Point, TX). SoilBuilder™ is prepared from a bioreactor system consisting of a continuously maintained microbial community (patent pending). The final product contains bacteria and bacterial metabolites derived from the bioreactor. Based on plate counts using tryptic-soy agar (TSA) (24 h at 25 °C incubation), the most commonly occurring bacteria within the final stabilized product are *Acidovorax facilis*, *Bacillus licheniformis*, *Bacillus subtilis*, *Bacillus oleronius*, *Bacillus marinus*, *Bacillus megaterium*, and *Rhodococcus rhodochrous*, each at $1x10^3$ cfu/cc. SoilBuilder™ filtered (SBF) consists of SoilBuilder™ product without microbial cells and was prepared by filtering SB through a 0.45 µm filter and then through a 0.22 µm filter. SBF contains microbial metabolites derived from the bioreactor production system that, in addition to other components, include organic acids, peptides, and enzymes. The PGPR Bacillus mixture (referred to as BM) included four Bacillus strains: *Bacillus safensis* T4 (previously called *B. pumilus* T4), *Bacillus pumilus* INR7, *Bacillus subtilis subsp. subtilis* IN937a

(previously called *B. amyloliquefaciens* IN937a), and *Lysinibacillus xylanilyticus* SE56 (previously called *Bacillus sphaericus* SE56). These strains were obtained from culture collections in the Department of Entomology and Plant Pathology, Auburn University (Auburn, AL, USA). These strains have been shown to have important plant growth-promoting effects (Enebak et al., 1998; Jetiyanon et al., 2003; Kokalis-Burelle et al., 2002; Kokalis-Burelle et al., 2003).

Microbial-based treatments were applied at a rate of 375 mL per pot. For the BM treatment, the bacterial mix was prepared by mixing each strain's spore suspension, which was previously quantified by plating on TSA and incubating for 48 h at 25 °C. The spore mix was then adjusted to a concentration of $l0^5$ cfu mL^{-1}. The final concentration in each pot was 6.2 1 x 10^3 cfu /g of dry soil. The SoilBuilder™ product (SB) solution was prepared according to the label instructions by mixing 16 mL of SoilBuilder™ in 1.0 L of distilled water immediately before setting up the experiment. SoilBuilder™ product contained 10^6 cfu mL $^{-1}$, so the final concentration in each pot was 10^3 cfu/g of dry soil. The treatment of SoilBuilder™ filtered (SBF) was prepared in the same way as SB, but before applying the 25 mL to the incubated sample, the solution was filtered. Sterility of the filtrate was confirmed by plating onto TSA (48 h at 25 °C incubation) and observing no bacterial growth. Non-filtered SoilBuilder™ population concentrations were also confirmed by plate count on TSA after incubation for 48 h at 25°C.

<u>*Experimental Setup*</u>

Pots (6 L, C600 Classic from Nursery supplies, Kissimmee, FL) were filled with 6 kg of soil: sand mix and adjusted to a 60% WHC with treatments and fertilizer additions. The greenhouse was maintained at 27 ±2 ºC throughout the experiment. Treatments were organized in a complete randomized block design (CRBD) with a 4 × 4 factorial arrangement with three microbial treatments and a water control and three N fertilizer sources and one unfertilized control, each with 20 replicates. The N fertilizer sources included (i) urea-ammonium nitrate (UAN) -32%, (ii) urea, and (iii) calcium ammonium nitrate (CAN)-17%. Nitrogen fertilization was calculated based on 168 kg ha^{-1}. The experiment was designed to

provide the same amount of N regardless of N fertilizer source. Other nutrients were applied in order to have the same concentration of nutrients in each pot. Calcium fertilization was applied as $CaCl_2$ at a rate of 6.6 g/pot (UAN-32, urea, and unfertilized control) and 5.9 g/pot (CAN-17). Phosphorus and K fertilization was applied as K_2HPO_4 at a rate of 1.3 g/pot. The fertilizer source corresponding to each treatment was added in liquid form, followed by the appropriate microbial treatment. Two seeds of corn hybrid DKC61-73 (Dekalb Seed Company, Cedar, Iowa) were sown per pot and thinned to 1 plant per pot 3 d after germination. Pots were watered daily to keep the same moisture (20% moisture) in all the pots and avoid leaching. Soil moisture content of each pot was maintained daily by measuring it with a WaterScout SM 100 soil moisture sensor (Spectrum technology, Inc, Aurora, IL.).

Plant Evaluation

Five plants from each treatment were harvested and evaluated at V2 (14 DAP), V4 (27 DAP), and V6 stage (43 DAP), and the remaining five replicates were evaluated later at VT stage (72 DAP). Plant parameters of fresh shoot and root weight, dry shoot and root weight determination (70 °C), chlorophyll content (SPAD 502 meter), plant height, and root biomass were measured at V2, V4, V6, and VT stages. Root morphology was determined only at the V2 and V4 stages. Plant nutrient analysis was performed on the dried shoot tissue from V4, V6, and VT stages. Ground tissue from each plant shoot was analyzed for N using the combustion method (LECO FP-528 Nitrogen Analyzer), and an Inductively Coupled Argon Plasma Emission Spectrophotometer/Vacuum (ICP) was used to measure P, K, S, Mg, Ca, S, B, Fe, Cu, Al, Zn, and Mn concentrations. Root biomass was determined by carefully washing roots on a sieve with 0.5 mm mesh screen. An extra sieve of 0.2 mm was placed at the outflow of the system to make sure that no fine root material was lost. Plant roots from the V2 and V4 stages only were analyzed for root morphology using the WinRHIZO Arabidopsis software v2009c 32 bit (Regent Instruments, Quebec, Canada) system connected to an Epson XL 10000 professional scanner. Each root system was evenly spread apart in a water layer on a transparent tray (30 cm x 40 cm) and imaged at a

resolution of 400 dpi (dots per inch) as described by (Bauhus and Messier, 1999, Costa, et al., 2000). The following root characteristics were determined: total root length (cm) (TRL), root surface area (cm^2) (RSA), root volume (cm^3) (RV), and total root length of very thin roots with diameter range of 0-0.5mm. A large root sample was subdivided into smaller sub samples before scanning to avoid a high scanning density. Once the root morphological characteristics were determined, root samples were oven dried (70 °C) to determine dry matter weight.

Statistical Analysis

Analysis of variance (ANOVA), using a general linear model (GLM), was used fisrt to analyze the effect of microbial-based treatment, fertilizer and the interaction of both on each plant parameter. Then the response of each variable per fertilizer type was analyzed using the least significant difference (LSD) test was used to identify significant differences between treatments (SB, SBF, BM, and control). Pearson correlations were also used to identify relations between variables (SPAD and N uptake).All statistical analyses were performed using SAS 9.2 (SAS Institute, 2010) and a significance level of $\alpha=0.05$ set *a priori*.

III. Results

Plant Growth Promotion

Plant growth parameters evaluated in this study are presented in Tables 1, 2, 3, and 4. At V2 (Table 1) statistical differences were observed in SPAD, height, and fresh root and shoot weight for some of the fertilizers. The highest SPAD and height measurements were observed with BM when CAN-17 fertilizer was applied. SB enhanced fresh shoot weight, and SBF significantly increased fresh root weight when urea was present. When no N fertilizer was applied, the three microbial-based treatments increased SPAD readings, which also occurred with results observed when urea was present. The SB and SBF

treatments significantly increased fresh shoot and root weight, respectively. Microbial-based treatments did not show any effect on the plant parameters when UAN was applied.

When plants were evaluated at V4 (Table 2),and compared to V2 stage, fewer significant effects of microbial-based treatments were observed. The SBF treatment increased SPAD readings and plant height when urea was applied, while higher shoot dry weight was observed with SB when UAN was present. However, BM and SBF treatments increased plant height only in the unfertilized treatment. None of the parameters evaluated when CAN-17 was applied showed a significant increase with the application of microbial-treatments.

Table 1. Effect of inoculation with microbial-based treatments on corn parameters: SPAD, height, fresh shoot weight, shoot dry weight, fresh root weight, and dry root weight under different fertilizer treatments evaluated at V2 stage of growth (average of five replicates)

Fertilizer treatment	Microbial-based treatments	SPAD†	Height† (cm)	Fresh shoot w† (g)	Shoot dry w† (g)	Fresh root w† (g)	Dry root w † (g)
CAN-17	BM	49.87 a	20.9 a	2.69 a	0.188 a	37.3 a	0.059 a
	SB	46.65 b	19.5 ab	2.04 a	0.141 a	35.1 a	0.046 a
	SBF	48.82 ab	19.2 ab	2.34 a	0.182 a	35.0 a	0.062 a
	Control	47.15 b	15.5 b	2.07 a	0.173 a	38.4 a	0.048 a
UAN-32	BM	47.81 a	18.3 a	2.19 a	0.177 a	41.8 a	0.042 a
	SB	47.45 a	16.9 a	1.94 a	0.157 a	39.2 a	0.046 a
	SBF	46.98 a	16.2 a	2.15 a	0.183 a	38.2 a	0.049 a
	Control	46.63 a	18.2 a	1.73 a	0.146 a	35.5 a	0.042 a
Urea	BM	42.07 a	14.3 a	1.48 b	0.139 a	38.1 ab	0.038 a
	SB	46.27 a	18.7 a	2.12 a	0.171 a	35.7 ab	0.044 a
	SBF	45.61 a	17.2 a	1.79 ab	0.152 a	41.8 a	0.045 a
	Control	46.87 a	15.4 a	1.83 ab	0.156 a	33.3 b	0.052 a
Unfertilized control	BM	39.85 a	15.5 a	2.04 ab	0.123 a	38.5 a	0.060 a
	SB	41.91 a	17.8 a	2.41 a	0.143 a	32.0 ab	0.063 a
	SBF	39.91 a	14.6 a	2.16 ab	0.126 a	36.8 a	0.061 a
	Control	35.16 b	16.3 a	1.81 b	0.126 a	29.7 b	0.068 a

†Means within a column followed by the same letter are not significantly different at the 0.05 level using LSD values. SB (SoilBuilder™), SBF (SoilBuilder™ filtered), BM (Bacillus PGPR mix), and Control (no product applied).

Microbial treatments showed a greater effect for plant growth parameters at the V6 stage compared to that observed during V2 and V4 (Table 3). When CAN-17 was applied, differences in fresh shoot and dry weights were observed for the microbial-based treatments, with SBF having a greater enhancement on both parameters. When UAN-32 was applied, plant height, SPAD, and shoot and root dry weight were significantly different for the microbial treatments compared to control (no microbial treatments).

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Table 2. Effect of inoculation with microbial-based treatments on corn parameters: SPAD, height, fresh shoot weight, shoot dry weight, fresh root weight, and dry root weight under different fertilizer treatments evaluated at V4 stage of growth (average of five replicates)

Fertilizer treatment	Microbial-based Treatments	SPAD†	Height† (cm)	Fresh shoot w† (g)	Shoot dry w† (g)	Fresh root w† (g)	Dry root w† (g)
CAN-17	BM	46.90 a	37.3 a	13.46 a	1.08 a	4.70 a	0.34 a
	SB	44.98 a	35.1 a	13.36 a	1.08 a	4.17 a	0.33 a
	SBF	45.08 a	35.0 a	12.53 a	1.19 a	4.91 a	0.36 a
	Control	47.32 a	38.4 a	10.99 a	1.05 a	3.71 a	0.29 a
UAN-32	BM	48.47 a	41.8 a	8.41 a	0.76 b	3.49 a	0.24 a
	SB	46.77 a	39.2 a	10.79 a	1.00 a	3.31 a	0.26 a
	SBF	42.62 a	38.2 a	9.11 a	0.74 b	3.18 a	0.18 a
	Control	45.80 a	35.5 a	9.93 a	0.64 b	3.13 a	0.24 a
Urea	BM	49.12 ab	38.1 ab	10.11 a	0.86 b	3.35 a	0.23 a
	SB	49.52 ab	35.7 ab	13.60 a	1.21 ab	4.18 a	0.29 a
	SBF	50.2 a	41.8 a	10.11 a	1.04 ab	3.65 a	0.27 a
	Control	47.82 b	33.3 b	10.49 a	1.29 a	3.14 a	0.29 a
Unfertilized control	BM	40.45 a	38.5 a	9.33 a	0.79 a	3.11 a	0.28 a
	SB	41.62 a	32.0 ab	11.36 a	1.00 a	4.15 a	0.34 a
	SBF	40.30 a	36.8 a	9.49 a	0.82 a	3.79 a	0.30 a
	Control	37.42 a	29.7 b	9.00 a	0.77 a	3.48 a	0.27 a

†Means within a column followed by the same letter are not significantly different at the 0.05 level using LSD values. SB (SoilBuilder™), SBF (SoilBuilder™ filtered), BM (*Bacillus* PGPR mix), and Control (no product applied).

SB treatment showed the most consistent increase in the four growth parameters of the three microbial-based treatments. Significant increases were also observed when urea fertilizer was applied. In this case, microbial-based treatments showed a significant effect on SPAD, fresh root, and dry weight. It was found that when no N fertilizer was applied, microbial-treatments also increased SPAD and height values. When looking at all fertilizer treatments, BM consistently increased SPAD readings the most, with the exception of CAN-17. No effect of microbial treatments was observed when fresh root weight was evaluated.

Table 3. Effect of inoculation with microbial-based treatments on corn parameters: SPAD, height, fresh shoot weight, shoot dry weight, fresh root weight, and dry root weight under different fertilizer treatments evaluated at V6 stage of growth (average of five replicates)

Fertilizer treatment	Microbial-based Treatments	SPAD†	Height† (cm)	Fresh shoot w† (g)	Shoot dry w† (g)	Fresh root w† (g)	Dry root w† (g)
	BM	46.60 a	49.5 a	30.49 b	2.93 c	18.29 a	1.66 a
	SB	44.66 a	47.9 a	31.89 b	4.31 b	16.55 a	1.43 a
	SBF	47.93 a	48.6 a	40.72 a	5.88 a	17.55 a	1.57 a
CAN-17	Control	45.80 a	43.4 a	22.77 c	3.22 c	13.96 a	1.17 a
	BM	48.10 a	47.3 b	32.63 a	3.99 ab	20.37 a	1.31 b
UAN-32	SB	45.10 a	58.4 a	34.52 a	5.07 a	20.38 a	2.08 a
	SBF	44.40 a	57.9 a	32.88 a	4.78 ab	19.47 a	1.71 ab
	Control	37.16 b	48.2 b	23.59 b	3.81 b	15.81 a	1.40 b
	BM	45.73 a	46.31 a	32.63 a	4.52 ab	19.81 a	1.42 a
Urea	SB	41.45 b	52.61 a	34.52 a	4.79 a	16.88 a	1.44 a
	SBF	43.0 ab	52.88 a	32.89 a	4.05 ab	18.25 a	1.31 a
	Control	40.63 b	46.81 a	23.59 b	3.42 b	17.76 a	1.32 a
	BM	38.93 a	40.94 ab	24.82 a	2.93 a	14.77 a	1.37 a
Unfertilized control	SB	37.70 a	42.41 a	27.33 a	3.28 a	16.68 a	1.68 a
	SBF	37.73 a	42.01 a	26.56 a	3.54 a	13.79 a	1.32 a
	Control	31.28 b	36.94 b	27.61 a	3.21 a	16.25 a	1.38 a

†Means within a column followed by the same letter are not significantly different at the 0.05 level using LSD values. SB (SoilBuilder™), SBF (SoilBuilder™ filtered), BM (*Bacillus* PGPR mix), and Control (no product applied).

Table 4. Effect of inoculation with microbial-based treatments on corn parameters: SPAD, height, stem width, fresh shoot weight, shoot dry weight, fresh root weight, and dry root weight under different fertilizer treatments evaluated at VT stage of growth (average of five replicates)

Fertilizer treatment	Microbial-based Treatments	SPAD†	Height† (cm)	Stem width† (mm)	Fresh shoot w† (g)	Shoot dry w† (g)	Fresh root w† (g)	Dry root w† (g)
CAN-17	BM	45.00 a	156.0 a	15.53 a	173.32 b	48.21 a	86.36 b	13.95 a
	SB	42.90 ab	168.0 a	17.87 a	185.16 a	46.23 a	101.06 a	16.65 a
	SBF	39.34 ab	156.0 a	15.37 a	177.16 ab	44.47 a	100.2 a	15.84 a
	Control	37.10 b	124.0 b	11.63 b	148.68 c	30.62 b	91.66 b	12.76 a
UAN-32	BM	44.00 a	160.67 a	17.63 a	182.99 a	44.57 ab	103.12 a	22.25 a
	SB	45.18 a	162.17 a	17.71 a	191.21 a	47.38 a	99.57 ab	18.72 ab
	SBF	39.26 a	160.01 a	18.43 a	184.06 a	41.54 b	101.28 ab	19.51 ab
	Control	37.06 a	142.33 b	12.52 b	138.38 b	29.66 c	94.06 b	14.93 b
Urea	BM	46.80 a	162.33 a	17.62 a	176.41 a	44.29 a	108.03 a	22.31 a
	SB	39.36 b	157.01 a	17.67 a	179.37 a	45.19 a	100.21 b	14.81 b
	SBF	46.05 a	165.33 a	17.29 a	184.77 a	41.71 a	102.42 ab	18.72 ab
	Control	37.31 b	131.33 b	11.99 b	138.13 b	31.08 b	90.97 c	14.29 b
Unfertilized control	BM	30.03 a	81.01 b	11.23 a	77.41 a	18.83 b	44.48 a	7.11 a
	SB	29.50 a	83.67 ab	11.97 a	79.61 a	16.84 bc	43.11 a	8.18 a
	SBF	30.10 a	93.67 a	11.91 a	80.85 a	22.54 a	45.25 a	7.95 a
	Control	28.22 a	68.67 c	8.86 b	58.33 b	13.98 c	34.84 b	6.10 a

†Means within a column followed by the same letter are not significantly different at the 0.05 level using LSD values. SB (SoilBuilder™), SBF (SoilBuilder™ filtered), BM (*Bacillus* PGPR mix), and Control (no product applied).

The greatest effects of microbial-based treatments were observed at VT (Table 4). All parameters where positively affected by the application of microbes. However, SPAD measurements were significantly increased only when CAN-17 and urea were applied. At this observation time, the BM treatment was once again responsible for the highest readings. Consistent significant effects were observed with all three microbial treatments for the following parameters: height, stem width, fresh and dry shootweight, and fresh root weight. In these last three parameters, the average increased effect by microbial-based treatments was 31%, 55%, and 17%, respectively, while dry root weight was only increased by BM when UAN-32 and urea were applied.

Root Morphology Evaluation

Root growth and morphological measurements made in this study are presented in Tables 5 and 6. Plant roots evaluated at V2 showed an increase in all parameters evaluated when BM was applied with UAN-32 fertilizer (Table 5). The BM treatment also increased RSA and RV in plants fertilized with CAN-17. In contrast, SBF and SB treatments performed better with the unfertilized control, increasing all four root morphology parameters.

Table 5. Effect of inoculation with microbial-based treatments on early root morphology parameters: Total root length (TRL), root surface area (RSA), root volume (RV), and total root length of fine roots under different fertilizer treatments evaluated at V2 stage of growth (average of five replicates)

Fertilizer	Microbial-based treatment	TRL† (cm)	RSA† (cm^2)	RV† (cm^3/m^3)	Total length 0< D < 0.5 (mm) Fine roots†
CAN-17	BM	701.99 a	187.86 a	4.14 a	435.44 a
	SBF	698.77 a	170.51ab	3.51 ab	436.83 a
	SB	589.67 a	142.07 bc	2.78 bc	346.13 a
	control	557.88 a	122.21 c	1.91 c	313.07 a
UAN-32	BM	680.82 a	162.68 a	2.58 a	328.19 a
	SBF	415.44 bc	94.32 b	1.82 b	167.12 b
	SB	469.07 b	106.81 b	2.05 ab	179.56 b
	control	323.22 c	89.72 b	1.78 b	203.31 b
Urea	BM	368.16 a	94.45 a	1.36 a	159.31 a
	SBF	401.15 a	96.31 a	1.77 a	114.48 a
	SB	312.92 a	94.64 a	1.94 a	173.09 a
	control	347.14 a	99.93 a	1.78 a	170.58 a
Unfertilized control	BM	1004.1 ab	181.64 b	2.69 b	798.9 ab
	SBF	1156.5 a	279.15 a	6.79 a	857.4 ab
	SB	1151.2 a	221.17 b	5.31 a	919.9 a
	control	682.1 b	177.53 b	2.77 b	598.6 b

†Means within a column followed by the same letter are not significantly different at the 0.05 level using LSD values. SB (SoilBuilder™), SBF (SoilBuilder™ filtered), BM (*Bacillus* PGPR mix), and Control (no product applied).

Table 6. Effect of inoculation with microbial-based treatments on early root morphology parameters: Total root length (TRL), root surface area (RSA), root volume (RV), and total root length of fine roots under different fertilizer treatments evaluated at V4 stage of growth (average of five replicates)

Fertilizer	Microbial-based treatment	TRL† (cm)	RSA† (cm^2)	RV† (cm^3/m^3)	Total length 0< D < 0.5 (mm) Fine roots†
CAN-17	BM	1837.3 ab	390.79 a	6.27 ab	963.9 bc
	SBF	1959.2 a	404.88 a	6.50 ab	1244.9 a
	SB	2008.3 a	430.75 a	6.95 a	1233.6 ab
	control	1308.2 b	301.14 b	5.14 b	725.6 c
UAN-32	BM	1319.7 ab	297.24 a	5.60 a	739.9 a
	SBF	1602.0 a	323.24 a	5.26 ab	997.5 a
	SB	1382.9 ab	307.77 a	4.31 b	840.8 a
	control	1143.1 b	275.20 a	4.90 ab	787.1 a
Urea	BM	1198.3 b	275.05 a	5.06 b	694.8 a
	SBF	1738.7 a	370.74 a	6.47 ab	1006.7 a
	SB	1115.5 b	305.13 a	6.37 ab	809.9 a
	control	1291.1 b	381.84 a	7.62 a	980.8 a
Unfertilized control	BM	2249.1 a	389.18 a	5.36 a	1672.4 a
	SBF	2276.3 a	433.56 a	6.63 a	1742.1 a
	SB	2278.1 a	411.33 a	5.93 a	1548.1 a
	control	1819.1 a	355.32 a	5.54 a	1106.3 b

†Means within a column followed by the same letter are not significantly different at the 0.05 level using LSD values. SB (SoilBuilder™), SBF (SoilBuilder™ filtered), BM (*Bacillus* PGPR mix), and Control (no product applied).

When urea was applied,none of the microbial-based treatments had an effect on the root morphology parameters evaluated. Root morphology parameters evaluated at the V4 stage (Table 6) showed different results than at the V2 stage. In this case, microbial-based treatment application with the CAN-17 fertilizer presented a significant difference in the four parameters evaluated. The SB treatment produced better results in three of the parameters, except TRL of fine roots, in which SBF showed the greatest improvement in length of fine roots. Plants treated with UAN-32 showed the highest TRL with SBF and RV with BM. Few significant differences were observed with microbial treatments when applied with urea (Table 6). When no N fertilizer was applied, the total root length of fine roots was increased by all three microbial-based treatments.

Nutrient Uptake

Nutrient uptake as affected by microbial treatments for each of the fertilizer applications is shown in Figures 1, 2, and 3. Positive effects of microbial treatments on N uptake were first observed at V6 for urea and CAN-17 and were more noticeable at VT stage for all three N fertilizers and the unfertilized control (Fig. 1). Beginning at stage V4, unfertilized treatment and UAN-32 showed a significant difference in N uptake with SB. The largest increase in N uptake for all four fertilization treatments was observed at VT growth stage. No significant difference was observed between the three microbial treatments, indicating that the plant's capacity to increase N uptake was not dependent on fertilizer type. The average increase in plant N uptake by microbial-based treatments compared to the control was 72% for CAN-17, 61% for UAN-32, 72% with urea, and 54% in the unfertilized treatment.

Phosphorus uptake showed a similar pattern as N uptake (Fig.2). Significant increases were observed at V4 for UAN-32, urea, and unfertilized control. However, larger differences were observed at VT stage for all four fertilization treatments. When CAN-17 was applied, BM had the greatest impact, increasing P uptake by 138%. SB and SBF also increased P uptake by 85% on average, while increases of P by microbial-based treatments were on average 50% greater compared to the control when UAN-32 was used. Application of microbial-based treatments resulted in higher average values of 75% and 80% for urea and unfertilized control, respectively.

Potassium uptake was also affected positively by the application of microbial-based treatments (Fig. 3). In this case, significant differences at V4 were only observed when urea was applied. For CAN-17 and UAN-32, significant differences were observed at V6. With the unfertilized control, treatment differences were only observed at VT. Greater significant differences were observed once again at VT, with SBF increasing K uptake the most (100%) in the unfertilized control treatment. In contrast, BM increased K uptake the most with CAN-17 application (95%) compared to the SBF and SB treatments, which showed an increase of 65% compared to control. When UAN-32 was applied, the three treatments showed an average of 45% more K uptake. Meanwhile, with urea, the average increased uptake for the three microbial treatments was 71% more than the control. Nitrogen uptake and SPAD

readings were correlated in plants evaluated at V4 (r=0.483, p=<0.0001) and V6 (r=0.563, p=<0.0001). Higher correlation values were observed between these two parameters at the VT growth stage (r=0.754, p=<0.0001).

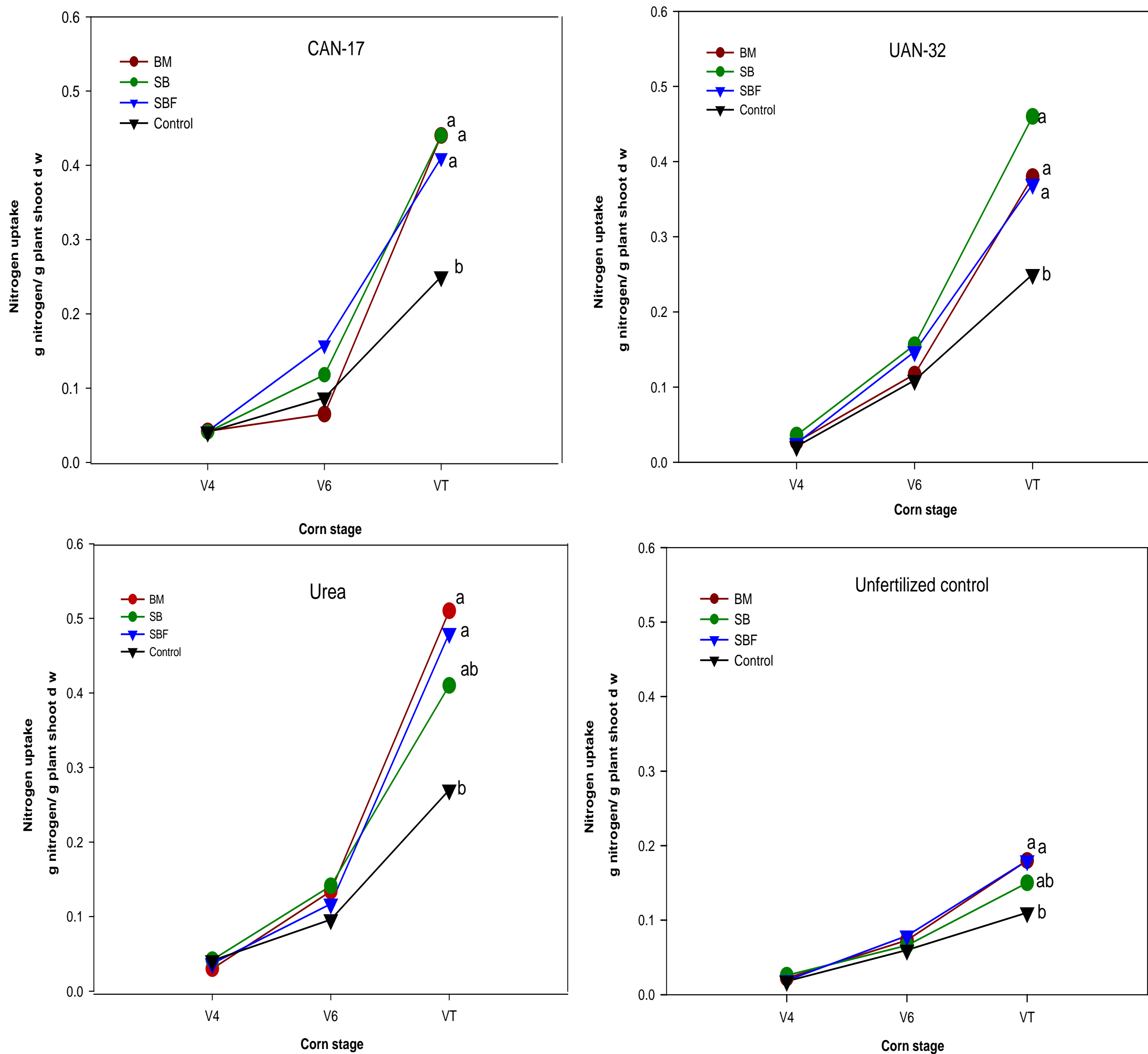

Figure 1. Effect of microbial-based treatments on corn shoot nitrogen uptake at three different stages (V4, V6, and VT) under three different types of nitrogen fertilizers (CAN-17, UAN-32, and urea) and an unfertilized control.

Symbols within the same growth stage followed by the same letter are not significantly different at the 0.05 level using LSD values.

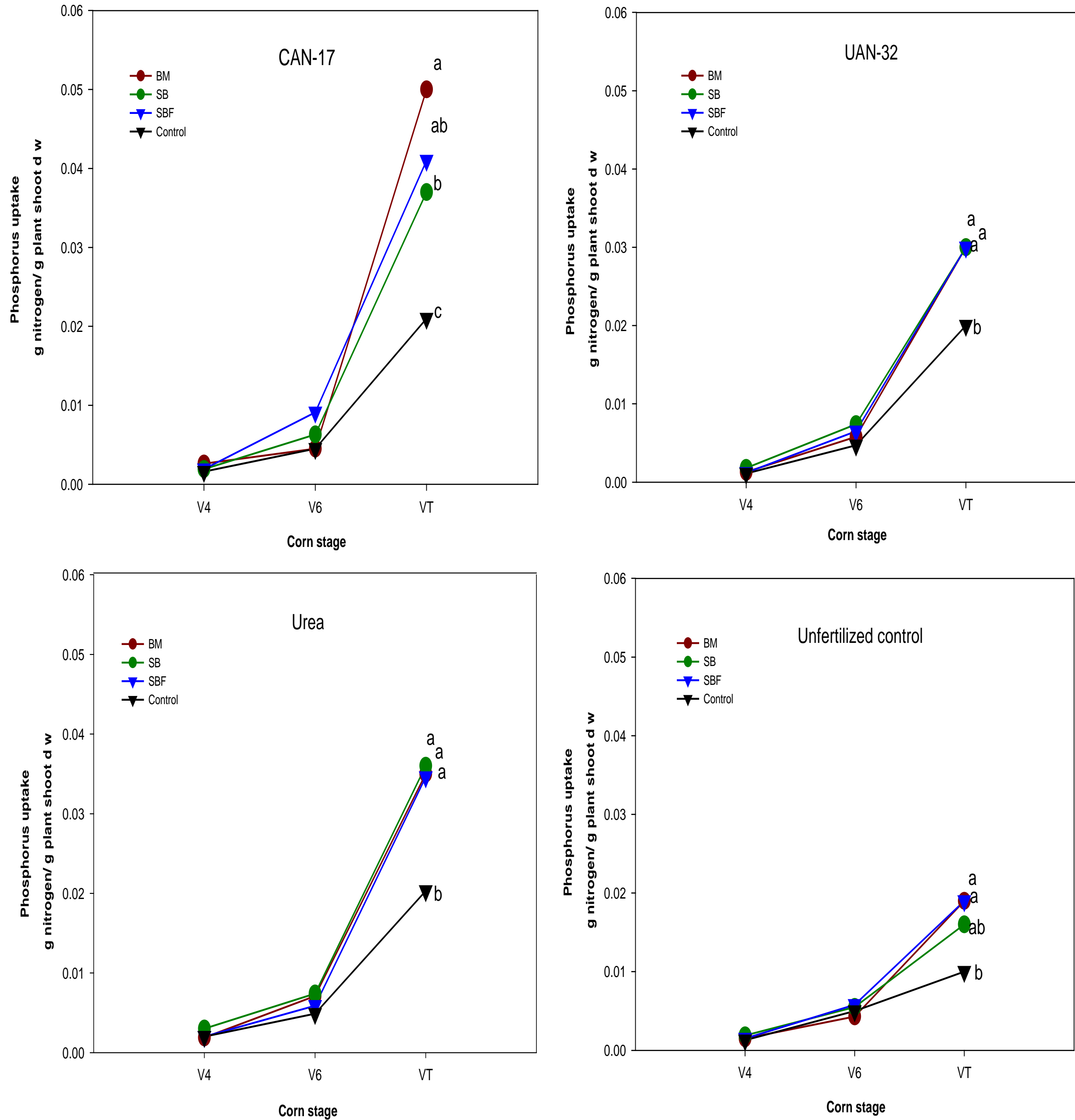

Figure 2. Effect of microbial-based treatments on corn shoot phosphorus uptake at three different stages (V4, V6, and VT) under three different types of nitrogen fertilizers (CAN-17, UAN-32, and urea) and an unfertilized control.

Symbols within the same growth stage followed by the same letter are not significantly different at the 0.05 level using LSD values.

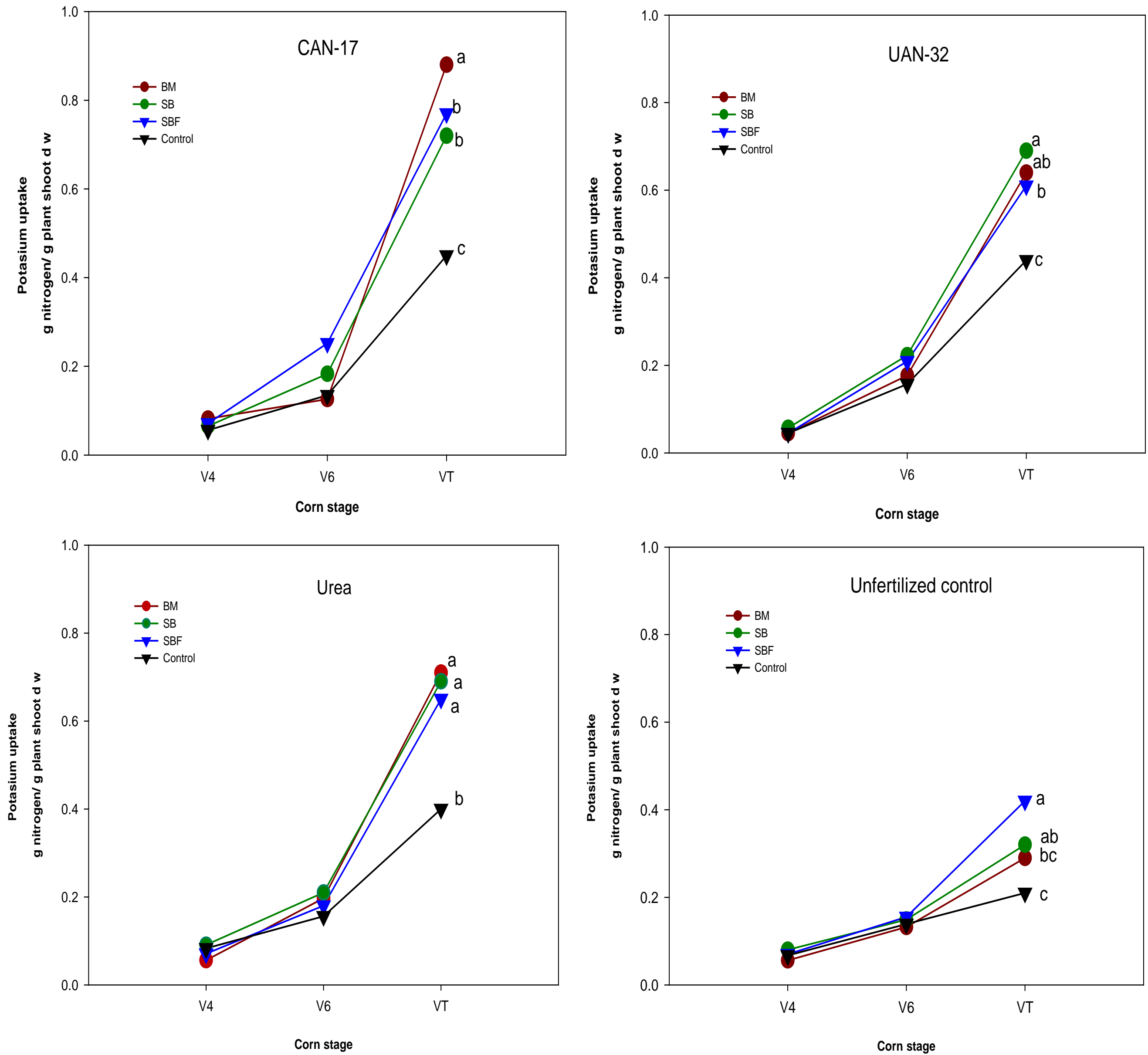

Figure 3. Effect of microbial-based treatments on corn shoot potassium uptake at three different stages (V4, V6, and VT) under three different types of nitrogen fertilizers (CAN-17, UAN-32, and urea) and an unfertilized control. Symbols within the same growth stage followed by the same letter are not significantly different at the 0.05 level using LSD values.

IV. Discussion

Responses of corn plants to microbial-based treatments depend on growth stage and type of N fertilizer applied. In all four growth stages evaluated, inoculation with microbial-based treatments significantly increased at least one plant growth parameterwith at least one N fertilizer type. However, greater and more consistent effects were observed at V6 and VT stages, with more parameters being significantly increased with all three N fertilizers and the unfertilized treatment. These findings suggest that plant age or stage of corn development affects nutrient demand. According to Shanahan et al. (2004), an active increase in dry matter and N accumulation was observed in corn between the V4 and V8 growth stages. After the V8 stage, the growth rate and nutrient uptake observed increased even more. Yu-kui et al. (2009) reported that with no N application differences in plant growth parameters, such as plant height, were not observed before the V8 growth stage. Thus, the lack of microbial-based treatments' effects on plants evaluated at V2 and V4 could be explained by the low rate of biomass accumulation and nutrient uptake during the early corn development.

A significant impact of PGPR was reported by Çakmakçi, et al. (2006) in the early growth stages of beetroots, which later resulted in higher yields. These findings differ from our findings in which early growth of corn (V2 and V4) was not highly affected by microbial-based treatments. However, significant differences were found when plants were evaluated at V6 and especially at VT stages. Similar observations were reported by Katulanda and Rajapaksha (2012), who found thatplants inoculated with PGPR and evaluated near harvest showed significant increases in plant height and shoot dry weight. Historically, improvements in corn grain yield have been accompanied by increased total biomass yield. This potential for biomass accumulation provides the driving force

for mineral nutrient uptake and assimilation (Lorenz, et al., 2010). Hence, microbial-based treatments tested in the present study that increased biomass-related parameters could potentially increase corn yield. Biari et al. (2008) reported that the application of PGPR strains not only increased corn growth parameters, such as shoot dry weight, but also increased grain dry weight and ear dry weight. They reported that this positive effect of PGPR application was related to the strains' capacity to promote better absorption of essential nutrients that are responsible for high rate of photosynthesis.

Application of microbial-based treatments has also been shown to affect plant root morphology and nutrient uptake. In the first three stages, fresh and dry root parameters were less affected by microbial-based treatments compared to other plant parameters. At VT, fresh root growth was increased by the three microbial-based treatments. However, dry root growth was only significantly increased by BM treatment when UAN-32 and urea were present. Even though the biomasses of fresh and dry roots were not affected by microbial-based treatments in the first two stages, there were significant differences when root morphology parameters were evaluated (V2 and V4). Root morphology parameters play an important role in plant development; it has been demonstrated that the capture of belowground resources by plants is more dependent upon root length or root surface area than total root biomass (Kramer and Boyer, 1995, Sattelmacher, et al., 1990). It was demonstrated in previous studies (Gamalero, et al., 2004, Lemanceau, et al., 2005) that root structure and morphology could be influenced by beneficial microorganisms, such as PGPR, that colonized roots. PGPR's effect on root morphology
(e.g., increasing total root length and branching) could be, in some cases, a consequence of hormone production, which results in improvements in plant mineral nutrition (Canbolat, et al., 2006).

Increases in root length and surface root area of corn plants evaluated at different growth stages when the PGPR *Azospirillum lipoferum* was applied was previously reported (El Zemrany, et al., 2007). Root surface area has been shown to be a good predictor of nutrient capture from soil zones (Hodge, et al., 1999).Besides root length and root surface area, total lengths of fine roots

(0<D<0.5 mm) were also increased by microbial-based treatments in all fertilizer types except urea. While working with three different corn genotypes that differed in canopy architecture and system morphology characteristics, Costa et al. (2000) observed that the greatest contribution for total root length came from roots with diameter classes between 0.2 and 0.4 mm. Furthermore, there is now considerable evidence linking root architecture and morphology with water and nutrient acquisition efficiency (Lynch, 2007).

Increased nutrient uptake of N, P, and K by inoculated corn plants was observed in the present study. Greater differences compared to control started at the V6 stage and increased by VT. This observation agrees with a previous report by Al-Kaisi and Yin (2003) who showed a small effect of lower N rates on N plant uptake when plants were evaluated at V6 compared to plants evaluated at VT. Under normal nutrient uptake patterns reported for corn, the rate of N uptake is relatively slow before the plant enters the period of rapid growth around the V6 growth stage, and large N accumulation occurs during the mid to late vegetative growth stages (Subedi and Ma, 2009). In the same way, other corn nutrient uptake curves, such as for P, K, Mg, S, and Cu, followed sigmoidal or linear uptake curves that began to increase significantly from V5 to V6 (Bender, et al., 2013). Besides giving information on the nutritional status of plants, nutrient uptake parameters have also been found to predict future yield potential. A close association between corn productivity and greater total plant nutrient uptake (N, P, K) as well as increased nutrient removal has previously been documented (Bender, et al., 2013, Ciampitti, et al., 2013, Setiyono, et al., 2010).

Important increases in N uptake that ranged between 54% and 72% on average for the three N fertilized and the unfertilized treatments were observed at VT, when microbial-based treatments were applied. Increases in N uptake at tasseling were also reported in corn plants treated with PGPR *Azospirillum* spp., *Bacillus* spp., and *Pseudomonas* spp., suggesting that microbial inoculants could improve N acquisition by the corn crop (Biari, et al., 2008, Egamberdiyeva, 2007). Enhanced N uptake could be explained by modified root growth or increased root biomass (Katulanda and

Rajapaksha, 2012). In the present study, significant effects on fresh root biomass at VT and increased RL, RSA, and RV at V2 and V4 were observed when microbial-based treatments were applied, indicating that root growth could be responsible for the increased nutrient uptake. Wood et al. (1993) found that N uptake at V10 is a good predictor of grain yield. The increased N uptake found in the microbial-based treatments of the present study could also indicate a potential for increased corn yield. A positive correlation between SPAD and N concentration was also reported by Zhu et al (2011), who found a higher correlation between these two parameters 53 days after seeding of corn plants in a greenhouse environment. Subedi and Ma (2009) also found SPAD readings that were highly correlated with N concentration and N uptake. However, they emphasized that the readings made before V6 stage were less effective in predicting crop nutritional status than readings made after V8 stage. In a similar way in the present study, SPAD readings at VT had a higher correlation with plant N uptake compared to previously evaluated stages.

The present study showed that P uptake was increased by microbial-based treatments. Increases reached 138% when BM treatment was applied in the presence of CAN-17 and evaluated at VT stage. Increase in P uptake by PGPR was previously reported for corn evaluated at V6. The increase was related to the capacity of the bacteria to efficiently mobilize P from soil and make it more available to the plant (Katulanda and Rajapaksha, 2012, Singh, et al., 1998). Schachtman et al. (1998) found that surface area and root length are the main characteristics responsible for P use efficiency because this nutrient has low mobility in soil and is transported primarily by means of diffusion (Horst et al., 2001). These observations agree with the present study's results in which significant differences in root length and surface area were observed at V2 and V4 when microbial treatments were applied. Coincidently, the BM treatment that enhanced P uptake the most with CAN-17 also showed significantly higher surface area at V2 and V4 stages. Length of fine roots (0-0.5 mm) was also enhanced by microbial-based treatments at V2 and V4 stages, which has been reported to be associated with elevated efficiency in the acquisition and use of soil nutrients especially in corn

(Rosolema, et al., 1994). Parentoni and Souza (2008) reported that roots with a diameter smaller than 0.5 mm play an important role in absorption kinetics and are believed to be a strategy that the plant uses, in low P availability conditions, to allow for an increase of root surface area that favors increased efficiency in the absorption of nutrients.

Potassium uptake followed the same trend as N and P. Microbial-based treatments increased K uptake on an average of 65% in all fertilizer treatments. Similar to P uptake, BM was the treatment that enhanced K uptake the most in the presence of CAN-17. This significant increase in the uptake of these two nutrients by BM could be associated with the capacity of some PGPR to solubilize phosphate and K. K-solubilizing bacteria may enhance mineral uptake by plants through solubilizing insoluble P and releasing K from silicate in soil (Goldstein and Liu, 1987).

When comparing the general effect of the three microbial-based treatments on increasing plant parameters, nutrient uptake, and root morphology, all three microbial-based treatments showed a very similar effect. The effect of SB and BM could be explained by the presence of PGPR that increased some of the plant growth parameters and promoted nutrient acquisition. On the other hand, the mechanisms of SBF could be explained in a different way. SBF treatment did not contain bacteria; it only contained bacterial fermentation products. Therefore, the secondary microbial metabolites would be responsible for the plant growth effect. Among the metabolites that have been related to the plant-growth promotion effect are hormones, such as indol acetic acid (IAA) which promotes root growth (Aloni, et al., 2006, Araújo, et al., 2005), organic acids, which have the capacity to solubilize nutrients in soil like phosphate, siderophores, and other enzymes such as nitrogenase, chitinases, and glucanases (Cattelan, et al., 1999, Vassilev, et al., 2006). Another potential reason for the positive effect from fermentation products present in SBF is the enhancement in proliferation and activity of the microorganisms already present in the soil that could also positively affectplant growth and root development.

When selecting microbial-based treatments for their potential to increase crop yield, greenhouse experiments are an important starting point for the screening process so that a greater number of treatments can be evaluated. In the specific case of corn, it is important toconsider that the potential effect of microbial-based treatments could be masked during early growth stages in which the corn plant is not actively growing compared to future stages. Furthermore, the living bacteria in BM and SB treatments may need a minimum period of time to colonize the root system and fully realize effects on plant development. Thus, an evaluation of plant growth and nutrient uptake close to the end of the vegetative growth stage in corn would be more appropriate. Based on the present results, when corn was evaluated at VT, a significant increase in plant growth parameters and also nutrient uptake by microbial-based treatments was observed.

V. **Conclusions**

Application of microbial-based inoculants increased plant growth and nutrient uptake of corn plants. However, the plant growth promotion effect depended on the type of N fertilizer applied and on the vegetative stage in which the plant was evaluated. In early stages like V2 and V4, plant growth promotion was not consistent among some plant parameters. With some of the fertilzers, plant growth promotion by microbial-based treatments was not observed. In later stages, like V6 and especially VT, increased plant growth by microbial-based treatments was more consistent. At VT, the differences were noticable. Microbial-based treatments were capable of altering root morphology parameters in both corn growth development stages evaluated. The effect on root morphology (total root length, root surface area, root volume, and total length of fine roots) by microbial-based treatments also depended on the type of nitrogen fertilizer applied. For this reason an interaction between nitrogen fertilizer and microbial-based treatments was observed and should beconsidered in future studies that include microbial-based treatments. Nutrient uptake by the plant was also increased when microbial-based treatments were applied

and was dependent on the type of nitrogen fertilzer and the stage in which the plant was evaluated. Plants evaluated at VT stages had significantly higher increases in the upatke of N, P, and K. On average, microbial-based treatments increased N uptake by 65%, P uptake by 86%, and K uptake by 78%. Increases in nutrient uptake could be related to increases of some root morphology parameters at early stages, which would be an advantage as the plant could absorb more nutrients. The fact that plants at VT stage had better nutrient content due to application of microbial-based treatments has proven to be a good predictor oft future corn yields (Walker and Peck, 1974).

The results indicate that microbial-based inoculants have the potential to be tools in many nutrient use efficiency practices. Furthermore, the fact that they could increase the nutrient uptake of plants opens the door to decreases in some fertilizer rates without affecting the final yield. Further studies are needed to confirm the effects of microbial-based inoculants on corn yield under field conditions.

VI. Literature review

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Reduction of nitrogen fertilization and increased nutrient uptake by application of PGPR mixtures

Abstract

Excessive application of nitrogen (N) fertilizer and low efficiency of N uptake by plants have led to a wide variety of negative effects for the producer and for the environment. Plant growth-promoting rhizobacteria (PGPR) have been reported to exert positive effects on plant growth, yield, and nutrient uptake by an array of mechanisms. Due to their capacity to increase nutrient uptake, PGPR inoculants could be potential tools to ehance nutrient use efficiency. The objectives of the present study were (1) to select PGPR mixtures that would allow a decrease in N fertilization by promoting plant growth to a level statistically equivalent to 100% N fertilization without PGPR in chinese cabbage; (2) study the effect of spore concentrations of PGPR mixtures on plant growth promotion and nutrient uptake in cabbage plants; and (3) predict how much N fertilization could be reduced in the presence of PGPR to obtain plant growth equivalent to 100% N fertilization without PGPR. in cabbage and pepper plants. The overall hypothesis is that select mixtures of PGPR could be used in reduced N fertility regimes to give statistically equivalent plant growth to treatment with full rates of N without PGPR All experiments were performed at the greenhouse using a randomized complete block design (RCBD). Ten different PGPR *Bacillus* spp. mixtures were used in the first screening experiment. From all PGPR mixtures evaluated, 3 PGPR mixtures +80% N fertilization gave results that were equivalent to 100% N fertilization + no PGPR mixture. There was no effect from increasing the concentration of the 3 PGPR mixtures from 10^6 spores/ml to 10^7 spores/ml on plant growth and nutrient uptake. The three PGPR mixtures were capable of reducing N fertilization on average of 16%,in fresh weight, 1.5% in dry weight, and 6.64% in plant N uptake, in cabbage. The same PGPR mixtures were capable of reducing N fertilization an average of 30.46% in fresh weight, 30.03% in dry weight, and 26.93% in plant N uptake in pepper PGPR mixtures

have the potential to reduce N fertilization without affecting plant growth parameters. Further studies are needed to elucidate the specific mechanisms involved and how the reduction effect varies according to crop.

I. Introduction

The green revolution came with a tendency toward increased use of fertilizers, especially N , in order to increase productivity. The excessive use of N chemical fertilizers eventually became a serious threat to the environment. Furthermore, fertilizer prices have been rising in the last 10 years, which also presents a problem for agriculture production (Ott, 2012). Thus, there a growing focus has developed on more "environmentally friendly and sustainable" fertilization management tools that could help optimize N fertilizer use by increasing the efficiency of plant nutrient uptake (Esitken, Karlidag, et al., 2003). Across the world, only about 30% to 50% of N fertilizer is taken up by crops (Cassman, 2002).The supply of N more than any other nutrient in soil limits crop production. For this reason, it is important to find ways to make plants more efficient in the absorption of N without compromising plant yield. In this context, microbial inoculants like plant growth-promoting rhizobacteria (PGPR) represent a possible solution that would allow a reduction of N fertilization by enhancing plant nutrient uptake and plant growth.

Microbial fertilizers have been reported to increase crop yields, improve biodiversity and soil fertility, reduce the need for chemical fertilizers, recycle organic wastes, and, consequently, abate environmental pollution (Li and Zhang, 2001).Wu et al. (2005) reported that microbial inoculation with *Bacillus megaterium* and *Bacillus mucilaginous* not only increased plant growth but also improved nutritional assimilation in the plant (total N, P, and K). Application of PGPR showed significant increases in NPK uptake and root and shoot dry weight of several crop such as cotton (Egamberdiyeva and Höflich 2004) and wheat (Shaharoona et al. 2008). The mechanisms used by microbial inoculants such as PGPR

to stimulate plant growth and nutrient uptake are several. Among the mechanisms reported are (i)asymbiotic N fixation (Boddey and Dobereiner 1995b; Dobereiner 1997); (ii) nutrient solubilization (de Freitas et al. 1997); (iii) sequestering of iron by production of siderophores; (iv) production of volatile compounds (VOCs); (v) phytohormone (indol acetic acid, gyberellins, and cytokinins) production; and (vi) lowering of ethylene concentration by production of ACC-deaminase (Frankenberger and Arshad, 1995, Gamalero and Glick, 2011, Glick et al., 1999).

Another proposed explanation for the beneficial effects of PGPR is the increase in the root surface area and the volume of soil foraged by the root, which leads to enhanced nutrient and water uptake and shoot biomass accumulation (Abbasi et al., 2011, Mantelin and Touraine, 2004). This rationale is consistent with the observation that oil palm plants inoculated with PGPR strains of *Azopsirillum* spp. and *Bacillus* spp. take up N, P, K, and microelements more efficiently from the soil due to higher root dry weight (Amir et al., 2005). Besides the already know plant growth promotion mechanisms of phytohorme production and increase on root growth (Mantelin and Touraine, 2004). by PGPR, there new evidence that plant growth promotion could also involve plant signinalling. Bertrand et al. (2000) reported that the uptake rates of NO_3^- in roots (measured using ion-specific electrodes) increased in response to the PGPR *Achromobacter* sp. These findings indicate that PGPR affect mineral ion uptake due to the stimulation of the proton pump ATPase activity.

Initially, experimental applications of PGPR inoculants consisted of only one strain of bacteria. However, recent attention has been given to the application of mixtures (combinations of microorganisms) of different PGPR strains and/or species that interact synergistically. Previous studies of mixtures of bacteria cultures indicate that bacteria interact with each other synergistically and provide nutrients, remove inhibitory products, and stimulate each other through physical or biochemical activities that may enhance some beneficial properties of their physiology (Bashan, 1998). Bashan and Holguin (1997) reported that inoculation with mixtures of PGPR microorganisms increased growth, yield, and nutrient absorption compared to a single strain inoculation. The application of a microbial fertilizer

compounded with *Bradyrhzobium* sp. and *Bacillus subtilis* to peanut showed an increase in yield of 34.5% (Li and Zhang, 2001). In another study, Wu et al. (2005) showed that application of a mixture of *Azotobacter chroococcum*, arbuscular mycorrhizal fungus, and two different species of *Bacillus* spp. increased plant biomass, height, and nutrient (N,P, and K) content of corn. Belimov et al. (1995) reported significantly greater uptake of P in shoot of barley with co-inoculation of *Azospirillum lipoferum* 137 and *Arthrobacter mysorens* 7 or *A. lipoferum* 137 and *Agrobacterium radiobacter* 10 than with a single inoculation of any of the three organisms.

The objectives of this study were to (1) to select PGPR mixtures that would allow a decrease in N fertilization by promoting plant growth to a level statistically equivalent to 100% N fertilization without PGPR in chinese cabbage; (2) study the effect of spore concentrations of PGPR mixtures on plant growth promotion and nutrient uptake in cabbage plants; and (3) predict how much N fertilization could be reduced in the presence of PGPR to obtain plant growth equivalent to 100% N fertilization without PGPR. in cabbage and pepper plants. The overall hypothesis is that select mixtures of PGPR could be used in reduced N fertility regimes to give statistically equivalent plant growth to treatment with full rates of N without PGPR.In order to achieve these objectives, Chinese cabbage was chosen as the main model plant to study the reduction of N fertilization by PGPR applicationbecause it produces substantial leaf biomass during a short growth period. This rapid growth results in a high nutritional demand, particularly for N.

II. Materials and methods

Study Site and Soil Characteristics

All experiments were performed in a climate-controlled greenhouse at the Plant Science Research Center in Auburn, Alabama. Minimum and maximum air temperatures were 24 and 30 °C, respectively.

The soil medium used for this study consisted of a soil: sand mixture. Sand was mixed with soil in order to improve water infiltration and minimize anaerobic conditions during the study. Briefly, a sandy loam soil with a texture of 72.8% sand, 10.4% clay, and 16.8% silt was mixed 3:1 (soil: sand; v:v) with white brick/Mason sand (particle size: 1/8 mm -1/4 mm). The mixture resulted in a soil medium with the texture of loamy sand (85.2 % sand, 4.8% clay, and 10% silt). The soil: sand mixture had a pH of 6.4, CEC of 1.15cmol kg^{-1}, total N concentration of 2.9 g kg^{-1}, organic matter concentration of 4.9 g kg^{-1}, total C concentration of 3.8 g kg^{-1}, NO_3 concentration of 6.77 µg g-1, Mg concentration of 47 µg g^{-1}, Ca concentration of 487.5 µg g^{-1}, P concentration of 37 µg g-1 , and K concentration of 39.5 µg $g\text{-}^{1}$. Initial soil analysis was performed by Auburn University Soil Testing Laboratory as described by Hue and Evans (1986). Briefly, total C and N were analyzed using an Elementar vario Macro C-N analyzer (Elementar Americas, Inc. Mt. Laurel, NJ, USA). Soil pH was determined on 1:1 soil/water suspensions with a glass electrode meter. Concentrations of P, K, Mg, and Ca were determined using Melich 1 (double acid extracting solution) (Olsen and Sommers, 1982) and measured using an ICAP 9000 (Thermo Jarrell Ash, Franklin, MA, USA). The Cation Exchange Capacity (CEC) was determined by base summation (Ca, Mg, K, and Na) according to procedures of Hue and Evans (1986).

Plant Source and Fertilization

Chinese cabbage “Kaboko” (*Brassica rapa* var. pekinensis) (Park Seed Wholesale organic cabbage,Greenwood, SC) and bell pepper (*Capsicum annuum*) “California Wonder” (Park Seed Wholesale, Greenwood, SC) were planted using pot seedlings produced in multicell trays filled with Sunshine Professional Growing Mix (Sun Gro Horticulture Canada Ltd.). Optimum fertilization was calculated based on the recommendation of Maynard and Hochmuth (1997) for vegetables grown in sandy soils. In addition to this recommendation, a preliminary plant growth curve using different rates of N fertilizer was provided to select the correct N rates for each plant type.

Bacterial Strains and Microbial Source Preparation

A total of ten different PGPR Bacillus mixtures were tested (Table 1) using strains from culture collections at the Department of Entomology and Plant Pathology, Auburn University (Auburn, AL, USA). These strains have been shown to have important plant growth-promoting effects (Enebak et al., 1998, Jetiyanon et al., 2003, Kokalis-Burelle et al., 2003, Kokalis-Burelle et al., 2002, Niranjan-Raj et al., 2003). All strains were previously tested and were negative for N fixation based on the tests with JNFbN media. The bacterial mixtures were prepared by mixing each strain's spore suspension, which was previously quantified by plating the spore mix suspension on TSA and incubating for 48 h at 25 °C. The spore mix was then adjusted to a specific concentration.

Experiment 1: Selection of PGPR Mix

The experiment was designed to select the best mixtures from a total of ten mixtures of *Bacillus* spp. PGPR that will be capableto increase nutrient uptake and plant growth when N fertilization was reduced by 20%. The experiment was designed as a complete randomized block design (CRBD) with 12 treatments, each with 8 replicates. The experiment was repeated twice. The treatments consisted of ten different PGPR mixtures + 80% of the N applied, a control (no PGPR) + 80% of the N fertilization, and a positive control (no PGPR) + 100% of the N fertilization. N was applied as calcium nitrate at the doses of 224 N mg/kg (80%) and 280 N mg/kg (100%) in four equal split doses once a week. Potassium and phosphorus were applied only once, at transplanting, as 300 mg/kg of K_2HPO_4. Calcium chloride ($CaCl_2.2H_2O$) was applied in the 80% N N treatments in order to match the amount of Ca presented in 100%N. Hoagland's micronutrients solution was applied once a week (1ml/L). After 4 weeks in trays, Chinese cabbage plants were transplanted to 32 oz. styrofoam cups filled with 1.2 kg of the soil: sand mix adjusted to 60% WHC. Immediately after transplanting, a 5 ml spore suspension with a concentration of

10^6spores/ml was applied with a 10 ml syringe to each transplant's roots. Watering was controlled by measuring the soil moisture daily with a WaterScout SM 100 soil moisture sensor (Spectrum technology, Inc.). Cups were watered daily to keep the same moisture (20% moisture) in all the cups and avoid leaching. Four weeks after transplanting, plants were harvested and the following plant parameters were measured: plant diameter, fresh (SFW) and dry (DSW) shoot weight (oven dry at 70°C), chlorophyll content (SPAD 502 meter), and nutrient concentration of the shoot. Nutrient uptake by the plant shoot was calculated by multiplying each shoot dry weight by each nutrient concentration.

Table 1. Strains and bacteria species present in the PGPR mixtures used in Experiment 1.

PGPR Mix #	Original Strain #	Identification
1	INR-7, IN937a, T4, SE56	*B. altitudinis, B. subtilis, B. safensis, Lysinibacillusxylanilyticus*
2	INR-7, SE56, SE76, FZB42	*B. altitudinis, Lysinibacillusxylanilyticus, B. safensis, B. amyloliquefaciens*
3	INR-7, IN937a, SE56, E681	*B. altitudinis, B. subtilis, Lysinibacillusxylanilyticus, Paenibacilluspeoriae*
4	SE56, E681, FZB24, FZB42	*Lysinibacillusxylanilyticus, Paenibacilluspeoriae, B. amyloliquefaciens, B. amyloliquefaciens*
5	SE52, INR-7, SE56, E681	*B. safensis, B.altitudinis, Lysinibacillusxylanilyticus, Paenibacilluspeoriae*
6	SE34, SE56, E681, FZB24, FZB42	*B. safensis, Lysinibacillusxylanilyticus, Paenibacilluspeoriae, B. amyloliquefaciens, B. amyloliquefaciens*
7	2RA-17, 99-101, 33B-9, IN937a	*B. cereus*(group)*, B. amyloliquefaciens, B. mojavensis, B. subtilis*
8	17A-3, 33B-9, 1PC-11, 1PN-19	*B. amyloliquefaciens, B. mojavensis, B. solisalsi, B. amyloliquefaciens*
9	99-101, 17A-3, 1PJ-32, EXTN-1	*B. amyloliquefaciens, B. amyloliquefaciens, B. amyloliquefaciens, B. amyloliquefaciens*
10	S8-G6, IN937b, SE34	*B. subtilis, B. subtilis, B. safensis*

Experiment 2: Effect of PGPR Inoculum Concentration

The experiment was designed to determine if a higher concentration of bacteria would perform better than a lower concentration. Three mixtures showing the best results in experiment 1 were tested. Treatments were organized in a CRBD with a 2×5 factorial arrangement with 8 replicates. Treatments consisted of two concentrations-application regimens: (i) 5 ml of 10^6 spores/ml (transplanting) and (ii) 5 ml of 10^7 spores/ml (transplanting) + 10 ml of 10^6 spores/ml (2 weeks after transplanting). The second factor consisted of three microbial mixtures + 80% of the N fertilization, a control (no PGPR) + 80% of the N fertilization, and a positive control with 100% of the N fertilization. Plants were evaluated at 4 weeks after transplanting, fertilization, experimental setup, and parameters evaluated were the same as experiment 1.

Experiment 3: Nitrogen Fertilization Rates Study

The experiment was designed to predict how much N fertilization could be reduced when selected PGPR mixtures were applied. Three of the best mixtures obtained in experiment 1 and used in experiment 2 were tested. Treatments were organized in a RCBD with a 4×5 factorial arrangement with 8 replicates. Treatments consisted of three PGPR mixtures (1,2, and 5) and a control (no PGPR) and five N fertilization regimens: 100% (280 mg/kg), 80% (224 mg/kg), 70% (196 mg/kg), 60% (168 mg/kg), and 50 % (140 mg/kg). Fertilization, inoculum concentration, experimental setup, and parameters evaluated were the same as experiment 1. This experiment was performed in cabbage and pepper plants which were evaluated both at 4 weeks after transplanting.

Nutrient analysis

Plant nutrient analysis was performed on the dried shoot tissue. Ground tissue from each plant shoot was analyzed for N using the combustion method (LECO FP-528 Nitrogen Analyzer), and an Inductively Coupled Argon Plasma Emission Spectrophotometer/Vacuum (ICP) was used to measure P, K, S, Mg, Ca, S, B, Fe, Cu, Al, Zn, and Mn concentrations.

Statistical analysis

Analysis of variance (ANOVA), using a general linear model (GLM), was used to analyze each response variable in experiments 1 and 2. The least significant difference (LSD) test was used to identify significant differences among treatments. All statistical analyses were performed using SAS software version 9.2 (SAS Institute, 2010) with a significance level of $\alpha=0.05$ set *a priori*.

The data from experiment 3 were analyzed using general linear regression models, by the means of ANCOVA in which N applied (%), PGPR mixture, and interactions were the independent variables. ANCOVA is a more efficient procedure as it tests if the means of the dependent variable are the same among treatments (ANOVA) while controlling for the effect of the continuous variable (covariate). Three multiple regressions per crop were estimated as there were three different measures of dependent variable in each crop. For each one, the observations of all PGPR mixtures were pooled together in one regression in order to have more degrees of freedom and to avoid possible t and z distributions incongruence due to the low number of observations in each bacteria mix experiment. Each regression had dummy (binary) variables for each treatment, which means that if the observation corresponded to the bacteria PGPR mixture 1, the dummy variables for mixture 2 and mixture 5 would be zero, thus not having any effect on the dependent variable. When using dummies, one treatment (usually the control) was left out to avoid singularity, serving as the base for comparison purposes. The exponential functional form (Cobb-Douglas) has been long used in crop production regression models as it better fits fertilizer response

(Nelson, Voss, et al., 1985). Moreover, it was chosen over its linear counterpart as it fit the data better. The exponential function transformed into a linear form is as follows:

$$\log y = \log a + \beta \log N + \gamma_i \, trt_i + \delta_i \log N * trt_i$$

Where y is the dependent variable (fresh shoot weight, dry shoot weight, or N uptake), N is the amount of N applied, and trt_i are the dummies for each bacteria mix. β, γ_i, and δ_i are the parameters to be estimated. A property of this model is that it allows for prediction along the range of N applied (140 mg/kg – 280 mg/kg) making these regressions quite useful.

III. Results and discussion

Experiment 1

From all the plant parameters evaluated (Table 2), it was not possible to identify only one mixture that had a consistently greater effect. PGPR mixtures 1, 2, 5, 7, and 9, used with 80% N,showed significantly higher plant diameters compared to the 80% N control. PGPR mixtures 1 and 2 were not significantly different from the 100% N positive control, and mixture 2 + 80% N was the only treatment that was capable to enhance significantly the plant diameter compared to a 100% N fertilization. Plants treated with mixtures 1, 2, 4, 5, 7, and 10 showed higher chlorophyll content compared to the 80% N control. SPAD values obtained with mixtures 1, 2, 5, and 7 (mixtures + 80% N) were not significantly different from the 100% N positive control. Shoot fresh weight of all plants treated with the ten different PGPR mixtures (mixtures + 80% N) showed significantly higher values compared to the 80% N control.

Among the ten PGPR mixtures, mixture 2 was the only one capable of significantly increasing plant shoot fresh weigh compared to 100% N treatment. Finally, all PGPR mixtures except mixture 8

resulted in higher plant shoot dry weight compared to 80% N control. Also, with 80% N, all PGPR mixtures resulted in SDW values that were not significantly different from 100% N.

Table 2. Effect of inoculation with 10 different PGPR mixtures on Chinese cabbage parameters: plant diameter (Plant D), chlorophyll content (SPAD), shoot fresh weight (SFW), and shoot dry weight (SDW).

Treatments	Plant D (cm)	SPAD	SFW(g)	SDW(g)
MIX 1 +80% N	30.8 ab	38.41 abc	58.15 ab	4.37 b
MIX 2 + 80% N	30.08 bcd	38.98 ab	60.43 a	4.93 a
MIX 3 + 80%N	28.01 fg	37.12 cde	56.80 b	4.45 b
MIX 4 +80%N	28.37 fg	38.01 bcd	56.91 b	4.34 b
MIX 5 +80% N	31.93 a	39.08 ab	58.13 ab	4.43 b
MIX 6 + 80% N	28 00 g	37.55 cde	56.89 b	4.41 b
MIX 7 +80%N	29.42 cde	38.35 abc	57.57 b	4.41 b
MIX 8 + 80%N	28.02 fg	36.88 de	56.52 b	4.18 bc
MIX 9 + 80%N	29.03 ef	37.51 cde	57.32 b	4.31 b
MIX 10+ 80%N	28.01 fg	37.73 bcd	56.62 b	4.44 b
80% N	27.48 g	36.17 e	51.56 c	3.9 c
100% N	30.27 bc	39.55 a	56.99 b	4.49 b

†Means within a column followed by the same letter are not significantly different at the 0.05 level using LSD values.The data are means of two experiments.

None of the PGPR mixtures+ 80% N was capable to increase SDW more than the 100% N treatment. In general, all PGPR mixtures showed a plant growth promotion effect at one point. This finding indicates that a reduction of 20% of the recommend N fertilization + PGPR mixture has the same results as full fertilization without PGPR for some of the plant parameters. Regarding which PGPR mixtures showed consistently better results, mixtures 1, 2, and 5 (mixtures + 80% N) had a consistent increase in all the plant parameters compared to 80% N fertilization. Moreover, mixture 2 was capable to increase significantly plant diameter and SFW compared to the 100% N control. Hence, an application of 80% N recommended fertilization + PGPR mixture 2 resulted in higher plant diameter and SFW values than the 100% N recommended fertilization control without PGPR.

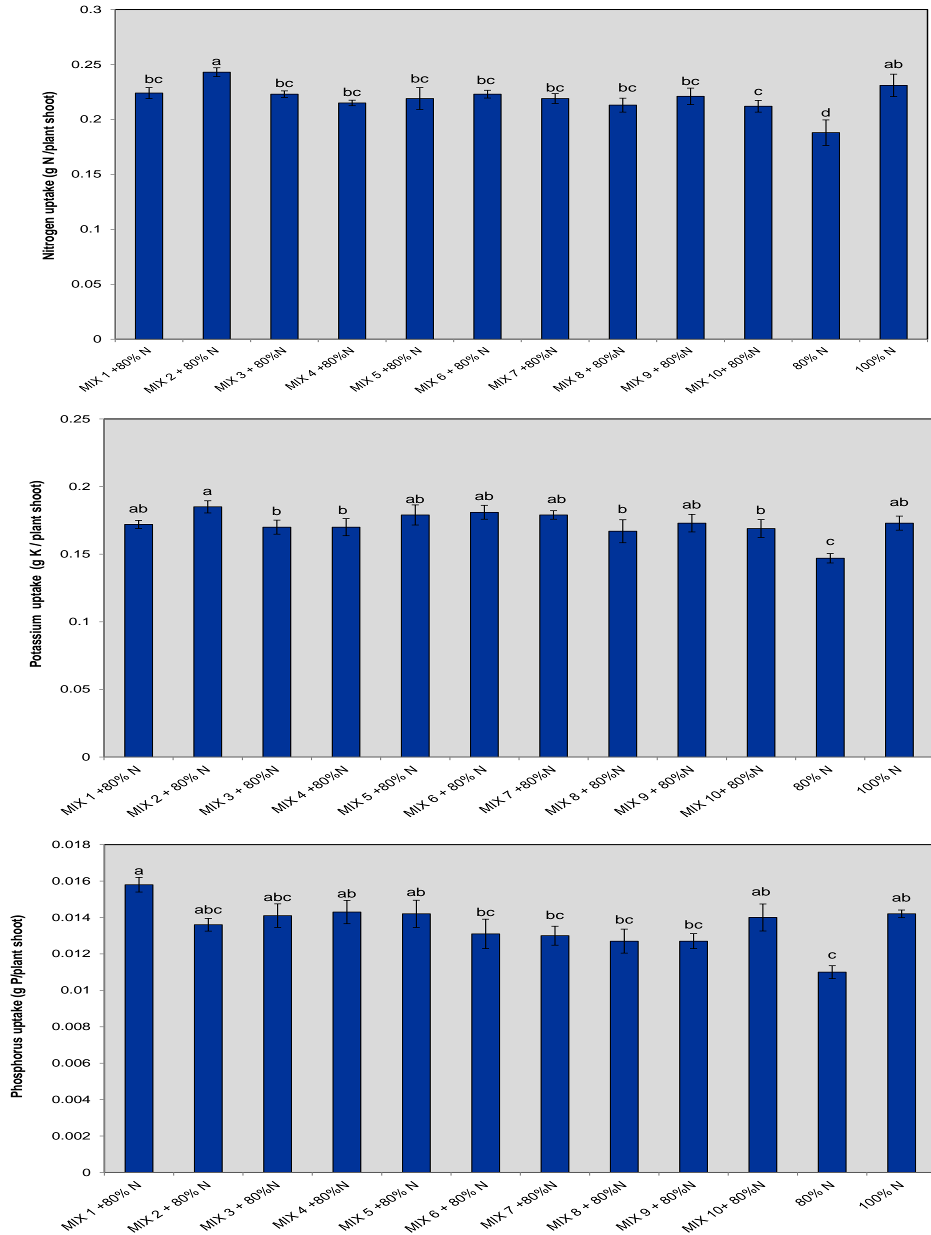

Figure 1. Total plant uptake of N, phosphorus and potassium. Uptake average of each of the 10 PGPR mixtures+ 80%N, control 80 %N, and positive control 100%N. Error bars represent the standard deviation of each treatment (n=8). Bars followed by the same letter are not significantly different at the 0.05 level using LSD values.

All treatments that contained PGPR mixtures + 80% N fertilization significantly increased N uptake compared to the control with 80 % N and no PGPR (Fig 1). All PGPR mixtures + 80% N treatments, except PGPR mixture 10, were not significantly different from the positive control that contained 100% N of the recommended fertilization. Among all PGPR mixtures, mixture 2 was the one that increased the N uptake the most. These results agree with the above results where PGPR mixture 2 increased plant growth parameters significantly. Even though only the 80% N fertilization was applied, the application of PGPR mixtures increased the uptake of N comparable to fully recommended N fertilization.

Even though P and K fertilization were maintained at the same levels in all treatments, PGPR mixtures significantly increased the uptake of these two nutrients compared to the 80% N recommended fertilization. The decreased N fertilization also indirectly affected the uptake of P and K as shown in the values observed in the 80% N control without PGPR mixtures. This treatment had significantly lower uptake values for P and K compared to the 100% N recommended fertilization. However, when PGPR mixtures were applied, P and K values were similar to the ones obtained with the 100% N recommended.

Results for plant growth and nutrient uptake in the present study agree with previous experiments that have shown a positive effect of PGPR inoculation on plant fresh and dry weight and on uptake of N,P,K of different types of crops such as pea, cotton (Egamberdiyeva and Hoflich, 2004), raspberry (Orhan et al., 2006), rice (Biswas et al., 2000), corn (Biari et al., 2008), and peanut (Dey et al., 2004) among others. Reduction of N fertilization due to the application of PGPR has been reported before in the production of vegetables in Cuba, where inoculation with the PGPR *Azotobacter chroococcum* reduced the need for N fertilizer by 40% (Martınez Viera and Dibut, 1996). The application of a mixture of PGPR species on rice fields also allowed a 40% reduction of N fertilization, resulting in the same level of yields compared to a 100% N fertilization (Martınez Viera, 2001). Our results also confirmed previous observations made by Adesemoye et al. (2009) who used two strains presented in PGPR mixture 1 (T4, IN937a), which, when applied to tomato plants, allowed the reduction of 75% of the total fertilization.

and maintained plant parameters and plant nutrient content similar to the full fertility rate without inoculants. Hence, the fact that two strains that previously showed fertilizer reduction effect in tomato also showed very similar reduction effects on cabbage demonstrated that this fertilization reduction capacity is consistent.

Experiment 2

From experiment 1, PGPR mixtures 1, 2, and 5 were selected. In experiment 2, the effect of two different spore concentrations were tested with these three mixtures. The ANOVA analysis of the effect of PGPR concentration on the different plant parameters did not show any significant differences (Table 3). Hence, there is no difference in applying a lower concentration of PGPR mixture spores than applying a higher concentration. Only an application of 5 mL of 10^6 spores /ml inoculum at transplanting was enough to have a plant growth promotion effect.

Table 3. Analysis of variance for the effects of PGPR mixture concentration, PGPR mixture, and the interaction of both factors on plant parameters: Plant diameter (cm) (Plant D), chlorophyll content (SPAD), shoot fresh weight (SFW), shoot dry weight (SDW), N uptake per plant shoot (N uptake), phosphorus uptake per plant shoot (P uptake), and potassium uptake per plant shoot (K uptake).

Factor†	**ANOVA *P>F* LSD (0.05)**						
	Plant D(cm)	**SPAD**	**SFW (cm)**	**SDW (cm)**	**N uptake (mg N/plant shoot)**	**P uptake (mg N/plant shoot)**	**K uptake (mg N/plant shoot)**
PGPR mixture (M)	0.0199	0.7927	0.042	0.0247	0.03815	0.0245	0.0761
Concentration (C)	0.0899	0.7633	0.8488	0.6003	0.9216	0.6533	0.1126
M x C	0.97	0.5527	0.3452	0.767	0.8052	0.8822	0.356

† PGPR mixture factors include: PGPR mixture 1, PGPR mixture 2, and PGPR mixture 5; concentration factors include: 10^6 spores/ml (transplanting) , and 10^7 spores/ml (transplanting) + 10^6 spores/ml (2 weeks after)

The results observed agree with previous results reported by Poupin et al.(2013) who found that higher inoculum concentration of the PGPR *Burkholderia phytofirmans* did not result in significant

differences in plant growth parameters of *Arabidopsis thaliana* than lower inoculum concentrations. Belimov et al. (2007) also observed that a lower concentration of 10^6ufc/ml of PGPR *Pseudomonas* spp. strains had a greater effect on root length than a higher concentration (10^7 and 10^9ufc/ml). Similarly, Nihorimbere et al. (2010) observed that there was no difference between the application of *Bacillus subtilis* at the concentrations of 10^5, 10^6, 10^7, and 10^8 spores/ml. All four concentrations resulted in the same growth promotion results (number of fruits, plant height, and fruit weight) of tomato plants. Even though our results concur with previous studies, we found that it is difficult to establish an optimum dose of bacteria to elicit a plant growth promotion effect. There are also many other variables that should be taken into account when choosing the correct doses and/or number of applications. For example, it is important to consider that the optimum doses might be specific to each type of crop, soil type, or environment. The optimum bacteria concentration will be the one that guarantees optimum bacteria colonization of plant roots.

Experiment 3

All Chinese cabbage regressions (shoot fresh weight, shoot dry wweight, and N uptake) were statistically significant at a 1 % level (F-test) withthe N uptake equation being the one that best explained the data (r-squared of 0.80 compared to 0.67 and 0.63 for fresh weight and dry weight, respectively) (Fig 2). In general, when analyzing the effect of N fertilization on the three different equations (SFW, SDW, and N uptake), we observed that the amount of N fertilization played an important role in the results (Fig 2). For example, for the control treatment (no PGPR mixture) when N fertilization was increased by 1%, fresh weight, dry weight, and N uptake measures were increased by 0.22%, 0.40%, and 0.83%, respectively.

Fresh weight regression showed that PGPR mixtures had a significant and positive effect regardless of the amount of N applied compared to the control (Table 4). When PGPR mixtures 1, 2, and 5 were applied, fresh weight increased by 5%, 4.9%, and 2%, respectively, compared to the control. This

can be observed in all the curves sharing the same slope but differing in the intercepts. For dry weight and N uptake regressions, the interactions were also statistically significant, meaning that there was an intercept effect but also a slope effect (Table 4). The negative sign of the interaction effect indicates that when a PGPR mixture was applied, the effect of increasing N application was lower compared to the control (Fig 2). Hence, it is possible to conclude that PGPR mixtures (1, 2, or 5) behave differently depending on the amount of N fertilization applied. In the dry weight graphic, it could be observed that when using between 50% and 65% of N fertilization, PGPR mixture 5 was the one that increased the dry weight the most compared to the other two treatments. However, when N applied was higher than 75%, mixture 2 was the one that increased the parameter the most. When N fertilization was higher than 95%, PGPR mixture 1 was as effective as mixture 2. For N uptake, when N applied was below 65%, PGPR mixture 1 was the one that increased N uptake the most. However, if the amount applied was higher than 65%, PGPR mixture 2 performed better.

Table 4. Estimated coefficients of the multiple regression models. Models include shoot fresh weight (SFW), shoot dry weight (SDW), and N uptake per plant shoot (N uptake) of Chinese cabbage.

Parameter	Variable	Parameter Estimate	Standard Error	t for H0: Param=0	Prob>|t|
log (SFW)	Intercept	2.734406	0.0836865	32.67	0.000
	log (N fertilization)	0.224666	0.0157918	14.23	0.000
	PGPR mixture 1	0.0500163	0.010608	4.71	0.000
	PGPR mixture 2	0.0492055	0.010608	4.64	0.000
	PGPR mixture 5	0.0208285	0.010608	1.96	0.052
log (SDW)	Intercept	-0.8190368	0.2312867	-3.54	0.001
	log (N fertilization)	0.4049623	0.0437763	9.25	0.000
	PGPR mixture 1	1.603782	0.3270888	4.9	0.000
	PGPR mixture 2	1.862156	0.3270888	5.69	0.000
	PGPR mixture 5	2.200271	0.3270888	6.73	0.000
	Interaction 1	-0.284255	0.0619091	-4.59	0.000
	Interaction 2	-0.3305174	0.0619091	-5.34	0.000
	Interaction 5	-0.3942343	0.0619091	-6.37	0.000
Log (N uptake)	Intercept	-6.235214	0.3222654	-19.35	0.000
	log (N fertilization)	0.8308729	0.0609962	13.62	0.000
	PGPR mixture 1	2.374637	0.4557521	5.21	0.000
	PGPR mixture 2	1.863022	0.4557521	4.09	0.000
	PGPR mixture 5	2.122985	0.4557521	4.66	0.000
	Interaction 1	-0.418356	0.0862616	-4.85	0.000
	Interaction 2	-0.3204196	0.0862616	-3.71	0.000
	Interaction 5	-0.3723636	0.0862616	-4.32	0.000

Using the multiple regression equations, it is possible to calculate the percentage of N fertilization that could be reduced in the presence of the different PGPR mixtures in order to have results equal to those from a 100% N fertilization without PGPR mixture. For fresh weight, N fertilization could be reduced by 20 %, 19.67%, and 8.85% when PGPR mixtures 1, 2, and 5 were applied, respectively. For dry weight, N fertilization can be reduced by only 1.5% when PGPR mixture 1 was applied. For N uptake, N fertilization can be reduced by 4.03%, 10.67%, 5.22% when mixtures 1, 2, and 5 were applied, respectively. Even though N fertilization could not be greatly reduced to obtain the same results as

100%N for dry weight and N uptake, a greater effect of PGPR mixtures was observed at lower N fertilization. This can be visualized by the greater differences on the PGPR mixtures curve compared to the control curve at lower N fertilization. As fertilization application increased the difference between the curves was reduced.

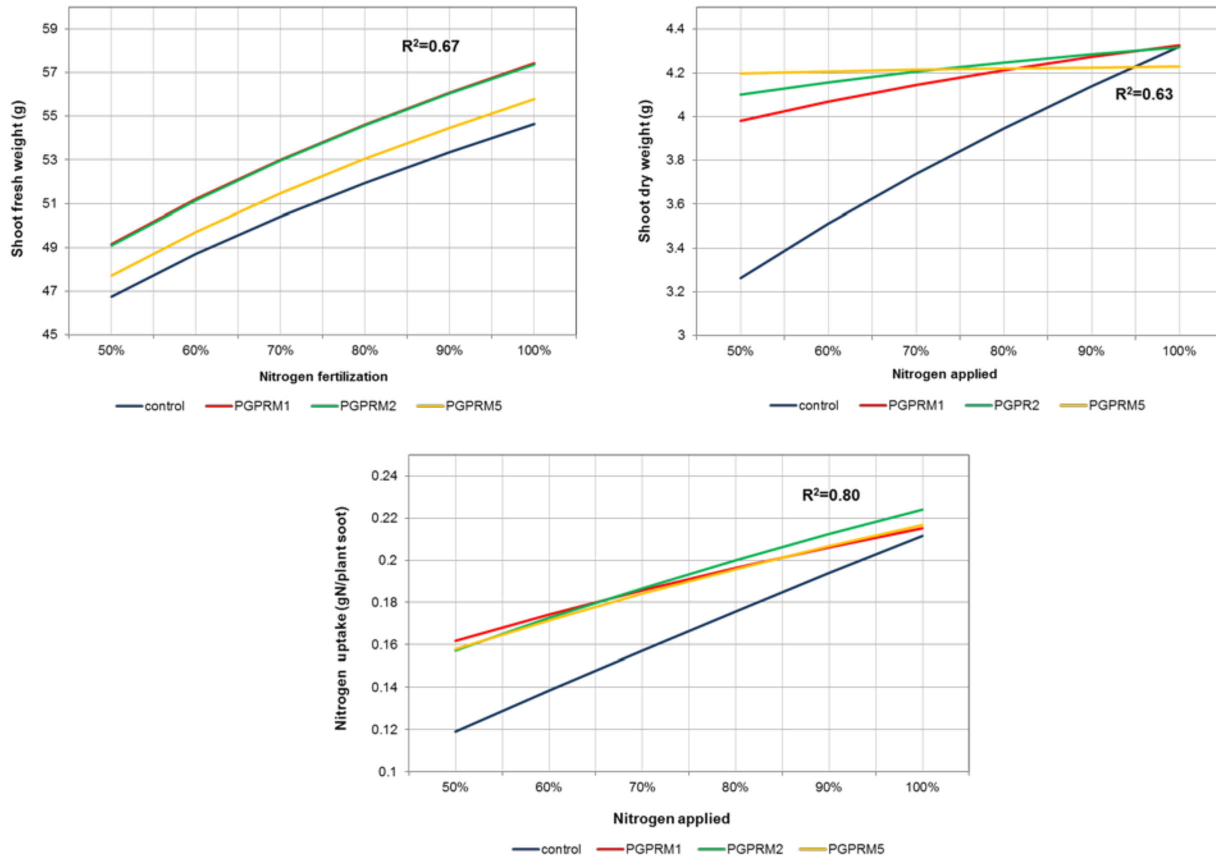

Figure 2. Mean shoot fresh weight, shoot dry weight, and N uptake (expressed as total g N in per plant shoot dry weight) regressions of chinese cabbage with three different PGPR mixtures (1 ,2, and 5) and a control(no PGPR) as a function of % N recommend fertilization.

All pepper regressions were shown to be statistically significant at a 1 % level (F-test). The N uptake equation was the one that best fit the data, showing a 0.69 r-squared compare to 0.56 and 0.68 for fresh weight and dry weight, respectively (Fig 4). These regressions showed again that N is an important factor in determining outputs. In this case, for the three parameters evaluated (SFW, SDW, N uptake), a PGPR mixture effect (intercept effect) and an interaction effect between PGPR mixture and N applied (slope effect) were present (Table 5).

Because of the negative sign of the interactions, the rate of output (SFW, SDW, and N uptake) increase was reduced as N fertilization increases (Fig 3). Similar to the Chinese cabbage results, a specific PGPR mixture was preferred depending on the amount of N that was applied. For example, with fresh weight, when the rate of applied N was below 75%, PGPR mixture 2 had the greater effect. Howeverwhen N applied was greater than 75%, PGPR mixture 5 had better results. For the dry weight there was not much difference between mixtures below 60%; however, after that point, mixture 5 or mixture had better results than mixture 2. Lastly, for the N uptake, PGPR mixture 1 had a better effect all along the range of N applied. Its effect became similar to the application of PGPR mixture 5 when approaching higher fertilization values (Fig 3).

Using the multiple regression equations, it is possible to calculate the percentage of N fertilization that could be reduced in the presence of the different PGPR mixtures in order to have results equal to a 100% N fertilization without PGPR mixture. For fresh weight, N fertilization could be reduced by 29.42 %, 33.51%, and 28.45% when PGPR mixtures 1, 2, and 5, respectively, were applied. For dry weight, N fertilization can be reduced by 33.67%, 25.01%, and 31.42% when PGPR mixtures 1, 2, and 5, respectively, were applied. For N uptake, N fertilization can be reduced by 32.90%, 19.10%, 28.79% when mixtures 1, 2, and 5, respectively, were applied. The magnitude of reduction of N fertilization by the three PGPR mixtures for the three parameters was greater in pepper than in cabbage.

Table 5.Estimated coefficients of the multiple regression models. Models include shoot fresh weight (SFW), shoot dry weight (SDW), and N uptake per plant shoot (N uptake) of pepper.

Parameter	Variable	Parameter Estimate	Standard Error	t for H0: Param=0	Prob>|t|
log (SFW)	Intercept	0.0870118	0.3148866	0.28	0.783
	log (N fertilization)	0.4928015	0.0595394	8.28	0.000
	PGPR mixture 1	1.266038	0.5450329	2.32	0.022
	PGPR mixture 2	1.581083	0.5450329	2.9	0.004
	PGPR mixture 5	0.1652664	282808	5.84	0.000
	Interaction 1	-0.2073214	0.1031253	-2.01	0.047
	Interaction 2	-0.2637379	0.1031253	-2.56	0.012
log (SDW)	Intercept	-2.388574	0.3577896	-6.68	0.000
	log (N fertilization)	0.5680238	0.0677199	8.39	0.000
	PGPR mixture 1	1.905205	0.5059909	3.77	0.000
	PGPR mixture 2	2.051265	0.5059909	4.05	0.000
	PGPR mixture 5	1.73868	0.5059909	3.44	0.001
	Interaction 1	-0.3200546	0.0957705	-3.34	0.001
	Interaction 2	-0.3525414	0.0957705	-3.68	0.000
	Interaction 5	-0.2898314	0.0957705	-3.03	0.003
log(N uptake)	Intercept	-0.9648997	0.3723196	-2.59	0.011
	log (N fertilization)	0.5689006	0.0704701	8.07	0.000
	PGPR mixture 1	1.337288	0.5265395	2.54	0.012
	PGPR mixture 2	1.49843	0.5265395	2.85	0.005
	PGPR mixture 5	1.050916	0.5265395	2	0.048
	Interaction 1	-0.2123076	0.0996597	-2.13	0.035
	Interaction 2	-0.253646	0.0996597	-2.55	0.012
	Interaction 5	-0.161999	0.0996597	-1.63	0.107

The negative interaction effect between PGPR mixture and N applied was greater in cabbage than in pepper, which explains why, especially for dry weight and N uptake, there was more effect of PGPR mixtures at lower N fertilization rates than at higher fertilization. As a result, for dry weight and N uptake, the reductions of N fertilization that could be made using PGPR mixtures, in order to have the same results as 100% N, were lower. It seems that plants benefit more from PGPR bacteria when the N fertilization is lower. This observation agrees with previous studies that showed that the effects of inoculation with the PGPR *Azospirillum* spp. are higher in fields moderately fertilized with N, P, and K

than in fields with high fertilization levels (Okon, 1985, Puente et al., 2009).Even though it was observed that PGPR mixtures performed better at lower fertilization rates, application of PGPR inoculants for plant growth promotion as part of a nutrient use efficiency program should be considered as a complement of chemical fertilization rather than a substitution.

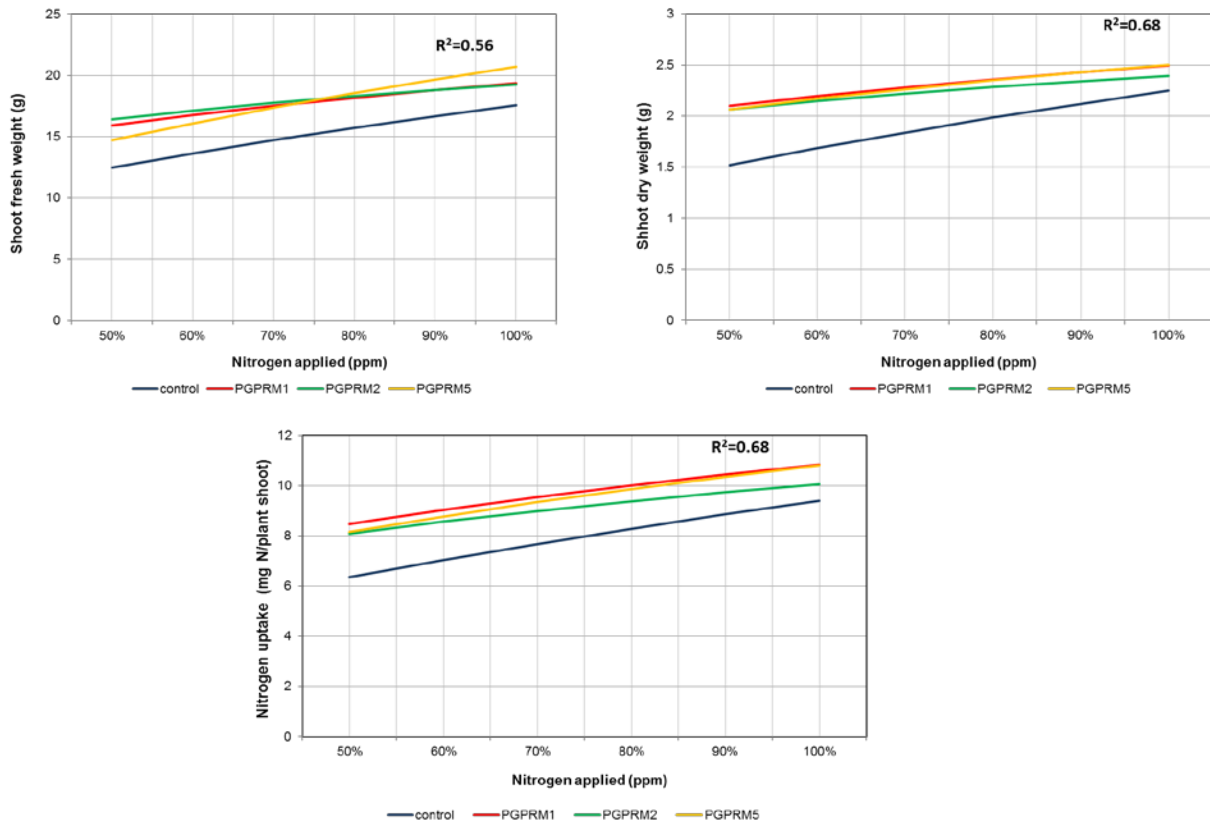

Figure 3. Mean shoot fresh weight, shoot dry weight, and N uptake (expressed as total g N in per plant shoot dry weight) regressions of pepperwith three different PGPR mixtures (1,2, and 5) and a control (no PGPR) as a function of % N recommend fertilization.

Even though it has been reported that the effect of PGPR inoculants depends on plant species or even plant cultivar, the effect of the three PGPR mixtures in cabbage and pepper followed the same trend. For plant fresh weight, it was possible to reduce N fertilization between 8% and 33% when PGPR mixtures were applied in both plant species. However, for dry weight and N uptake in cabbage and pepper, it seems that PGPR mixtures have a slightly different effect. . Higher reductions in N fertilization

for dry weight and N uptake were observed in pepper. This effect could be due to the different types of root exudates, which may support the activity of the PGPR inoculum or serve as substrate for the formation of biologically active substances by the inoculum (Khalid et al., 2004).

Because none of the *Bacillus* spp. strains was positive for N fixation, the mechanisms used by PGPR mixtures to increase nutrient uptake even at less N fertilization conditions could be related to hormone production , solubilization of soil nutrients, enhancement of root growth or root surface and/ or with a direct enhancement of plant nutrient uptake system as reported by Mantelin and Touraine (2004). It has also been proposed that microbial inoculation could impact soil mineralization proceses, thereby affect the direct availability of nutrients in soil solution that could potentially be absorbed by the plant (Brimecombe et al., 1999). Nevertheless, it is important to consider that the increased nutrient uptake by PGPR inoculants could be due to a combination of mechanisms more than the result of a single one.

IV. Conclusions

Overall, the results observed in the present study support the idea that PGPR inoculants can allow a decrease in N fertilization and still maintain plant growth at a level equivalent to a that obtained with full N fertilization rates. In the first screening with Chinese cabbage, three PGPR mixtures (1, 2, and 5) + 80% N fertilization showed results that were equal to 100% N fertilization without bacteria when SFW,SDW, plant diameter and chlorophyll content were evaluated. Furthermore, N uptake results when these three PGPR mixtures were applied were not significantly different from the 100% N fertilization without PGPR. Even though phosphorus and potassium fertilization was constant in all treatments, significant increases in uptake of P and K were observed by PGPR mixtures 1, 2, and 5 compared to the uninoculated 80% N fertilization control. The concentration effect experiment performed with the three selected PGPR mixtures showed that a higher concentration of 10^7 spore/mL (transplanting) + a reinoculation of 10^6 spores/mL (2 weeks after transplanting) did not result insignificant differences from a

single application of 10^6 spores/ml (transplanting). Multiple regression equations helped to predict the amount of N fertilization that could be reduced when the selected PGPR mixtures were applied in order to obtain the same fresh weight, dry weight, and N uptake as with 100% N fertilization in cabbage and pepper plants. In cabbage the three PGPR mixtures reduced N fertilization on average of 16%, 1.5%, and 6.64% when fresh weight, dry weight, and N uptake, respectively, were evaluated . On the other hand, reductions of N fertilization were greater when PGPR mixtures were applied in pepper. The average reductions were 30.46%, 30.03%, and 26.93% for fresh weight, dry weight, and N uptake, respectively. The two PGPR mixtures selected performed well in both crops, but the degree of this effect was different depending on the crop. In addition to the potential for N fertilization reduction by PGPR mixtures, it was also observed that in general PGPR inoculation had a greater effect compared to the non-inoculated control at moderate levels of N fertilization. This finding supports the idea that higher amounts of chemical fertilization could mask the potential effect of PGPR inoculants. PGPR inoculants should be considered as tools that will allow improvednutrient efficiency practices by increasing the plants' nutrient uptake efficiency and therefore reducing N losses. Further greenhouse and field studies should be performed in order to know the threshold of N fertilization reduction that could be achieved when PGPR inoculants are applied in different crops and with different types of N fertilizers.

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Plant Growth-Promoting Rhizobacteria Increase Plant Growth and Induce Changes in *Arabidopsis thaliana* Gene Expression of Nitrate and Ammonium Uptake Genes

Abstract

Plant growth-promoting rhizobacteria (PGPR) enhance plant growth by direct and indirect mechanisms. Recent evidence points out that PGPR may also affect nitrate and ammonium uptake genes, which could potentially explain the improvement in plant nutrition and plant growth. The objectives of this chapter are to determine if the application of three different PGPR *Bacillus* mixtures increases overall plant growth compare to a non-inoculated control and to evaluate the effect of these PGPR mixtures on expression of nitrate and ammonium uptake genes in root tissue. *A. thaliana* plants were grown in a soil-like substrate under growth chamber conditions, The results and three PGPR mixtures significantly increased plant shoot fresh weight, root fresh and dry weight, chlorophyll content, and plant diameter in plants at 21 days after transplanting (DAT).

Gene expression analysis revealed that the transcript levels of five of six nitrate genes evaluated were significantly higher on plants treated with PGPR mixtures. Specifically, the expression level was higher in AtNRT2.1 gene (62 folds) on plants treated with one of the PGPR mixtures. Among the five ammonium uptake genes evaluated, the transcript levels of four genes were significantly higher on the roots of plants treated with PGPR mixtures, and the highest transcript level increment of 21-fold was recorded for the AtAM1.3 gene. Mechanisms by which specific PGPR strains exert their positive effects are continually being investigated and studies typically identify specific bacterial metabolites that promote plant growth. The results of the present study suggest that plant growth promotion by PGPR can also be associated with up-regulation of plant genes involved in nitrate and ammonium uptake.

I. Introduction

Microbial associations with roots growing in soil are complex and can enhance the capacity of plants to acquire nutrients from soil through a number of mechanisms. Nitrogen (N) availability represents a limiting factor in crop production. Low N use efficiency promotes excessive N chemical fertilization, which increases production costs (Kaiseret al., 2002) and can negatively impact the environment. . In this respect, plant growth-promoting rhizobacteria (PGPR) are of particular importance and have been widely studied as agricultural additives. PGPR can stimulate plant growth through either a ”biofertilizing” effect or a ”biocontrol” effect. The biofertilizer effect results from several mechanisms including production of volatile compounds (Zhanget al., 2007), increased mineral and nitrogen availability in the soil (Linet al., 1983), and production of plant growth regulators/hormones (indole-3-acetic acid [IAA]), gibberellins, and cytokinins) which directly increase plant growth by increasing root surface area and number of root hairs (Bottiniet al., 2004, Khalidet al., 2004, Ryu, et al., 2005, Tsavkelovaet al., 2006).

In recent years, biofertilizers have been increasingly reported as alternatives or supplements to, fertilizers to stimulate improved uptake of nutrients as a possible solution to agro-environmental problems (Dibut, 2003, Martinez Viera and Dibut Alvarez, 2006). Many PGPR inoculants have demonstrated enhanced nutrient uptake in various crops, including rice (Meunchang et al., 2006), cotton and pea (Egamberdiyevaa and Hoflich, 2004), tomato (Kirankumar et al., 2008), maize (Biari et al., 2008) and peanut (Dey et al., 2004). Some PGPR-mediated increases in nutrient uptake are related to increases in root surface area and root morphology; as a result there is an indirect enhancement of nutrient uptake by the plant (Bákonyi et al., 2009). Increased root development leads to an increased root surface that could improve plant nutrient uptake thus would be a key factor for plant growth promotion by PGPR. It is also possible that PGPR have more direct effects on the root’s nutrient transport systems. For example, Bertrand et al. (2000) showed that an *Achromobacter* spp. enhanced nitrate (NO_3^-) uptake rate per unit

root area in *Brassica napus*. While the plant responses to PGPR are well described, the underlying signaling mechanisms triggered in plants by these bacteria that could potentially help enhance plant nutrient use efficiency are not yet well identified.

A first step in improving N use efficiency of plants would be to increase the primary acquisition process occurring at the root/soil interface. In Arabidopsis, NO_3^- and NH_4^+ transporters are located mainly at the root plasma membrane and, therefore, are positioned to uptake N from the soil (Forde and Clarkson, 1999). Uptake mechanisms of N in roots are specific and rely on high-affinity and low-affinity transport systems (Touraine et al., 2001). The "high –affinity transport systems" (HATS) transport ions when the concentration in the medium is low, in the range of 10 µM/L. The "low-affinity transport systems" (LATS) operate only when this substrate is present in relatively high concentrations in the medium, in the range of mM/L (Mort-Gaudry, 2001). Thermodynamic calculations and the association of both the HATS and the LATS with membrane depolarization suggest that both systems require energy potentially provided by proton gradients (Wanget al., 2012).

Nitrate transporters in *A. thaliana* have been classified as NRT1 transporters (also known as AtNRT1 in Arabidopsis), which include 53 members, and NRT2 transporters (also known as AtNRT2 in Arabidopsis), which include 7 members (Orsel et al., 2002). The relative contribution of these transporters to nitrate uptake is dependent on the developmental stages of the root and the N status of the plant (Wang, et al., 2012). In Arabidopsis, two AtNRT1 genes (AtNRT1.1 [CHL1] and AtNRT1.2) and two AtNRT2 genes (AtNRT2.1 and AtNRT2.2) have been implicated most often due to their involvement in nitrate uptake (Hoet al., 2009, Krouket al., 2010). In addition, these two genes are also expressed the most among 7 members, and their expression was identified mainly in root tissue (Forde, 2000). Expression of CHL1 (NRT1.1), NRT1.2, NRT2.1, NRT2.2, and/or NRT2.4 is regulated at the transcriptional level by nitrate, nitrite, ammonium, glutamine, N starvation, light, sucrose, diurnal rhythm, and/or pH (Krouk, et al., 2010). A strong correlation between the transcript abundance of these nitrate transporter genes and

nitrate uptake activities suggests that transcriptional regulation plays a key role in modulating nitrate uptake activities (Krouk et al., 2010, Wanget al., 2012).

The uptake of ammonium (NH_4^+) has been studied less intensively than that of NO_3^-. Many plant species that normally use NO_3^- also have an efficient system(s) for absorbing NH_4^+, which is constitutively expressed at high levels. An ammonium transporter has been identified as AMT (AtAMT in Arabidopsis) (Yuan et al., 2007). There are uncertainties regarding the exact chemical species transported by AMT, which can be in the form of either hydrophobic NH_3 or charged ammonium. The AMT1 family of high-affinity NH_4^+ transporters contains five members, of which AtAMT1.1, AtAMT1.2, AtAMT1.3, and AtAMT1.5 have been studied in detail (Glass et al., 2002). The location in which the transporter genes are expressed in the plant seems to be dependent on the plant species and type of transporter. AtAMT1.2, AtAMT1.3 and AtAMT1.5 genes are expressed in roots, while only AtATM1.1 is expressed in root and leaf tissue (Forde and Clarkson, 1999, Glass et al., 2002, Khademiet al., 2004). Furthermore, the AtAMT transporters are localized in different types of root tissue. While AtAMT1.1, AtAMT1.3, and AtAMT1.5 are localized in the plasma membrane of rhizodermis cells, AtAMT1.2 is found to be localized in the plasma membrane of endodermal and cortical cells (Ludewig et al., 2007). These locations indicate a spatial arrangement of AMT1-type transporters that assures ammonium uptake for efficient radial transport across the root tissue via the symplastic and apoplastic routes (Yuan et al., 2007).

Among all PGPR, species in the genus *Bacillus* have been the most often commercialized as biofertilizer and biological control agents in agriculture. An important characteristic of those bacteria is their ability to form thermo-stable and chemically-resistant endospores that allow them to survive for a long period of time in soil. The underlying signaling mechanisms triggered in plants by this specific bacterial genus have not yet been fully identified. Currently, there is little information about the potential effect of *Bacillus* PGPR on NO_3^- and NH_4^+ plant uptake genes and the connection that they may have with the increased nutrient uptake effect. Furthermore, none of the few studies available have been performed in soil or soil-like substrate, which limits the information available to *in vitro* environments. The main

objective of the present study is to determine the effects of selected *Bacillus* PGPR mixtures on plant development and the expression of NO_3^- and NH_4^+ uptake genes on *Arabidopsis thaliana* roots. In order to examine these effects, a soil-like system (*in vivo*), instead of an *in vitro* system, was used to grow *A. thaliana* plants. This method allowed conditions that mimic the relationship between PGPR and roots under agricultural conditions.

II. Materials and methods

Bacterial strains and microbial Source Preparation

A total of three different PGPR *Bacillus* mixtures were tested (Table 1). They were obtained from culture collections at the Department of Entomology and Plant Pathology, Auburn University (Auburn, AL, USA). These strains have shown important plant growth-promoting effects (Enebak, Wei, et al., 1998, Jetiyanon, Fowler, et al., 2003, Kokalis-Burelle, Vavrina, et al., 2003, Niranjan-Raj, Chaluvaraju, et al., 2003) and have been prevoiusly selected for their potential to increase nitrogen uptake in cabbage and pepper (results shown in the prevoius chapter). The bacterial mixtures were prepared by mixing each strain's spore suspension, which was previously quantified by plating the spore mix suspension on TSA and incubating for 48 h at 25 °C. The spore mix was then adjusted to a specific concentration.

Table 1. Strains and bacteria species present in the PGPR mixtures used in *Arabidopsis thaliana* experiment

PGPR Mix #	Original Strain #	Identification
1	INR-7, IN937a, T4, SE56	*Bacillus altitudinis, Bacillus subtilis, Bacillus safensis, Lysinibacillusxylanilyticus*
2	INR-7, SE56, SE76, FZB42	*Bacillus altitudinis, Lysinibacillusxylanilyticus, Bacillus safensis, Bacillus amyloliquefaciens*
5	SE52, INR-7, SE56, E681	*Bacillus safensis, Bacillus altitudinis, Lysinibacillusxylanilyticus, Paenibacilluspeoriae*

Plant source, growth conditions, treatments, and experimental design

Seeds of wild-type *A. thaliana* (ecotype Columbia 0, seeds originally provided by ABRC, Ohio State University, OH, USA, and propagated in the laboratory growth chamber) were maintained at 4°C for at least 2 days before sowing. Seeds were then surface-sterilized by immersion in 70% ethanol(v/v) for 5 min, washed subsequently four times with sterile distilled water, immersed in 1mL solution that contained 0.5 ml 0.1% Triton X-100 + 0.5 ml of calcium hypochlorite for 3 min, and subsequently washed four times with sterile distilled water. Approximately 100 seeds were sown in 4 separate plastic trays (22 cm (L) x 14 cm (W) x 5 cm (H)) containing 70 g of sieved (pass through a 0.4 cm sieve and through a 0.2 cm sieve) Sunshine Professional Growing Mix (Sun Gro Horticulture Canada Ltd.). To assess the effect of the PGPR mixtures, the experiment consisted of PGPR mixture 1, PGPR mixture 2, PGPR mixture 5, and a control with no bacteria. A total amount of 175 ml of bacteria spore suspension with a concentration of 10^5 spores/ml was applied before sowing to each tray. Enough additional water was applied to each tray in order to reach the WHC of the sunshine mix. After seeds were sown, each traywas covered with a plastic wrap and placed into a growth chamber (Percival Scientific E-41L2) with a photoperiod of 16 h of light(from 6 am to 10 pm), day/night temperatures of 23°C/22 °C, and an average of 100 μmol m^{-2} s^{-1} light intensity at the rosettes level. At 15 days after sowing (DAS), uniform seedlings were selected and transferred to individual pots containing 70 g of sunshine mix plus 200 ml water (amount of water to reach WHC). The experimental design was a complete randomized design (CRD) with 4 treatments (PGPR mixture 1, PGPR mixture 2, PGPR mixture 5, and control) and 10 replicates per treatments. Pots were placed in a growth chamber with the same conditions described above. Three days after transplanting, a second bacteria inoculation of 3 ml containing 10^7 spores/ ml bacteria was applied to the base of the plant.Plants were watered every other day by weighting each pot and adding enough water to reach 270 g.

Evaluation of plants

Plant growth was measured at 8, 12, 14,16, and 19 days after transplanting (DAT), using digital photography and image processing via ImageJ 1.47v software to determine rossette areas. At 21 DAT, plants were harvested and evaluated. From the 10 replicates per treatments, five were used to evaluate plant diameter, chlorophyll content (SPAD 502 meter), number of leaves, fresh shoot weight(FSW), root fresh weight (RFW), root dry weight (RDW), and maximum root length.

Total RNA isolation and cDNA synthesis

Roots from the above experimental setup were quickly harvested, immediately frozen in liquid nitrogen, and kept at -80°C. A metal spatula was used to grind frozen samples to a fine powder in an Eppendorf tube containing liquid nitrogen. Total RNA was extracted from 50mg root, and each sample was obtained by using TRIzol® Plus Purelink RNA Mini Kit (Ambion®) according to the manufacturer's instructions. RNA concentration and purity were determined using a NanoDropTM Spectrophotometer ND-2000 (Thermo Scientific, Wilmington, USA), and the integrity of RNA was also assessed by 1% agarose gel electrophoresis and ethidium bromide staining. The presence of contaminant DNA in the RNA samples was verified by PCR, in which specific primers of a known gene and PCR master mix (Promega) were used for PCR reactions. PCR reactions were performed according to the manufacturer's instruction under the following conditions: 95°C for 3 min, 40 cycles (95°C for 30 s, 57°C for30 s, and 72°C for 1 min), and 72°C for 5 min. After the reaction, the complete PCR reaction mix was loaded onto 1% agarose gel, and the PCR products were separated by electrophoresis. In all samples tested in this work, no fragments of genomic DNA were identified. First strand cDNA was synthesized from 500 ng RNA using a Goscrpit^{TM}Reverse Ttranscription System Kit (Promega USA) according to the manufacturer's instructions. All experiments were repeated at least five times.

Real-Time PCR

The transcript levels of genes that are involved in regulation of nitrate and ammonium uptake and transport in *A. thaliana* were measured by quantitative RT-PCR (see list of primers used in Table 2 & 3). PCR was carried out on an ABI 7500 Real Time PCR System (Applied Biosystems) with a 96 well rotor. The amplification reactions were performed with 25 µl of mixture consisting of 12.5 µl of PerfeCTA® SYBR® Green Fastmix®LowROX qPCR Master Mix (Quanta Biosciences, Inc, USA), 0.5 µl of cDNA, and 100 nM primers (Integrated DNA Technologies, Coralville, IA, USA). Relative RNA levels were calibrated and normalized with the level of four housekeeping genes: Cytoplasmic Glyceraldehyde-3-Phosphate Dehydrogenase (GAPC2), Actin(ACT1), ubiquitin specific protease 6(UBP6), and 18S ribosomal RNA (18S rRNA). The stable housekeeping gene was selected based on delta cycle threshold (ΔCt) ratio to transform the Ct values to linear scale expression quantities with help of Normfinder software (Version 20, excel addin). The reactions were incubated at 95 °C for 2 min, followed by 40 cycles of 95°C for 15 s and 57 °C for 34 s, and a final cycle of 72 °C for 34 s. PCR conditions were determined by comparing threshold values in a dilution series of the RT product, followed by non-template control for each primer pair. Relative RNA levels were calibrated and normalized with the level selected stable housekeeping 18S (AT2G03810) mRNA. Relative expression levels of genes were calculated by using the Pfaffl method (Pfaffl, 2001).

Statistical analyses

Analysis of variance (ANOVA), using a general linear model (GLM), was used to analyze each plant parameter and gene expresion response. All statistical analyses were performed using SAS software version 9.2 (SAS Institute, 2010) with a significance level of α=0.05 set *a priori*. The least significant

difference (LSD) test was used to identify significant differences of plant paramters between treatments, and Tukey's test was used to analyze gene expression results.

Table 2. Summary of most important genes analyzed and involved in nitrate and ammonium uptake and transport as expressed in root tissue of *Arabidopsis thaliana*.

Name	Function	Response	Reference
AtNRT1.1	High and low affinity nitrate uptake, also plays an important role regulating root architecture and lateral root elongation	Induction	Krouk et al. (2010)
AtNRT1.2	Low affinity nitrate uptake, express more in root hair and root epidermis	Constitutive	Huang et al. (1999)
AtNRT1.5	Low affinity bidirectional transporter that transport nitrate from outer root tissue to xylem		Theologis et al. (2000)
AtNRT2.1	High-affinity nitrate uptake, also regulate lateral root development. Express in mature root tissue (transmembrane transport)	Induction	Zhuo et al. (1999)
AtNRT2.2	High- affinity nitrate uptake	Induction	Zhuo et al. (1999)
AtNRT2.3	High affinity nitrate uptake	Constitutive	Nakamura et al. (1998)
AtAMT1.1	High affinity ammonium transporter		Mayer et al. (1999)
AtAMT1.2	High affinity transporter. Involve in ammonium transfer into vascular tissue		Theologis et al. (2000)
AtAMT1.3	High affinity ammonium transporter		Salanoubat et al.(2000)
AtAMT1.5	High affinity ammonium transporter		Salanoubat et al. (2000)
AtAMT2.1	High affinity ammonium transporter		Lin et al. (1999)
At18S	Housekeeping gene that has protein modification of type, N-terminal protein myristoylation		Arabidopsis genome initiative (2000)

Table 3. Gene specific primers sets for real-time RT-PCR amplification

AGI Code	Name	Forward primer (5' → 3')	Reverse primer (5' → 3')	Produce size (bp)
At1g12110	AtNRT1.1	CATGATTCTTTGTATTGAGGCCGTGGAGA	ATGAACGGAATTGTTCAGTGTGTGGCA	800
At1g69850	AtNRT1.2	CCGCCTCTTTCGTCTTAGTG	TCCCAAAAACTCGATGGAAG	250
At1G32450	AtNRT1.5	CTTCCTAACTCGCGTTTTGC	CCCCACAACCTCTTGGTCTA	230
At1g08090	AtNRT2.1	TCATCCGGGAGAATCTCAAC	CACGAAGCTCATGGAGAACA	210
At1g08100	AtNRT2.2	ATGGTAGTGAGCCGACCAAC	CGTCGAAACAAAACATGTGG	110
At5g69780	AtNRT2.3	ATCAAACGCACATGCACAAT	CCATGACATCAATGCACACA	200
At4G13510	AtAMT1.1	TGGTTTGGATGGTACGGATT	CCAGTTATGGCTGCAAACC	260
At1G64780	AtAMT1.2	CTCCTCTTCTCCGCCTACCT	AGGTGTACCAAAGGCGAATG	220
At3G24300	AtAMT1.3	GCCACAATGGGAACACTCTT	TAGCTGATCGAGGGAAAGGA	230
At3g24290	AtAMT1.5	TTCAACCCTGGTTCCTTCAC	AGTAACCCGTTGCAAACGTC	240
At2G38290	AtAMT2.1	CTTTTGTGCCATTGTGGTTG	CTTAGGCCTTGGTCCTACCC	180
At2G03810	At18S	AAGTCTGCGGAAGACAGCAT	TTAGCCCATGTGTTGGTGAA	190

III. Results and Discussion

Plant growth

All *A. thaliana* parameters evaluated at 21 DAT were significantly increased by the three PGPR mixtures (Table 3). Fresh shoot weight, root fresh weight, and dry root weight were increased 81%, 52%, and 100%, respectively, on average when PGPR mixtures were applied. Chlorophyll content measured by SPAD also was significantly increased by bacterial inoculation. Ling et al. (2011) reported that measurements made with the SPAD meter are highly correlated to values of absolute units of chlorophyll concentration in *A. thaliana*. Hence, it is possible to conclude that chlorophyll content is also somehow increase by bacteria inoculation. Application of PGPR mixtures enhanced plant growth during the whole growing season (Fig. 1). Differences in rosette area could be observed from the 8th day after transplanting (8 DAT). Inoculated plants appeared bigger and had larger and greater numbers of leaves compared to the control plants. Rosette areas were significantly increased by the PGPR mixtures in all five stages evaluated. These results showed that the positive effects of the PGPR mixtures on plant development

started at the early plant stages. The growth promotion elicited by the PGPR mixtures was maintained constantly in all stages until the last harvest at 21 DAT, in which differences between the control and the PGPR treatments were greater than previous days.. Plant growth promotion effects were not only limited to an increased rosette area. PGPR mixtures also increased number of leaves and total leaf area (Table 3).

Table 3. Effect of PGPR mixtures on plant parameters: diameter, chlorophyll content (SPAD), fresh shoot weight (FSW), number of leaves, maximum root length, fresh root weight (FRW), dry root weight (DRW), total leaf area evaluated at 21 DAT.

Treatment	Diameter (cm)	SPAD	FSW (g)	Number of leaves	Max root length	FRW (g)	DRW (g)	Total leaf area
Control	8.86 b	20.36 b	0.85 b	17.40 c	11.35 b	0.152 c	0.0086 b	32.79 b
PGPRM1	10.58 a	22.92 a	1.54 a	22.60 ab	13.44 a	0.250 a	0.016 a	50.47 a
PGPRM2	10.40 a	23.39 a	1.64 a	24.40 a	13.86 a	0.214 ab	0.017 a	47.84 a
PGPRM5	10.30 a	23.00 a	1.45 a	21.80 b	12.90 a	0.178 bc	0.015 a	48.75 a
LSD (0.05)†	1.15	1.15	0.43	2.42	1.54	0.0425	0.0036	11.22

† LSD = least significant difference for each plant parameter. Means within a column followed by the same letter are not significantly different at the 0.05 level using LSD values.

Similar results of plant growth promotion (total leaf area, plant weight, chlorophyll content, and root length) during early and late stages of *A. thaliana* development were previously reported using other PGPR species such as *Burkholderia phytofirmans* (Poupin, Timmermann, et al., 2013), *Phyllobacterium brassicacearum* STM196 (Mantelin, Desbrosses, et al., 2006), and *Bacillus subtilis* GB03 (Xie, Zhang, et al., 2009). Ryu et al. (2005) reported that two of the strains tested in the present study (T4 and INR937a) increased foliar fresh weight and total leaf area of *A. thaliana* plants grown in soil under greenhouse conditions. It was previously hypothesized that the faster growth observed in all stages of inoculated plants could be explained by different effects of bacteria, which, when put together, result in improved availability and acquisition of nutrients and/or a direct effect on plant metabolism (Vacheron, Desbrosses, et al., 2013).

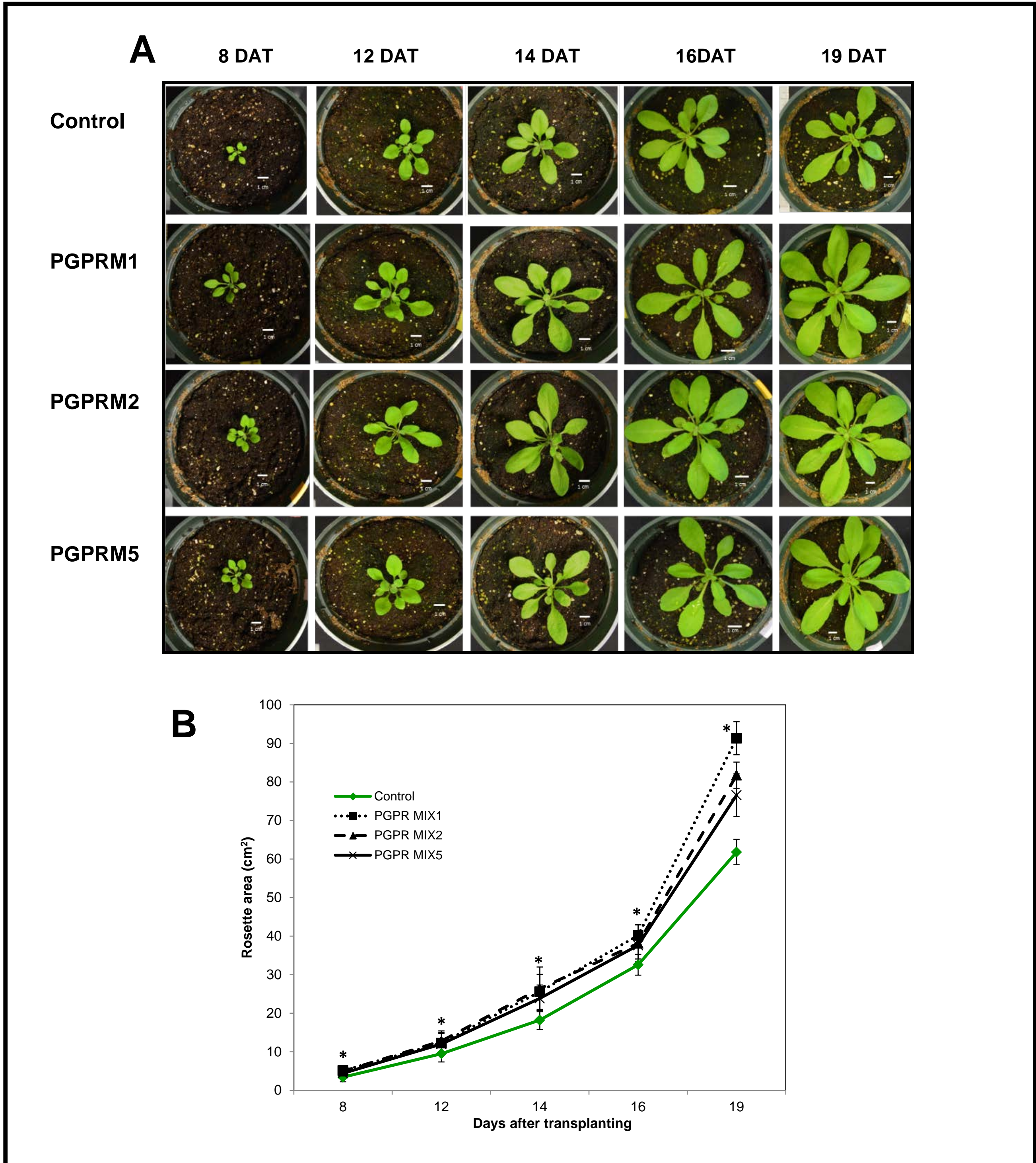

Figure 1. Effects of PGPR mixtures on rosette areas during long-term growth of *Arabidopsis thaliana* plants. A) Representative photographs of *A. thaliana* rosettes of plants exposed to the different treatments (control, PGPR mixture 1, PGPR mixture 2, and PGPR mixture 5) at 8, 12, 14, 16, and 19 days after transplanting (DAT), bars correspond to 1 cm. B) Graphic representation of rosette average of plants subjected to the different treatments at 8, 12, 14, 16, and 19 DAT. Media and SE were calculated with 10 plants per treatment. Asterisks indicate significant differences among the control treatment and the three other PGPR treatments at each time (One or two-way ANOVA, $p<0.05$).

Gene expression

Generally, the expression of genes that regulate nitrate uptake and transport were induced by treatment of *A. thaliana* with PGPR mixtures. The transcript levels of nitrate uptake genes were significantly higher when plants were treated with PGPR mixtures. However, the expression level of AtNRT2.2 gene in PGPR-treated plants was not significantly different from untreated plants (Fig 4). Apparently, the expressions of AtNRT2.1 and AtNRT1.1genes were higher in PGPR-treated plants. After treatment with PGPR-M1, PGPR-M2, and PGPR-M5, the transcript levels of AtNRT2.1 gene were significantly increased by 62, 45, and 29 folds, respectively. Likewise, the expression levels of AtNRT1.1 gene were increased by 39, 16, and 17 folds after treatment with PGPR-M1, PGPR-M2, and PGPR-M5, respectively. The expression level of the gene that regulates low affinity bidirectional transport of nitrate from outer root tissue to xylem AtNRT1.5 was increased significantly by 0.97 folds on plants treated by three PGPR mixtures. No single pattern of gene expression occurred following treatment with all three PGPR mixtures. For instance, AtNRT1.1 gene was more highly expressed in plants treated with PGPR-M1 than in plants treated with PGPR-M2 and PGPR-M5. AtNRT1.2 gene was more highly expressed in plants treated with PGPR-M2 than in plants treated with PGPR-M1 and PGPR-M5. Moreover, the expression level of AtNRT1.2 gene in PGPR-M5-treated *A. thaliana* plants was not significantly different from that found in plants in other treatments or the control (Fig 2). These results demonstrate that different PGPR strain mixtures can differentially affect genes involved in nitrate transport into the plant.

Similar gene expression studies were carried out for ammonium uptake genes, and the results were parallel to those of nitrate uptake genes. The transcript levels of ammonium uptake genes in all PGPR mixture-treated plants were significantly higher, except in AtAMT2.1 gene, which showed no significant differences among plants treated with PGPR mixtures and untreated ones. AtAMT1.1 genes showed significantly enhance expression only in PGPR-M1 treated plants (Fig 5). Plants treated with

PGPR-M2 and PGPR-M5 showed 35 and 42 fold significant increases, respectively, in transcript levels of AtAM1.5 gene. Similar to nitrate uptake genes, no pattern among PGPR mixtures was observed. However, plants treated with PGPR-M2 and PGPR-M5 showed very similar patterns of expression in all ammonium uptake genes, and in all cases there was no significant differences between their relative expressions (Fig 3).When comparing nitrate and ammonium genes, PGPR mixtures were able to induce higher level expressions of nitrate uptake genes than ammonium uptake genes. Forde and Clarkson (1999) reported that AtNRT 1.1, AtNRT1.2, AtNRT2.1, and AtNRT2.2 expressed the most among all nitrate uptake genes when *A. thaliana* plants were grown under optimal conditions (Forde and Clarkson, 1999). A strong correlation between the transcript abundance of these specific nitrate transporter genes and nitrate uptake activities suggests that transcriptional regulation plays a key role in modulating nitrate uptake activities (Krouk, Crawford, et al., 2010, Wang, Hsu, et al., 2012). In the present study, two of these genes, AtNRT1.1 and AtNRT2.1, were highly expressed in plants treated with PGPR mixtures, which indicates that select PGPR exerted a significant effect on two of the most important genes involved in nitrate uptake in roots.

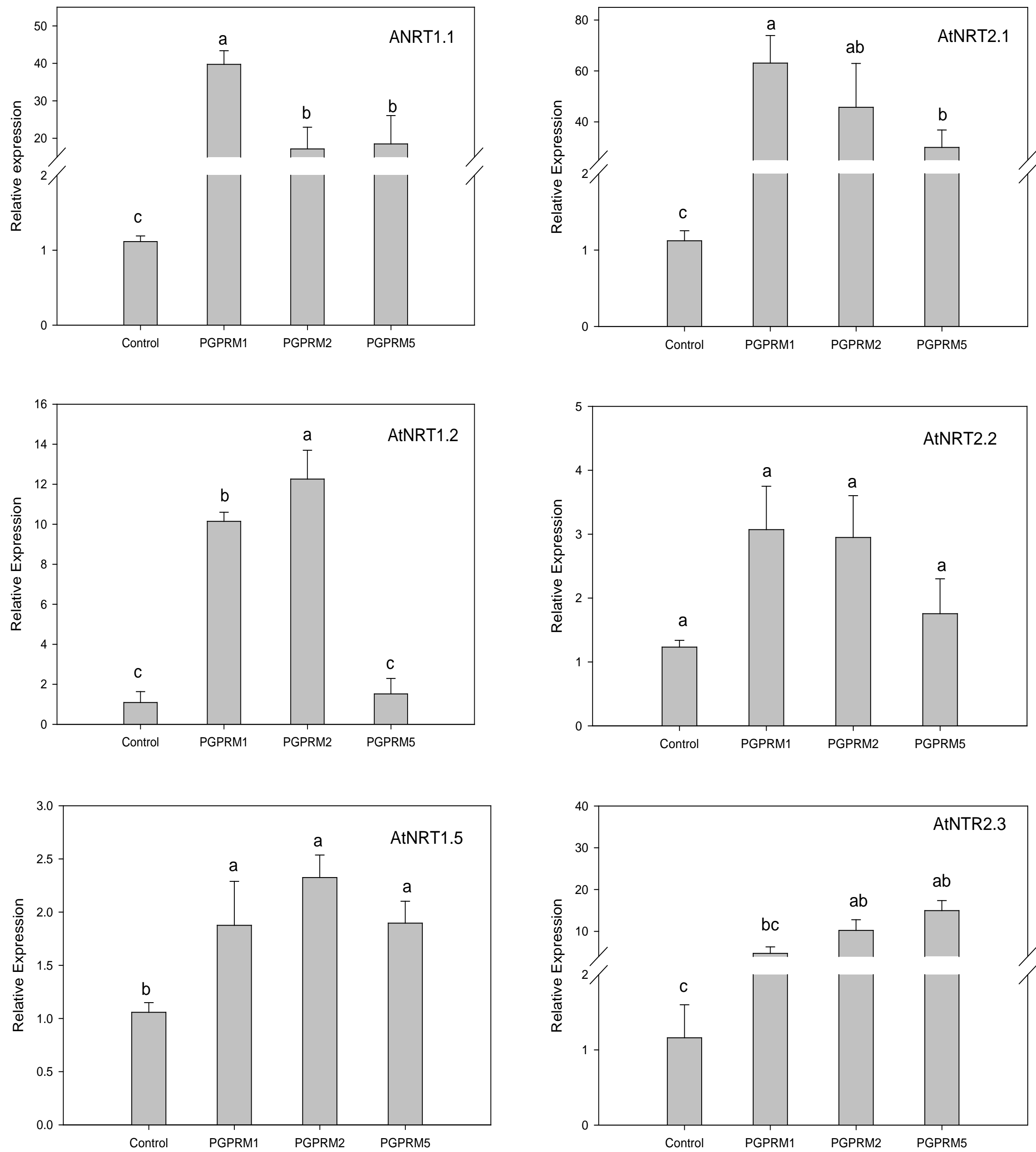

Figure 2. Quantitative real time PCR of selected nitrate uptake (AtNRT1.1,AtNRT1.2, AtNRT2.1, AtNRT2.2, and AtNRT2.3) and nitrate transport(AtNRT1.5) genes expressed in root tissue of *Arabidopsis thaliana* after inoculation with three PGPR mixtures. Quantitative RT-PCR determinations of relative levels of gene expression in complete plants at 21 DAT. Data are means ± SE. Columns labeled with the same letter are not significantly different based on Tukey's test (α =0.05).

Ammonium uptake transporters are all high affinity transporters. Unlike nitrate uptake genes, they are expressed in different parts of the root tissue. Ammonium can enter the symplastic route for radial transport toward the root stele via regulation of AMT1.1, AMT1.3, and AMT1.5, which are localized at the plasma membrane of rhizodermis cells, including root hairs. Ammonium can also bypass outer root cells via the apoplastic transport route and subsequently enter the root symplast by AMT1.2-mediated transport across the plasma membrane of endodermal (in the root hair zone) and cortical (in more basal root zones) cells (Yuan et al., 2007). In the current study, AMT1.1, AMT1.2, AMT1.3, and AMT1.5 genes showed significantly high transcript levels in *A. thaliana* plants treated with three different PGPR mixtures, which demonstrates that PGPR affect apoplastic and symplastic ammonium transport in different root tissues including root hair, endodermal, and cortical cells. It is important to note that the one ammonium uptake gene in which PGPR mixtures did not show any effect (AtAMT2.1) is distantly related to the AtAMT1 family (Sohlenkamp, Shelden, et al., 2000). It has not been characterized as well as the AtAMT1 family and has a lower activity in root cortex and meristematic root tissue. It may be expressed in leaves and other *A. thaliana* organelles. Because AtAMT2.1 is expressed in various tissues, it is likely that this transporter plays diverse roles in the plant (Sohlenkamp, Shelden, et al., 2000).

Only a few previous studies have focused on the impact of PGPR on regulation of nutrient uptakein plants. Inoculation of canola with *Achromobacter* sp.strain U80417 resulted in an increase of NO_3^- and K^+ net influx rates per root surface area unit (Bertrand, C, et al., 2000). Mantelin et al. (2006) reported that there was no effect on expression of nitrate and ammonium genes (AtNRT1.1, AtNRT1.2, AtNRT2.1, AtNRT2.2, AtNRT2.3, AtAMT1.1, AtAMT1.2, AtAMT1.3, AtAMT1.5, and AtAMT2.1) in *A. thaliana* roots inoculated with *Phyllobacterium brassicacearum* STM196 . However, the transcript levels of nitrate transporter genes AtNRT2.5 and AtNRT2.6 in *A.thaliana* shoot increased by 20 and 25 fold, respectively, in *P.brassicacearum* inoculated plants. Notably, the genus *Phyllobacterium* is a gram-negativebacterial genus,unlike *Bacillus* which is a gram-positive genus. Hence, the potential effect on specific nitrate or ammonium uptake genes may vary among genera of PGPR.

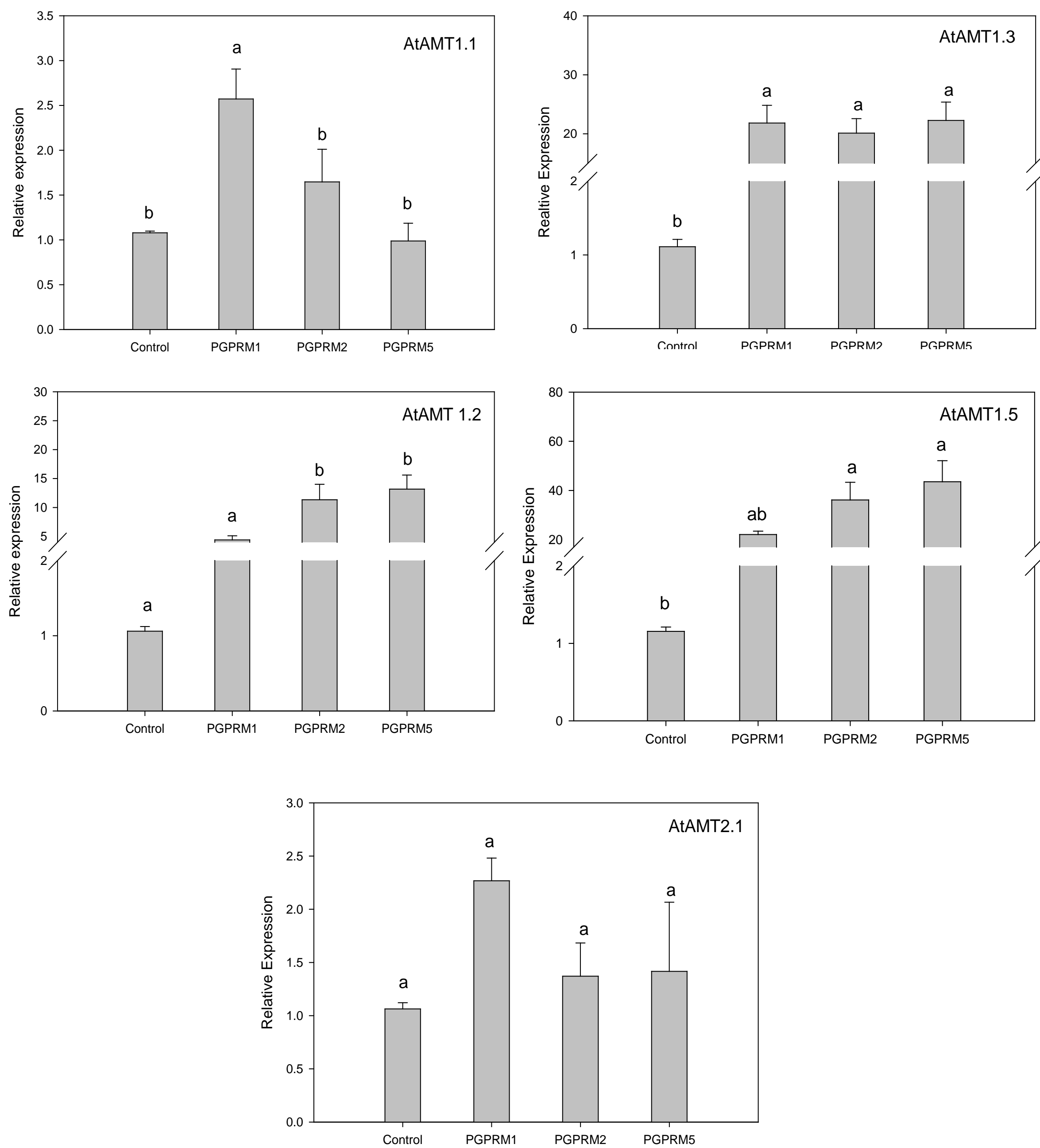

Figure 3. Quantitative real time PCR of selected ammonium uptake (AtAMT1.1, AtAMT1.2, AtAMT1.3, AtAMT1.5, and AtAMT2.1) genes expressed in root tissue of *Arabidopsis thaliana* after inoculation with three PGPR mixtures. Quantitative RT-PCR determinations of relative levels of gene expression in complete plants at 21 DAT. Data are means ± SE. Columns labeled with the same letter are not significantly different based on Tukey's test (α =0.05).

We could not find any previous studies that showed an effect of *Bacillus* PGPR on nitrate and ammonium uptake genes. Previous *in vitro* microarray studies by Lakshmanan et al. (2013) showed that inoculation with *Bacillus subtilis* FB17 resulted in upregulation of membrane transport genes in roots. They also found upregulation levels as high as 104 fold in unknown protein genes when bacteria were applied *in vitro* to *A. thaliana* plants. These results support the idea that PGPR significantly affect plant gene expression and confirm that PGPR have an effect that is not limited to production of metabolites.

With only few exception (Ryu et al., 2005), most studies involving *A. thaliana* and PGPR until now were performed under *in vitro* conditions. *In vitro* studies using *A. thaliana* are important for genetic research; however, when performing *in vitro* PGPR experiments with *A. thaliana*, the interaction between PGPR and microorganisms in the plant rhizosphere is not taken into account, which could affect the gene expression results. Through the release of root exudates in the rhizosphere, plants can affect bacterial colonization and also bacterial gene expression, especially genes encoding plant-beneficial traits. Moreover, PGPR also interact with bacteria naturally found in soil outside the rhizosphere. Thus it is important to consider the complexity of the interaction of PGPR with their rhizosphere environment (Vacheron, Desbrosses, et al., 2013). For this reason, there may be a difference in results from PGPR applications in plants grown *in vitro* and plants grown in a soil-like subtract (*in vivo*). In support of this hypothesis, Ryu et al. (2005) observed that the majority of PGPR strains that were more effective at promoting *A. thaliana* growth *in vitro* were only moderately effective *in vivo.*

Here we have reported for the first time *in vivo* effects of PGPR mixtures that are related to more than one bacterial species in the regulation of plant growth and expression of genes that are involved in nitrate and ammonium uptake and transport. When PGPR mixtures are applied as inoculants their effects on plant growth were the result of the contribution of all active individual cells from each bacterial species (Vacheron, Desbrosses, et al., 2013). Furthermore, a synergistic effect between bacterial species in the same mixtures could be occurring, meaning that the performance level might be higher than if only one type of strain was involved (Bashan, 1998).

IV. Conclusions

Understanding the mechanisms behind PGPR-plant interactions is important to improve strategies for the use of these beneficial bacteria in agriculture. Here we have reported for the first time *in vivo* effects of PGPR mixtures that are related to more than one bacterial species in the regulation of plant growth and expression of genes that are involved in nitrate and ammonium uptake and transport. These findings support the hypothesis that PGPR can impact gene expression of *A. thaliana*. This gene regulation could be induced directly by the bacteria present in plant roots or as a response to a bacterial metabolite produced in the rhizosphere.

The results of this study also show that PGPR mixtures positively affect plant growth and plant development. Plants inoculated with the three PGPR mixtures showed increases in plant parameters such as plant diameter, fresh shoot and root weights, dry weights, number of leaves, and chlorophyll content. These increases in plant growth parameters by PGPR mixtures were associated with the expression of several genes involved in nitrate and ammonium uptake and transport in roots of *A. thaliana*. This effect was seen in the significantly higher gene upregulation as greater as 62 fold, which was recorded when PGPR mixtures were applied. Further analyses will be useful to confirm the relation between metabolites produced by PGPR and expression of nitrate uptake genes. Overall, these findings contribute to a better understanding of plants and beneficial bacteria interactions and provide novel information about the effect of PGPR on plant development and nitrate uptake regulation.

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