HARNESSING THE CAPACITY OF PGPR WITH OR WITHOUT AMF FOR

IMPROVED PLANT USE EFFICIENCY OF CHEMICAL FERTILIZERS

AND ORGANIC MANURE

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HARNESSING THE CAPACITY OF PGPR WITH OR WITHOUT AMF FOR IMPROVED PLANT USE EFFICIENCY OF CHEMICAL FERTILIZERS AND ORGANIC MANURE

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DISSERTATION ABSTRACT

HARNESSING THE CAPACITY OF PGPR WITH OR WITHOUT AMF FOR IMPROVED PLANT USE EFFICIENCY OF CHEMICAL FERTILIZERS AND ORGANIC MANURE

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The basis for the application of fertilizers (manure and chemical) is to make up for soil nutrient deficiencies and maintain soil fertility towards improved crop yield. Fertilizers could exacerbate environmental problems such as pollution of groundwater, leaching, nutrient runoff, soil salinization, greenhouse effect, global warming, etc which are major concerns. Alternatives that will halt this trend and which will have applications in different parts of the world are needed. Plant growth-promoting rhizobacteria (PGPR) and arbuscular mycorrhiza fungi (AMF) are important biofertilizers that could be used in an environmentally benign manner to improve plant nutrient use efficiency.

My first objective was to determine if PGPR or PGPR plus AMF will enhance N, P, and K uptake in plants with: (i) inorganic fertilizer application and (ii) organic fertilizer (chicken litter) application. A three-year field study was conducted with field corn from 2005 to 2007 in Sand Mountain, Alabama. Microbial inoculants, which included a

formulated PGPR product, AMF, and their combination, were evaluated across two tillage systems (no-till and conventional till) and two fertilization regimes (poultry litter and ammonium nitrate). Data were collected on plant height, yield (dry weight of ears and silage), and nutrient content of corn grain and silage. In addition, nutrient content of soil was determined, and bioavailability of soil nutrient was measured with plant root simulator (PRS^{TM}) probes. Results showed that inoculants promoted plant growth and yield. For example, grain yield (kg ha^{-1}) in 2007 for inoculants were 7,717 for AMF, 7,260 for PGPR+AMF, 7,313 for PGPR, 5,725 for Control, and for fertilizer were 7,470 for Poultry litter and 6,537 for NH_4NO_3. Nitrogen content per gram of grain tissues was significantly enhanced in 2006 by inoculant, fertilizer, and their interactions. Significantly higher amounts of N, P, K were removed from the plots with inoculants, based on total nutrient content of grain per plot.

The second objective was to determine (i) if reduced rates of inorganic fertilizer coupled with microbial inoculants (PGPR or PGPR plus AMF) will produce plant growth, yield, and nutrient uptake equivalent to that obtained with full rates of the fertilizer and (ii) to what minimum level the fertilizer could be safely reduced. The microbial inoculants used in this greenhouse study were a mixture of PGPR strains *Bacillus amyloliquefaciens* IN937a and *Bacillus pumilus* T4, a formulated PGPR product, and the AMF, *Glomus intraradices*. Results showed that supplementing 75% of the recommended fertilizer rate with inoculants produced plant growth, yield, and nutrient (N and P) uptake that were statistically equivalent to full fertilizer rate without inoculants. When inoculants were used with lower rates of fertilizer, the beneficial effects were not noted; however, inoculation with the mixture of PGPR and AMF at 70% fertility consistently produced the same yield as the full fertility rate without inoculants.

My third objective was to use ^{15}N tracer techniques to demonstrate that a model PGPR system (*Bacillus amyloliquefaciens* IN937a and *Bacillus pumilus* T4) can enhance plant uptake of N using different rates of depleted ammonium sulphate $(^{15}NH_4)_2SO_4$. Results showed that the dry biomass of plants which received 70% to 90% of recommended N fertilizer with PGPR inoculation was comparable to plants that received full rates of fertilizer without PGPR. Also, atom % ^{15}N per gram of tomato tissues decreased as the amount of fertilizer increased and PGPR inoculation had significant impacts on the values. For example, the atom % ^{15}N abundance in plants that received 80% fertilizer plus PGPR was 0.1146, which was significantly lower than 0.1441 for plants that received 80% fertilizer without PGPR.

In conclusion, the results support the idea that inoculants can aid plant nutrient use efficiency. In the long-term, results will have applications in sustainable use of fertilizers. In the short term, the integrated system and models that were developed would have practical applications for farmers. I recommend further studies using the models developed in this study as a launch pad to understand better, the intricacies of the actual flow of N, P, and K in PGPR-AMF-plant interactions.

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I. INTRODUCTION AND LITERATURE REVIEW

Plant nutrient requirements, deficiencies, surpluses, and the impact on plant health

There are sixteen elements (nutrients) considered to be essential for plant growth, nine among these are macronutrients while seven are micronutrients. The sufficient concentration required by plants is the basis for classifying an element as a macronutrient or a micronutrient (Mills and Jones 1996). Macronutrients include nitrogen (N), phosphorus (P), potassium (K), carbon (C), hydrogen (H), oxygen (O), calcium (Ca), magnesium (Mg), and sulfur (S). Micronutrients include boron (B), chlorine (Cl), copper (Cu), iron (Fe), manganese (Mn), molybdenum (Mo), and zinc (Zn). Many other elements are considered nonessential because they have little to do with the health of the plant. Even though some of these nonessential elements are often beneficial to plant health, they do not meet all the criteria for elemental essentiality. However, low or high concentration of nonessential elements in plants may pose health problems when they are consumed by humans and animals through the food chain. Examples of nonessential but beneficial elements include nickel (Ni), silicon (Si), sodium (Na), aluminum (Al), cobalt (Co), and titanium (Ti) (Mengel and Kirkby 1987; Mills and Jones 1996).

Some soils contain less than sufficient amounts of the essential elements, and then chemical fertilizers or manures are typically added as nutrient supplements to optimize crop productivity. However, fertilization has been associated with environmental problems such as nitrate and phosphate contamination of surface and (or) ground waters (Gyaneshwar et al. 2002; Sharpley et al. 2003). Lower or higher than recommended amounts of fertilizer are

both risky. Excess amounts of fertilizer may be washed away in runoff while lower amounts especially during unfavorable conditions, like dry surface soils during drought, may lead to less availability of fertilizer, which may result in stunted plant growth and low yield (Wade et al. 1999).

The impacts of plant nutrition on plant health could be either positive or negative as have been demonstrated by many authors, some of which are discussed below. Pacumbaba et al. (1997) studied the impact of four types of fertilizer on five pathogens (bacterial blight of soybean, Phytophthora root rot, soybean stem canker, soybean mosaic virus, and soybean cyst nematode) of soybean in Northern Alabama, USA, and showed a high significant interaction of fertilizer types and the rate of application of all fertilizer types with the incidence of all or some of the diseases. Zarafi et al. (2005) demonstrated that too high concentrations of both N and P fertilizers significantly increased the incidence and severity of downy mildew in pearl millet. Owolade et al. (2006) reported significant reduction in the incidence and severity of brown blotch disease of cowpea in Ibadan, Nigeria with phosphorus fertilizer application. Olesen et al. (2003) found low susceptibility of winter wheat (*Triticum aestivum*) to both powdery mildew (*Blumeria graminis*) and Septoria leaf spot (*Septoria tritici*) with split N application strategies but the severity of both diseases increased with increasing leaf nitrogen concentration.

Although the focus of this dissertation is on nutrient as an essential factor in plant growth, particularly N, P, and K, it is important to mention that besides nutrient, plant growth could also be constrained by many other physical and chemical factors such as water, temperature, light, relative humidity, carbon dioxide, soil moisture, etc. The importance of physical and chemical factors is underscored by the Julius von Liebig's "Law

of Minimum" that plant growth progresses to the limit imposed by the factor in least relative supply (Mills and Jones 1996).

Biogeochemical cycles: nitrogen, phosphorus and potassium

The soil environment is a complex system of biogeochemical cycles and three of the essential macronutrients – N, P, and K – which are the major growth limiting nutrients, are very important in the biogeochemical cycles and processes. Organic matter is a very important part of the cycles and it affects various chemical, physical and biological properties that are related to plant behavior and availability of nutrients (Fan et al. 2005). Saprophytes, which are organisms that live on dead plant materials, play important roles in the biogeochemical cycles and mineralization (Lugtenberg et al. 2002). During mineralization of plant residues in the soil, N, P, and K are produced along with other elements and they enter the biogeochemical cycle; thus, affecting soil fertility (Steinshamn et al. 2004). The biogeochemical cycling of N, as an example, is specifically dominated by four major microbial processes, which include nitrogen fixation, nitrification, denitrification, and N mineralization (Ogunseitan 2005). Nitrification and denitrification are important processes in the regulation of ammonium and nitrate in the soil. The combination of the two processes is the primary basis for the regulation of N in the soil and this has great impact on the N cycle and the whole biogeochemical cycle and soil fertility (Briones et al. 2003; Jetten 2008). Plant utilization of fertilizer N is impacted by the N cycle in the plant-soil system. In a similar manner, plant use of P and K fertilizers are impacted by the biogeochemical cycles. Figure 1 below is a depiction of the interactions and flow in the nitrogen cycle.

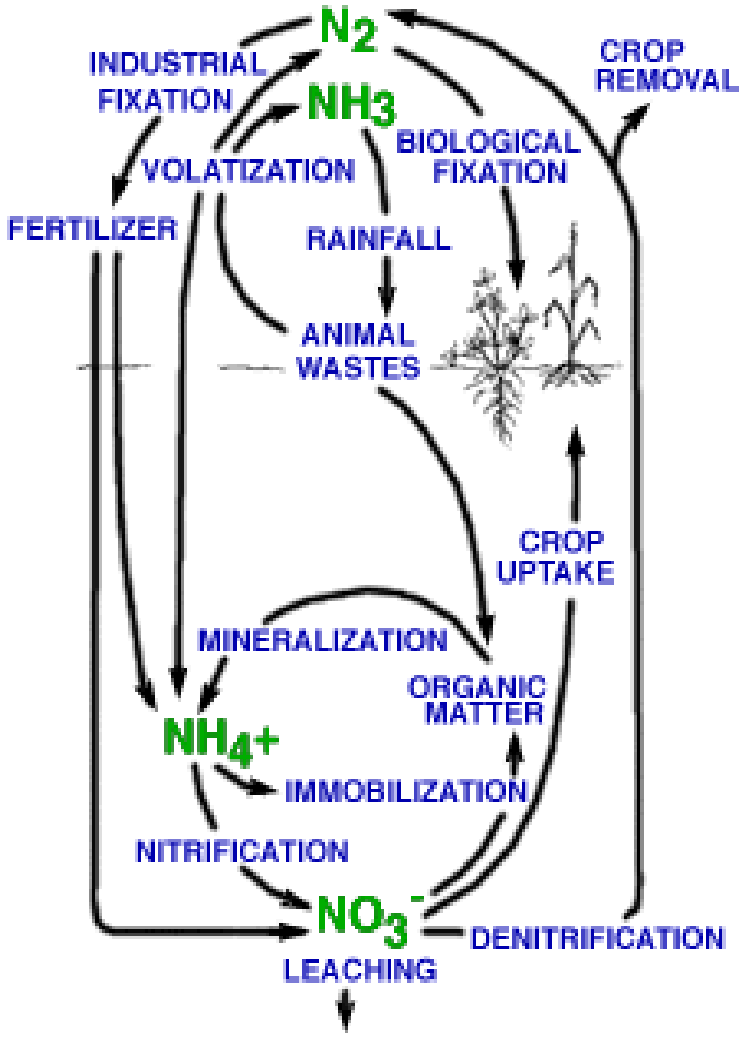


Figure 1: Nitrogen Cycle (Source: www.ncsu.edu).

Use of animal manure and chemical fertilizers

It cannot be overemphasized that the effort to make up for some soil nutrient deficiencies and maintain soil fertility towards improved crop yield is the basis for the application of both manure and inorganic (chemical) fertilizers. The rate of fertilizer usage has continued to increase over the years. The United States Department of Agriculture (USDA) reported that 30 million tones of N.P.K fertilizer used in 1960 across the world had risen to 154.8 million tons in 2005. In the U.S. alone, farmers used 7.46 million tons of

N.P.K fertilizers in 1960 compared to 22.15 million tons in 2005 (www.usda.gov). A publication of the Economic Research Services of the USDA (Gollehon et al. 2001), shows that in the U.S. in 1997, 68 counties had manure N exceeding assimilative capacities and 152 counties had manure P exceeding assimilative capacities (Figure 2A and B).

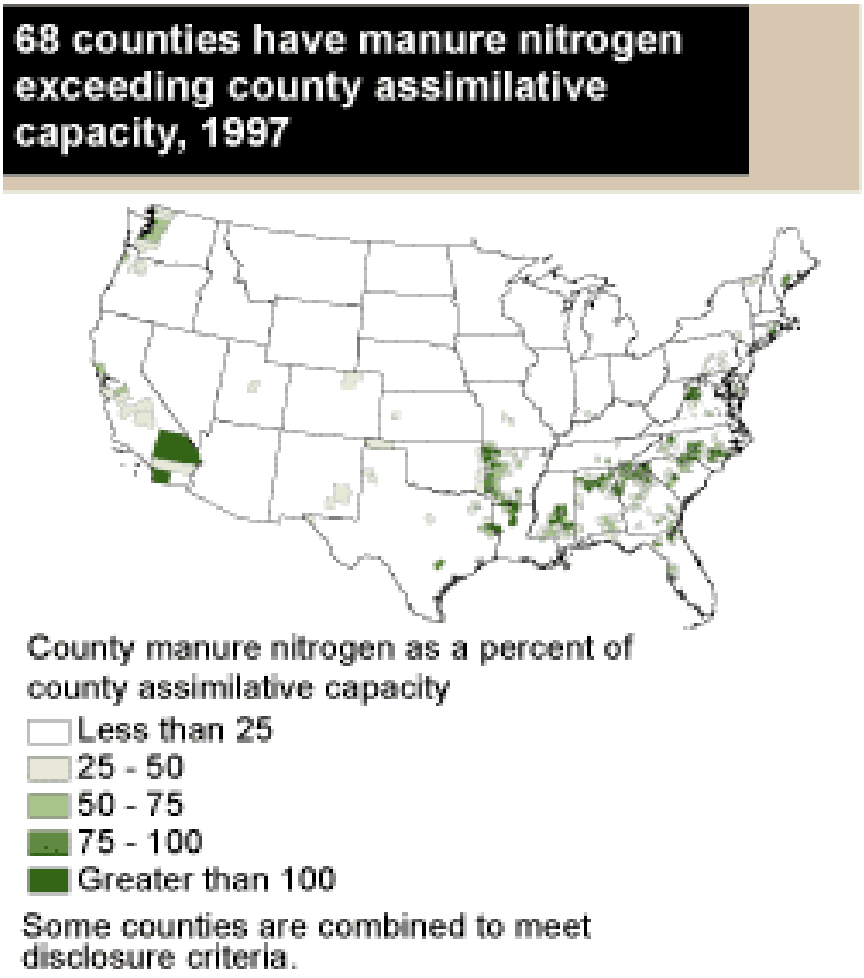


Figure 2A: Assimilative capacity of manure nitrogen in US counties in 1997 (Gollehon *et al*. 2001).

Mitchell and Tu (2005) reported that about 11.4 million tons of poultry broiler litter was produced yearly in the USA and 12% of all broilers produced in the USA came from Alabama. Relative to other animal manures, broiler litter contains high concentration of N,

P, K, Ca, micronutrient, and organic matter (Mitchell and Tu 2005). Although broiler litter is useful as plant nutrient sources, the excess nutrients from it constitute problems.

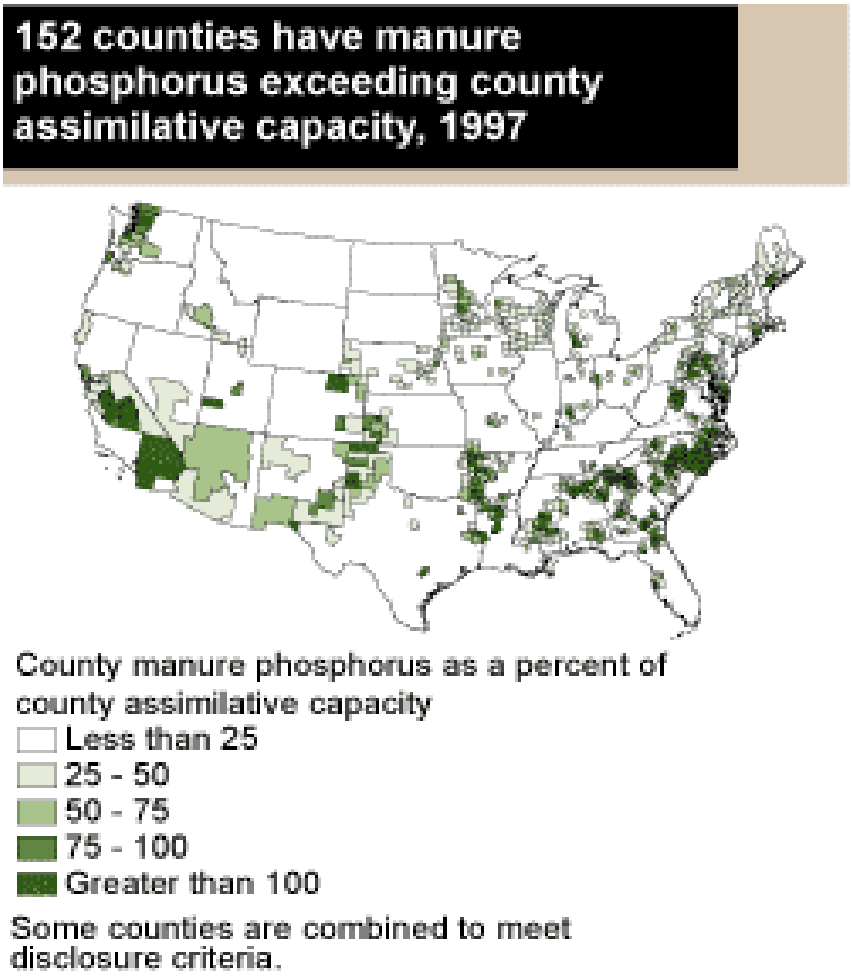


Figure 2B: Assimilative capacity of manure phosphorus in US counties in 1997 (Gollehon *et al*. 2001).

The issue of inconsistency, low efficiency and the influence of many factors in plant nutrient uptake when chemical fertilizers are used is a concern. Barlog and Grzebisz (2004) reported that winter oilseed rape plants (*Brassica napus* L.) showed year-to-year variability in maximum N uptake for whole plants and seeds in all the different types of nitrate

fertilizer that were used and within the growing season for some of the treatments. The yield of oilseed rape responded to the total amount of fertilizer N as well as the distribution pattern within the applied fertilizer rate and the timing of application (Barlog and Grzebisz 2004). The activity of N fertilizer is more pronounced from the soil surface to a depth of about 1 m but could sometimes continue below 1.5 m, especially when greater than recommended rates are applied. The type, rate, and timing of inorganic (chemical) fertilizers will affect the amount of fertilizer remaining in the soil after the season and this remnant could be subject to leaching (Ottman and Pope 2000). Many soils are P-deficient mostly because of the high reactivity of the soluble form with some metal complexes such as Fe, Al, and Ca leading to its precipitation. The reactivity of P to metal complexes has been reported to result in the precipitation or adsorption of between 75 – 90% of P (Gyaneshwar et al. 2002). Even when P fertilizers are added to such soils, their support to plants may remain little. When P-fertilizer is bound in soil, P is sparingly soluble to release sufficient P for high crop yield; the farmer may then have to add a large amount of fertilizers (Ohno et al. 2005; Gyaneshwar et al. 2002).

Discussing the importance of organic manure, Fan et al. (2005), observed that if inorganic fertilizer (e.g. N or P) was used alone, there was a tendency of a decline in soil chemical properties over time. Also, inorganic fertilizers cannot supply all required nutrients to plants, and hence, the addition of organic materials was needed for sustainability. Reactivity of P to soil is different in organic manures making bioavailability higher relative to inorganic fertilizers (Fan et al. 2005). For instance, dissolved organic carbon released from crop residue used for manure purposes can decrease P sorption, leading to greater soil P availability (Ohno et al. 2005). However, the mechanism for the bioavailability is not fully understood. Best management practices for fertilizers and

manures according to Alabama NRCS Nutrient Management code 590, is that they must be applied within 30 days of planting a crop or when a crop is actively growing.

Despite their usefulness and better modern application methods, problems arising from farm usage of fertilizers and organic manures are recurring across all regions of the world. In Southern USA, Mitchell and Tu (2005) reported that many cotton (the number one row crop in Alabama, Georgia, Mississippi, and Arkansas) producers have traditionally avoided animal manure as an alternative source of crop nutrient. With the information that Alabama ranks third in poultry production in the U.S (Mitchell and Tu 2005), then it becomes clearer why Alabama was affected that much in Fig. 2A and B, especially in areas where poultry production was higher. The negative impacts from poultry litter appear to be greater in the state relatively to many others.

Chemical fertilizers were an essential component of the Green Revolution. The overall success of the Green Revolution could actually be attributed in part to fertilizer use, because fertilizer along with improved seed and irrigation were considered to be an agricultural technological trinity at the time. Despite its benefits, the Green Revolution could be attributed in large part to the drastic increase in fertilizer use and the consequences. In modern day agriculture, one would be correct theoretically, to say that the use of chemical fertilizers has reached an optimum in many regions beyond which there are likely to be diminishing returns on yield. There is a great concern for the negative impacts of the low efficiency in the use of nitrogen and phosphorus on the environment throughout the world (Steinshamn et al. 2004).

Environmental impacts of the use of fertilizers and manure

Pollution of groundwater, phosphorus runoff, abnormal changes in soil pH, and changes in the salt concentration of soils are major environmental problems across the globe. The use of organic manures and chemical fertilizers can exacerbate these problems (Mosier et al. 1996; Frink et al. 1999). The enormity of these environmental issues will increase with time, so long as agricultural use of fertilizers and organic manure continue in order for food production to keep up with the demands of the growing world population (Vitousek et al. 1997; Frink et al. 1999; Fan et al. 2005). In the year 2000, the Food and Agricultural Organization reported that the world's population had exceeded 6 billion people (compared to 2.5 billion in 1950) and was consuming a daily average of about 2700 kcal per caput (www.fao.org).

Leaching and groundwater contamination

Nitrate leaching in agriculture has been documented for many crops. Ottman and Pope (2000) opined that leaching is inevitable due to high N fertilizer rates applied to farms and the need to leach salts periodically. However, the severity of leaching can be controlled by the farmer since it is influenced by nitrogen rate and timing (Ottman and Pope 2000). Concern over these issues has driven efforts towards finding measures that can lessen the use of fertilizers and manures; thus, reducing the negative effects. In the U.S, the Federal Clean Water Act of 1972 requires states to assess the impact of nonpoint sources of pollution on surface and ground waters and to minimize them (Sharpley et al. 2003; Maynard and Hochmuth 2007). Some states in the U.S. have enacted legislation targeted at the reduction of agro-environmental pollution and protection of groundwater quality (Sharpley et al. 2003). A specific example is the state of Arizona where all growers must

follow Best Management Practices (BMP) that include using soil and plant tissue testing as a guide to N fertilization (Ottman and Pope 2000).

Phosphorous runoff

Inorganic fertilizers and organic manures are now accepted universally as significant sources of P input into surface waters. Manures generated from livestock production have been reported to improve soil fertility but also lead to elevated concentration of P in runoff (Ohno et al. 2005). Ohno et al. (2005) warned that the practice of applying high amounts of P to soil often leads to eutrophication if P-rich soil particles erode from fields and reach surface waters. In some cases, nutrients may build up on the field. Fan et al. (2005) observed a substantial build-up of soil total phosphorus and available phosphorus following a long-term fertilization involving the combination of organic manure with inorganic fertilizer. As excess P contributes to eutrophication of surface waters, one of the effects could be death of aquatic organisms (Kleinman et al. 2005; Torbert et al. 2005). Part of the legislation in many states in the U.S now is that site assessment indexes must include P source coefficients (Sharpley et al 2003) so that fertilizers, manures, and biosolids applied to agricultural soils can be weighed on the basis of their relative availability to enrich runoff.

Other global phenomenon - greenhouse effect, global warming, ozone layer depletion, smog, and acid rain

Agro-environmental problems can be divided into two major groups – (i) those that are global in scale and (ii) those that occur in discrete locations without substantive impact at the global level (www.fao.org). Problems, which are theoretically localized, such as

leaching into groundwater; P runoff into surface water; build up N and P fertilizers; and salinization of irrigated lands were discussed above. The negative impacts of fertilizer on a global scale may include greenhouse gases, global warming, ozone layer depletion, and acid rain. Acid rain has high concentration of HNO_3 and H_2SO_4. Searchinger et al. (2008) listed fertilizer among the factors that could lead to increase in greenhouse gases especially when there is a change in land-use. The three most important greenhouse gases are CO_2, CH_4, and N_2O (Flessa et al. 2002; Jarecki et al. 2008). The release of CO_2, CH_4, and nitrogen compounds such as NH_3, N_2O, to the atmosphere may increase due to fertilizer application, especially when greater than recommended amounts are used. The release of gases could happen through gas fluxes from the soil surface or volatilization from plants (Mosier et al. 1997; Ottman and Pope 2000; Flessa et al. 2002; Jarecki et al. 2008), thus, contributing to global warming (Vitousek et al. 1997).

Effect on biodiversity and ecology of autochthonous microbes

All these environmental problems have effects on biodiversity of microorganisms in the soil. Undesirable effects associated with agricultural practices such as tillage, cropping methods, extensive use of pesticides, and fertilizer are far reaching on biodiversity, implying that the search for solution to the conflict between food production and environmental conservation must progress quickly (McLaughlin and Mineau 1995; Loneragan 1997; Frink et al. 1999).

"Dead zone" in the Gulf of Mexico: A case-in-point

A specific example of the negative impacts of fertilizer is the "dead zone" in the Gulf of Mexico where nutrients drain from fertilized farms across the Mississippi Basin

down to Louisiana and cause oxygen starvation (hypoxia or anoxia). This drives away aquatic life forms such as shrimps and fish leading to an almost lifeless area in the gulf. This "dead zone" is now seen as the largest in the Western Hemisphere (Malakoff 1998), with an estimated area of more than 25,900 km^2 in 2008 (Walsh 2008). Worldwide, "dead zones" have quadrupled in number in the last 30 years (Malakoff 1998; Diaz and Rosenberg 2008). More than 400 marine systems are now affected, resulting in a total area of more than 245,000 km^2 around the world (Diaz and Rosenberg 2008).

Rabalais et al. (1998) reported that each year, flood and spring rain wash nutrients including N and P into the Mississippi River and then to the Gulf of Mexico. It was estimated in the 1990s that about 1.82 million metric tons of the nutrients end up in the Gulf each year. Nitrogen fertilizer leaching from the Mississippi Basin's croplands, especially corn (maize) fields and livestock manure were identified as the leading sources of the nutrients (Malakoff 1998; Rabalais et al. 2002). With conducive conditions (e.g., sunlight), massive alga blooms occur near the surface. Then, in a sequence of events, hypoxia will set in when oxygen levels in the isolated bottom drops below 2 ml L^{-1}. Anoxia will occur when bacteria had used up the rest of the oxygen and bacteria are themselves suffocated (Malakoff 1998; Rabalais et al. 2002; Diaz and Rosenberg 2008). The *Time* magazine printed a picture in 2007 showing the rapid rate with which the dead zone has been expanding (see *Time* magazine of Thursday August 06, 2007).

Microbial inoculants (biofertilizers) as possible solutions: PGPR and AMF

Contamination from fertilizers and manures, which was described above, is not limited to any region of the world. The problem affects both developing and developed economies, though the enormity may be different (Ukpong and Moses 2001; Ottman and

Pope 2000; Wade et al. 1999; Yong and Jiabao 1999; Agrawal 1999). There is need to design alternatives that will halt this trend and which will have applications in different parts of the world. Biofertilizers are increasingly being reported as alternatives to, or a supplements with fertilizers to stimulate improved uptake of nutrient as possible solution to these agro-environmental problems (Vessey 2003; Egamberdiyeva and Höflich 2004; Aseri et al. 2008; Shaharooma et al. 2008). Lugtenberg et al. (2002) estimated that approximately 65% of N supplied to crops worldwide was from biofertilizers, among which *Rhizobiaceae* and mycorrhizae are the best known examples.

Nitrogen uptake from N-fixing bacteria associated with roots, iron uptake from siderophore producing bacteria, phosphorus from phosphate solubilizing bacteria, and sulfur uptake from sulfur-oxidising bacteria are all possible with biofertilizers (van Loon et al. 1998; Gyaneshwar et al. 2002; Saubidet et al. 2002; Vessey 2003; Lugtenberg et al. 2002). Among the biofertilizers with high prospects, according to Lucy et al. (2004), are free-living plant growth-promoting rhizobacteria (PGPR), which are not widely used by farmers. These microorganisms could enhance the efficiency of biological nitrogen fixation, the availability of trace elements, and the production of some plant growth substances (Kloepper 1989; Vessey 2003; Bashan et al. 2004; Osorio Vega 2007; Tariq et al. 2007). Some PGPR can enhance availability of phosphates (phosphorus solubilizers) for plant growth through P solubilization (Rodriguez and Fraga 1999; Gyaneshwar et al. 2002).

Another group of microorganisms that can positively affect plant growth by increasing nutrient and water uptake by plants is arbuscular mycorrhiza fungi (AMF). AMF are fungi with a high-affinity P-uptake mechanism that enhance P nutrition in plants. For instance, AMF scavenge the available P through their hyphae which have large surface areas. Extraradical hyphae of AMF act as a bridge between the soil and plant roots (Liu et

al. 2000; Bianciotto and Bonfante 2002; Stewart et al. 2005). The use of AMF is reportedly faced with two major problems. First, it is difficult to culture AMF *in vitro*, and the genetic basis of P solubilization and rhizosphere competence is not well understood (Amijee et al. 1989; Koide 1991; Gyaneshwar et al. 2002). Another problem is that a high concentration of the level of soil phosphate, for example above 100 parts per million (ppm) could lead to a reduction in hyphal growth and chlamydospore production by mycorrhizae, thus causing a reduction of the benefit to plant (Amijee et al. 1989; Koide and Li 1990; Koide 1991). A reduction in hyphal growth may affect both P and N uptake. For example, Ames et al. (1983) reported a correlation between mycorrhizal hyphal length and total N in mychorrizal plants, which were derived from applied ^{15}N-enriched ammonium sulfate $(^{15}NH_4)_2SO_4$, but no correlation was observed in non-mycorhizal plants.

Plant Growth-Promoting Rhizobacteria

Plant growth-promoting rhizobacteria (PGPR) can be described as bacteria having most of or all the following qualities - ability to colonize plant roots, adherence to soil in the rhizosphere, capacity to enter into root interior and establish endophytic populations with adaptability to the niche and benefit to the host plant (Kloepper and Schroth 1978; Hallman et al. 1997; Kloepper et al. 1999; Saubidet et al. 2002; Yan et al. 2003; Joo et al. 2004; Compant et al. 2005). In a review, Lucy et al. (2004) reported that some PGPR are able to reduce the negative impacts of irrigation after using high salty waters. The usefulness of PGPR in forestry as well as in environmental remediation has also been explored. In summary, PGPR have been reported as useful in many parts of the world and the beneficial effects are many, including biocontrol and management of soil and plant health (Kloepper et al. 1986; Thomashow and Weller 1988; de Freitas et al. 1997; Burd et

al. 1998; De Meyer et al. 1999; Parmar and Dadarwal 1999; Vazquez et al. 2000; Dobbelaere et al. 2002; Idriss et al. 2002; Lugtenberg et al. 2002; Mazzola 2002; Compant et al. 2005; Glick et al. 2007; Adesemoye et al. 2008a).

Benefits to plants when PGPR are used have been shown to include increases in seed germination rate, plant root growth, yield, leaf area, chlorophyll content, nutrient uptake, protein content, hydraulic activity, tolerance to draught, shoot and root weights, biocontrol, and delayed senescence (Mahaffee and Kloepper 1994; Glick 1995; Raaijmakers et al. 1997; Lucy et al. 2004; Mantelin and Touraine 2004). Also, some PGPR provide bioavailable phosphorus for plant uptake (Rodriguez and Fraga 1999; Saubidet et al. 2002; de Fraitas et al. 1997), nitrogen fixation for plant use (Bashan et al. 2004), sequestration of iron for plants by siderophores (Raaijmakers et al. 1997; Lugtenberg et al. 2002; van Loon et al. 1998), and production of plant hormones like auxins, cytokinins, and gibberellins and lowering of plant ethylene levels (Glick 1995; Glick et al. 2007). Some PGPR synthesize the enzyme 1-amino cyclopropane-1-carboxylate (ACC) deaminase which can lower ethylene level, a product of environmental stress in plants (Glick et al. 2007). Some of the important factors that can affect the performance of PGPR include the nutrient level of growth medium, adaptability of the PGPR strains to the environment, the population of PGPR (inoculum concentration) applied, and the capacity of the organism to bind and establish on or in seeds and roots (colonization) (Lucy et al. 2004; Yan et al. 2003).

The mechanisms of action of PGPR can be broadly divided into two - direct and indirect mechanisms. Direct mechanism of growth promotion in PGPR occur when bacteria produces metabolites or plant growth regulators/hormones (indole-3-acetic acid [IAA]), gibberellins, and cytokinins) (Glick 1999; Vessey 2003), which directly increase plant

growth and this could lead to improved root growth with large surface area and increased number of root hairs (Mahaffee and Kloepper 1994; Mantelin and Touraine 2004). Additionally, direct mechanism of PGPR include enhanced capacity of plant for uptake of nutrients (e.g., K, N, Fe, S) (Dobbelaere et al. 2002; Egamberdiyeva and Höflich 2004; Sheng and He 2006; Aseri et al. 2008; Shaharooma et al. 2008). Indirect mechanisms occur when PGPR exert deleterious effects on phytopathogens i.e., biological control. As reviewed by Kloepper (1993) and Glick et al. (2007), biocontrol can be achieved through antibiotic production, induced systemic resistance (ISR), parasitism, siderophore, competition for binding sites on the roots, cyanide production etc. The success of biocontrol of plant pathogens by PGPR may involve one or more of the metabolites or activities listed above (Kloepper et al. 1988; Elsheikh and Elzidany 1997; Kloepper et al. 1999; Park and Kloepper 2000; Alami et al. 2000; Ryu et al. 2003; van Loon et al. 2006**).**

Plant-microbe interaction on fertilizer use after a mixture of inoculants PGPR and AMF: synergism or antagonism?

The capacity of AMF to influence plant growth, water, and nutrient content has been widely reported over the years (Giovannetti and Mosse 1980; Gianinazzi and Gianinazzi-Pearson 1994; Barea et al. 2002; Giovannetti et al. 2006). However, the previously mentioned hindrances to the effectiveness of AMF must be considered. Considering all the attributes, benefits and weaknesses of PGPR and AMF discussed above, could their combination with reduced levels of inorganic fertilizer or organic manure be able to maintain the same plant growth, nutrient uptake, and yield achieved with full fertilizer rates? Could there be synergism between PGPR and mycorrhizae to maximize their benefits for plants in better uptake of N, P, and K? Would there be antagonism

between PGPR and AMF and so, would they have to be used separately? For example, Siddiqui et al. (2001) showed that using only PGPR strain *Pseudomonas fluorescens* GRP3 with organic manure performed better than the use of inorganic or organic fertilizers alone. Is it possible to replicate these results in a similar study elsewhere? Is it possible to get a reduction in the amount of fertilizer usually left in the environment after harvest through the combination of PGPR or PGPR plus AMF with fertilizer or manure?

Using ^{15}N isotope tracer technique to study N uptake

Isotope tracer techniques are increasingly being used in studying the different parts of the N cycle. Among the six known isotopes of N, ^{15}N and ^{14}N are the only isotopes that occur naturally and are stable, unlike isotopes ^{12}N, ^{13}N, ^{16}N, and ^{17}N, which are unstable. Isotopes ^{15}N and ^{14}N coexist in nature in every substance consisting N, with an almost constant abundance of 0.3663% for ^{15}N and 99.6337% for ^{14}N.

Isotope ^{15}N is commonly used in N tracer studies (Hauck and Bremner 1976). Any fertilizer having the percentage of ^{15}N below the 0.3663% natural occurrence is referred to as depleted, but if the percentage is higher than the natural occurrence, it is referred to as enriched. Enriched and depleted ^{15}N materials have both been used in tracer studies to monitor N recovery (Edwards and Hauck 1974; Ditsch et al. 1992; Hauck et al. 1994). The behaviors of the ^{15}N and ^{14}N isotopes are similar but their chemical identities are maintained in biological systems and the systems can hardly distinguish them. However, the isotopes can be differentiated with specialized equipment on the basis that some of their compounds behave differently in exchange or distillation columns (Hauck et al. 1994; Mulvaney et al. 1997). In a review, Hauck and Bremner (1976) reported that tracer methods offer several advantages over non-tracer methods for research on N cycle processes. One

advantage is that tracer N-fertilizer provides a definite result for studying both the behavior and fate of applied N because identification of labeled N is possible as it enters, is transformed, or leaves the system under study (Hauck and Bremner 1976; Saoud et al. 1992). Thus, relatively more accurate information can be obtained compared to non-tracer ^{14}N.

Goals, objectives, and overall concept of this study

This study will culminate in the development of new strategies to enhance nutrient bioavailability and improve nutrient uptake by plant roots from fertilizer and manure. The long-term goal of the study is sustainability in agriculture through the prevention of environmental pollution caused by fertilizer use. The short-term goal of the study is to develop an integrated system that combines bio-fertilizers (PGPR or PGPR plus AMF) and fertilizers (manure and inorganic) in an environmentally benign manner. Also anticipated is information on the combination of inoculants and appropriate reduced fertilizer rate that can maintain plant growth, yield, and nutrient content compared to full recommended fertilizer rates.

Specific objectives and hypotheses

The specific objectives and hypotheses tested in this study are as follows.

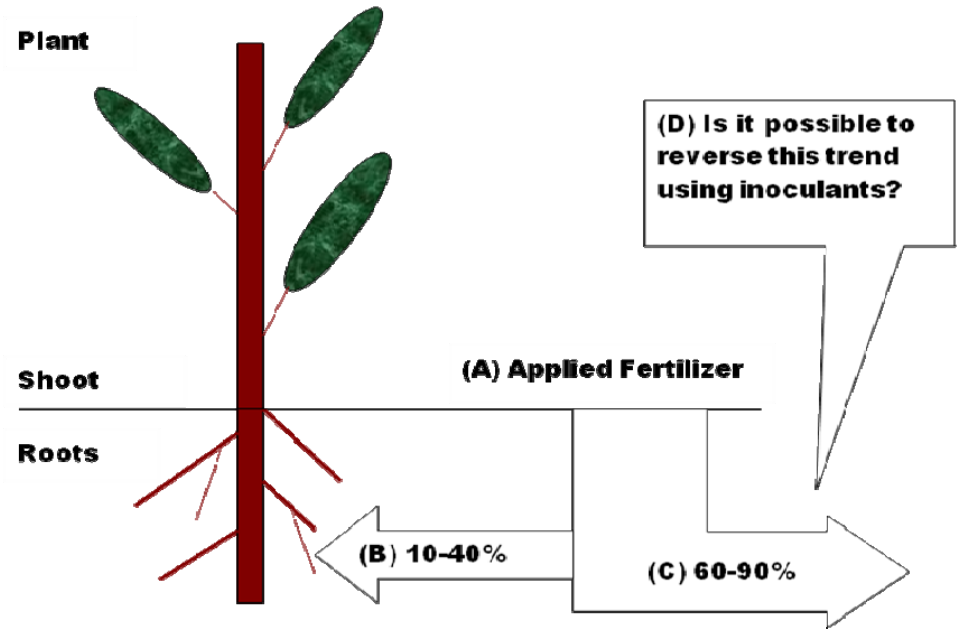
My first objective was to determine if PGPR or PGPR plus AMF will enhance growth, yield, and nutrient uptake in corn with: (i) inorganic fertilizer application and (ii) organic fertilizer (chicken litter) application. My hypothesis was that microbial inoculants that increase plant growth and yield will enhance nutrient uptake, and thereby remove more

nutrients, especially N, P, and K from the field as part of an integrated nutrient management (INM) system.

The second objective was to determine (i) if reduced rates of inorganic fertilizer coupled with microbial inoculants (PGPR or PGPR plus AMF) will produce plant growth, yield, and nutrient uptake equivalent to that obtained with full rates of the fertilizer and (ii) the minimum level that fertilizer could be reduced when inoculants were used.

My third objective was to use N tracer techniques to demonstrate that a model PGPR system can enhance plant uptake of N using different rates of depleted $(^{15}NH_4)_2SO_4$. The hypothesis was that PGPR will enhance plant uptake of N from ^{15}N-labeled fertilizer.

Fig. 3. A model for improved plant nutrient use efficiency with inoculants.



The concept of the study

Figure 3 above encapsulates the overall concept of this dissertation. In the figure, (A), the amount of fertilizer applied, is usually large; (B), the part of the applied fertilizer taken up by plant, is usually small, ranging between 10 to 40% depending on soil type, fertilizer type, plant, etc; and (C), the part of the applied fertilizer that is lost, which could be in the range of 60 to 90% of the original amount of fertilizer or manure applied (Rowarth 1997; Hood et al. 1999; Williams et al. 2001; Gyneshwar et al. 2002; Barlog and Grzebisz 2004; Kleinman et al. 2005). As have been discussed above, examples of the route of nutrient loss include N leaching, P fixation, P runoff, etc. Then, the overall question was asked in (D) - Is it possible to reverse this trend using inoculants while maintaining maximum plant growth and yield compared to the use of full recommended fertilizer rates? The application of this model is two-pronged. First, by getting more of the applied nutrient into the plant

tissues, fewer nutrients are left in the environment after the season, especially if crops are removed. Second, it will become possible to apply less amounts of fertilizer after achieving increases in the use efficiency of the applied fertilizers. In each case, reduction in agro-environmental pollution will be achieved.

II. ENHANCED PLANT NUTRIENT USE EFFICIENCY WITH PGPR AND AMF IN AN INTEGRATED NUTRIENT MANAGEMENT SYSTEM

Abstract

A 3 year field study was conducted with field corn from 2005 to 2007 to test the hypothesis that microbial inoculants that increase plant growth and yield can enhance nutrient uptake, and thereby remove more nutrients, especially N, P, and K from the field as part of an integrated nutrient management system. The field trial evaluated microbial inoculants, which include a commercially available plant growth-promoting rhizobacteria (PGPR), arbuscular mycorrhiza fungi (AMF), and their combination across 2 tillage systems (no-till and conventional till) and 2 fertilization regimes (poultry litter and ammonium nitrate). Data were collected on plant height, yield (dry weight of ears and silage), and nutrient content of corn grain and silage. In addition, nutrient content of soil was determined, and bioavailability of soil nutrient was measured with plant root simulator probes. Results showed that inoculants promoted plant growth and yield. For example, grain yield ($kg.ha^{-1}$) in 2007 for inoculants were 7717 for AMF, 7260 for PGPR+AMF, 7313 for PGPR, 5725 for the control group, and for fertilizer were 7470 for poultry litter and 6537 for NH_4NO_3. Nitrogen content per gram of grain tissues was significantly enhanced in 2006 by inoculant, fertilizer, and their interactions. Significantly higher amounts of N, P, K were removed from the plots with inoculants, based on total nutrient content of grain per plot. These results supported the overall hypothesis and indicate that application of inoculants can lead to reduction in the build up of N, P, and K in agricultural

soils. Further studies should be conducted to combine microbial inoculants with reduced rates of fertilizer.

Keyword: Plant growth-promoting rhizobacteria, arbuscular mycorrhiza fungi, integrated nutrient management, fertilizer, poultry litter.

Introduction

Fertilization is an essential practice to optimize crop productivity. However, fertilization has also been associated with nitrate and phosphate contamination of surface and (or) groundwaters, which can be attributed in large part to low efficiency in plant nutrient uptake. Phosphorus (P) is highly reactive with Fe, Al, and Ca leading to P precipitation at rates up to 90% (Requena et al. 1997; Gyaneshwar et al. 2002; Barlog and Grzebisz 2004), but overapplication of P can result in P runoff causing eutrophication of surface waters. Nitrogen (N) fertilization can also lead to runoff and leaching of nitrate into groundwater. In fact, nitrate leaching has been reported to be inevitable in agriculture production (Ottman and Pope 2000; Steinshamn 2004; Fan et al 2005; Kleinman et al. 2005; Ohno et al. 2005; Torbert et al. 2005).

Partly as a result of these problems, guidelines for P fertilization have been developed in some regions. For instance, many U. S. states include P source coefficients in site assessment indices so that materials applied to agricultural soils are evaluated on the basis of their relative availability to enrich dissolved reactive P in runoff (Sharpley 2003). Hence, integrated nutrient management (INM) is now being promoted to reduce negative impacts of P and N. The INM system promotes low chemical input but improved nutrient-use efficiency by combining natural and man-made sources of plant nutrients for increased

crop productivity in an efficient and environmentally prudent manner that will not sacrifice productivity of future generations (Gruhn et al. 2000).

Free-living plant growth-promoting rhizobacteria (PGPR) have shown promise as biofertilizers (Podile and Kishore 2007). Many previous studies and reviews had reported plant growth promotion, increased yield, solubilization of P or K, uptake of N and some other elements through inoculation with PGPR (de Freitas et al. 1997; Rodriguez and Fraga 1999; Joo et al. 2004; Sheng and He 2006; Glick et al. 2007). In addition, some studies have shown that treatment with PGPR enhances root growth, leading to a root system with large surface area and increased number of root hairs (Mahaffee and Kloepper 1994; Mantelin and Touraine 2004). Although, PGPR may be helpful in INM, they have not been evaluated as components of INM systems. Arbuscular mycorrhiza fungi (AMF) are another group of microbial inoculants that can influence plants growth, water and nutrient uptake. Extraradical hyphae of AMF act as a bridge between the soil and plant roots, however, AMF effectiveness is affected by soil P concentration (Liu et al. 2000; Bianciotto and Bonfante 2002; Stewart et al. 2005).

Our overall hypothesis was that microbial inoculants that increase plant growth and yield can enhance nutrient uptake, and thereby remove more nutrients, especially N, P, and K from the field as part of an INM system. In this study, we investigated PGPR, AMF, and their combination, as the microbial inoculants, for effects on growth and nutrition of corn grown in a long-term field study under 2 tillage systems (no-till and conventional till) and 2 fertilization regimes (poultry litter and ammonium nitrate).

Materials and methods

Experimental Design

The experimental design was a split-split plot in a randomized complete block with 4 replications. The main plot consisted of 2 tillage types (conventional till [CT] and no-till [NT]), 2 sub plots of either chemical fertilizer or manure (poultry litter), and sub-sub plots consisting of 4 types of inoculants (PGPR, a mixture of PGPR and AMF, AMF, and a water control). Each of the final sub-sub plots was 7.6 m (25 ft) long by 0.9 m (3 ft) wide. All treatments were applied to the same plots from year to year in order to confine treatment effects.

Field preparation and fertilizer application

This study was conducted on continuous corn plots within an existing long-term crop rotation field situated at the Sand Mountain Research and Education Center of the Alabama Agriculture Experiment Station in Crossville, Alabama. The initial split-plot had been in place for 25 years before the introduction of an additional split by 2005. Thus, the study period for this report spanned the summers of 2005, 2006, and 2007. We report here the results for 2006 and 2007. The test crop was field corn (CroplanTR1167RR), and seeding was done each year in April, with the specific date depending on weather conditions each year. The plots for conventional till were prepared by shallow disking, resulting in incorporation of crop residues, while no-till plots were planted by no-till planter. The manure used was dried poultry litter, applied at the rate of 427.5 $kg.ha^{-1}$. Crops received 57 kg $N.ha^{-1}$ as ammonium nitrate (NH_4NO_3, 32% N) and 171 kg $P.ha^{-1}$ as triple superphosphate at planting. They were then side-dressed with 171 kg N ha^{-1} as NH_4NO_3 between 4 and 5 weeks after planting. Also, 120.8 kg ha^{-1} of NPK 0-0-48, 22.8 kg

ha^{-1} of S, and 114 kg ha^{-1} of lime were applied based on the recommendations of Auburn University Soil Testing Laboratory, and no micronutrients were added.

Application of microbial inoculants – PGPR and AMF

One commercially available microbial PGPR and one AMF products were selected as models for the study. The selected PGPR product was Plant Growth Activator (PGA) (Organica, Norristown, PA) while the AMF product was *Glomus intraradices* (Becker Underwood, Ames, IA). The PGA is a mixture of many *Bacillus* strains and was prepared at the label rate of 1 tablespoon per gallon (1 tablespoon = 15 cm^3; 1 gallon = 3.785 411 784 dm^3) of water. The suspensions of both PGA and AMF were applied according to manufacturer's recommendation, around the base of each growing seedling at 2 weeks after seeding. In plots receiving single inoculant treatment, 100 ml suspension of the appropriate inoculant was applied per plant. For the plots receiving co-inoculation of PGPR and AMF, 50 ml suspensions of each inoculant were applied per plant. Controls were treated with 100 ml of water per plant.

Plant root simulator probes

Plant root simulator probes (PRS) (Western Ag Innovations Inc., Saskatoon, Saskatchewan, Canada) were buried in the plots. The probes estimate nutrient bio-availability by measuring nutrient supply rate through an ion exchange resin (IER). The probes are designed to be susceptible to all edaphic factors affecting nutrient uptake by plants, so that they mimic plant roots (Hangs et al. 2007). The pattern of nutrient availability over time was monitored, and the supply rate to the probe was compared to nutrient uptake in plants. The probes were used in pairs - one for anion exchange (orange

color) and the other for cation exchange (purple color). The first set of probes was removed 24 h after burial. Subsequent burials were made into the same location, and the probes were inserted for 2 week intervals before removal. On each sub plot, two pairs of the probes were installed. After being removed from the soil, probes were washed thoroughly with deionized water and placed in plastic bags under moist and cold conditions on ice for transporting to the lab. They were later sent to Western Ag Innovations Inc, Saskatoon, Canada for analysis. The details about washing and preparing the probes in the lab, including analysis procedures, were previously described by Hangs et al. (2004).

Monitoring plant growth, harvesting and estimation of yield

Plants within the middle 150 cm of each plot were chosen for data collection in order to avoid edge effects. Plant height was measured at about 8 weeks after planting (V7-8 growth stage). At physiological maturity (R6), destructive harvesting was done. Ears (cob plus grains) of corn within the middle 150 cm of each sub-sub plot were harvested from the stalk. Weights of ears were recorded in the field. Corn stalks were cut near the ground (at the crown of the roots), and total fresh weight of stalks from each sub-sub plot was recorded. The stalks were shredded with a chipper shredder (Briggs and Stratton, Wauwatosa, WI), after which a sub sample was taken at random from the silage, packed into a small bag, and weighed. Samples from all plots were then transported to the USDA-Agricultural Research Services, National Soil Dynamics Laboratory (USDA-ARS-NSDL) in Auburn for drying and further processing. Drying was done at 55ºC for 2 weeks, and dry weights of ears and silage were recorded. Ears were shelled with locally fabricated equipment to remove seeds which were then weighed. The seeds were ground with a Wiley Mill model No. 4 (Arthur Thomas Scientific, Swedesboro, NJ) and further grinding was

done with a Cyclone Sample Mill (Udy Corporation, Fort Collins, Colorado) to achieve a fine powder for nutrient analysis. Both mills were used for grinding the silage.

Nutrient content of plant tissues and soil

Tissues of ear and silage samples were ashed to analyze their nutrient contents. The samples were analyzed for N and carbon using TruSpec CN (LECO, St. Joseph, Michigan). Analyses for other elements, including P, K, S, Ca, Mg, Zn, Cu, Mn, and Fe, were done at the Soil Testing Laboratory, Auburn University, using inductively coupled plasma-atomic emission spectroscopy (ICP-AES) (Varian, Victoria, Australia). Only the steps involved in preparing the samples for analysis based on the procedure developed by Teem (1986) will be reported here because both ICP-AES and TruSpec CN are automated systems. For each sample, approximately 0.5 g of the dry fine powder (which can pass a 40 mesh, i.e. 0.60 mm stainless steel sieve) was placed into a 50 ml beaker, covered with a watch glass, and placed in a muffle furnace. After heating to 450ºC for 4 h, 10 ml of 1 $mol.L^{-1}$ HNO_3 was added to the grayish-colored ash and slowly evaporated to dryness on a hot plate, ensuring that it does not bake. Subsequently, 10 ml of 1 N HCl was added to dissolve the residue. It was warmed nearly to boiling and transferred to a 100 ml volumetric flask. The beaker was washed 3 times with small amounts of water, and the volume was made up to 100 ml followed by filtration. The elemental composition of the filtrate was then determined using ICP-AES. Nutrient uptake on per plot basis was estimated through uptake per gram of plant tissue multiplied by total yield per plot (i.e., yield × percent nutrient per gram of plant tissue).

Soil samples were collected from the plots, close to the plant roots but not the rhizosphere and analysed at the start and the end of the 3 year study period in order to

detect any changes. Mehlich 1 (double acid) extraction method, common in the southeastern USA (Mehlich 1953), was used for soil analysis in which 5 g of sieved air-dried soil was added to 150 ml extraction flask, followed by 25 ml of Mehlich 1 extracting solution (0.05 mol.L^{-1} H_2SO_4 + 0.05 mol.L^{-1} HCl) and then shaken for 5 min on a reciprocating shaker (Barnstead/Thermolyne, Dubuque, Iowa) at 180 oscillations.min^{-1}. It was centrifuged (International Equipment Co, Needham, MA) at 80% speed for 10 min, filtered through Whatman no. 2 filter paper, and analyzed using ICP-AES.

Data analysis

Data for plant growth, yield, and nutrient uptake were analyzed using the mixed procedure of the Statistical Analysis System (SAS) 9.1 (SAS Institute Inc., Cary, North Carolina). Mixed procedure was recommended for designs such as split-split plot randomized complete block due to the method used in fitting linear mixed models, including its ability to apply likelihood methods to complex mixed models (Littell et al. 2006). Slices test was done to determine equality of simple effects of factors for each level of other factors. Pairwise comparisons of the least square means were obtained with the 'Diff' option, while the 'adjust = sim' option provided a family-wise error rate protection. Analysis of variance (ANOVA) was based on each year to allow yearly comparisons and to avoid introduction of another factor (year), which could violate the requirement of independence of the residuals. Treatment effects and the interactions among treatments were tested. The Glimmix procedure was used to plot diffograms (mean-mean scatter plot) (Littell et al. 2006). Gplot and Boxplot procedures were used for the data on soil nutrient content and PRS probes, respectively. Unless otherwise stated, statistical significance was considered at $\alpha = 0.05$.

Results

Growth and yield promotion

Inoculation of AMF, PGPR, and the combination of the 2 (PGPR+AMF) resulted in significantly greater plant height compared to the non-inoculated control (Fig. 1). The mean height of plants in each of the 3 inoculants was not different. Comparing the 2 fertilizers, plants from plots that received poultry litter were higher than those on plots with ammonium nitrate. Tillage effect was not significant. The overall growth across all treatments in 2006 was greater than 2007 due to the severe drought in Alabama in 2007; however, the trend of the treatment effects was generally similar.

Fig. 1. Plant height for 2006 and 2007. AMF, arbuscular mycorrhiza fungi; PGPR, plant growth promoting rhizobacteria; PG+AM, co-inoculation of AMF and PGPR; WTR, water (no inoculation); PL, poultry litter; and NH_4, ammonium nitrate.

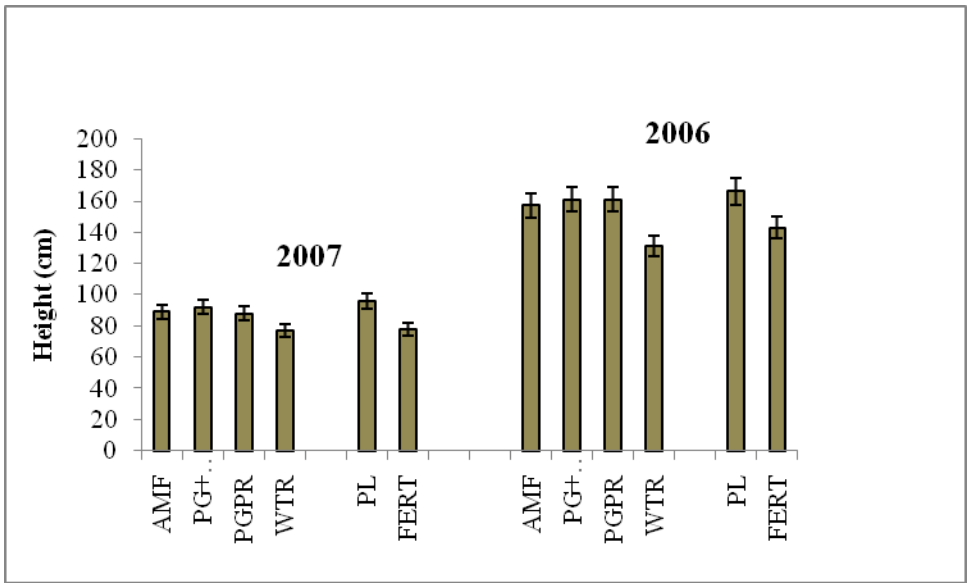
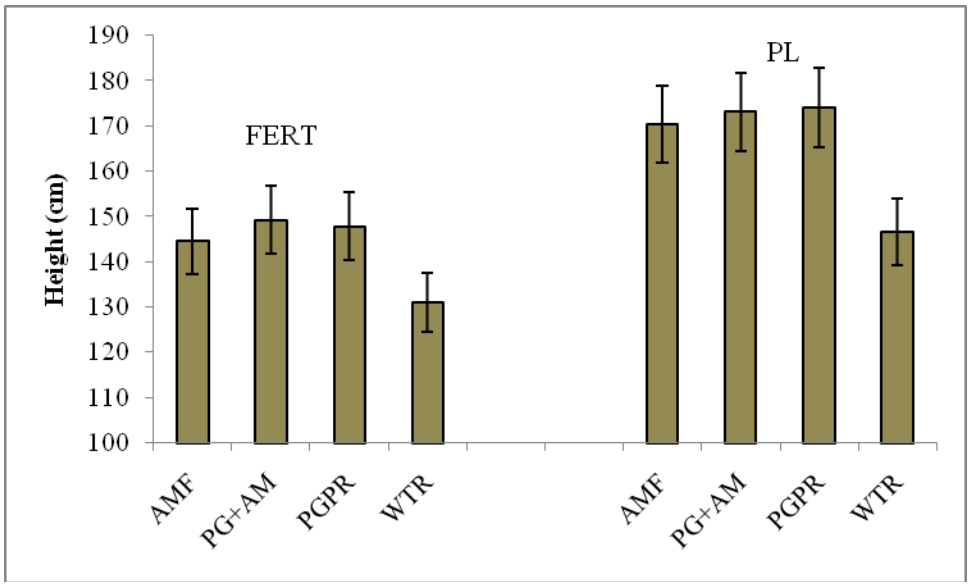


Fig. 2. Significant interactions between inoculant and fertilizer on plant height in 2006. AMF, arbuscular mycorrhiza fungi; PGPR, plant growth promoting rhizobacteria; PG+AM, co-inoculation of AMF and PGPR; W, water (no inoculation); NH_4, ammonium nitrate; and PL, poultry litter.



There was a significant interaction effect among inoculant and fertilizer in 2006 (Fig. 2). Height of plants on plots that received inoculants within plots of ammonium nitrate was greater than plants that received no inoculant. A similar trend was observed for inoculants on plots with poultry litter. All plots with inoculants within the poultry litter treatment showed relatively greater height than those of their corresponding inoculants within ammonium nitrate treatments.

Analysis showed that fertilization and inoculation affected corn yields (including grain and silage) significantly, but tillage did not affect yield (Table 1). For both grain and silage, plants from plots that received poultry litter yielded more than those that received ammonium nitrate (Table 1). A comparison of grain yield among different inoculant treatments in 2006 and 2007 revealed that yield for both PGPR and AMF were similar to each other, but all were generally greater than the non-inoculated plots. It is interesting that

despite the drought in 2007, inoculants still produced better yield (Fig. 3) than the non-inoculated control. Although the effect of tillage alone was not significant, there was a significant interaction effect of inoculant by tillage (Fig. 4).

Table 1. (A) Mean yield and (B) ANOVA for yield in 2007

(A) Mean yield (kg ha^{-1})

Treatment	Grain[a]	Silage[a]
Inoculants		
AMF	7717	8994
PGPR+AMF	7260	8534
PGPR	7313	8517
WTR	5725	6671
Fertilizers		
PL	7470	8751
NH_4	6537	7607

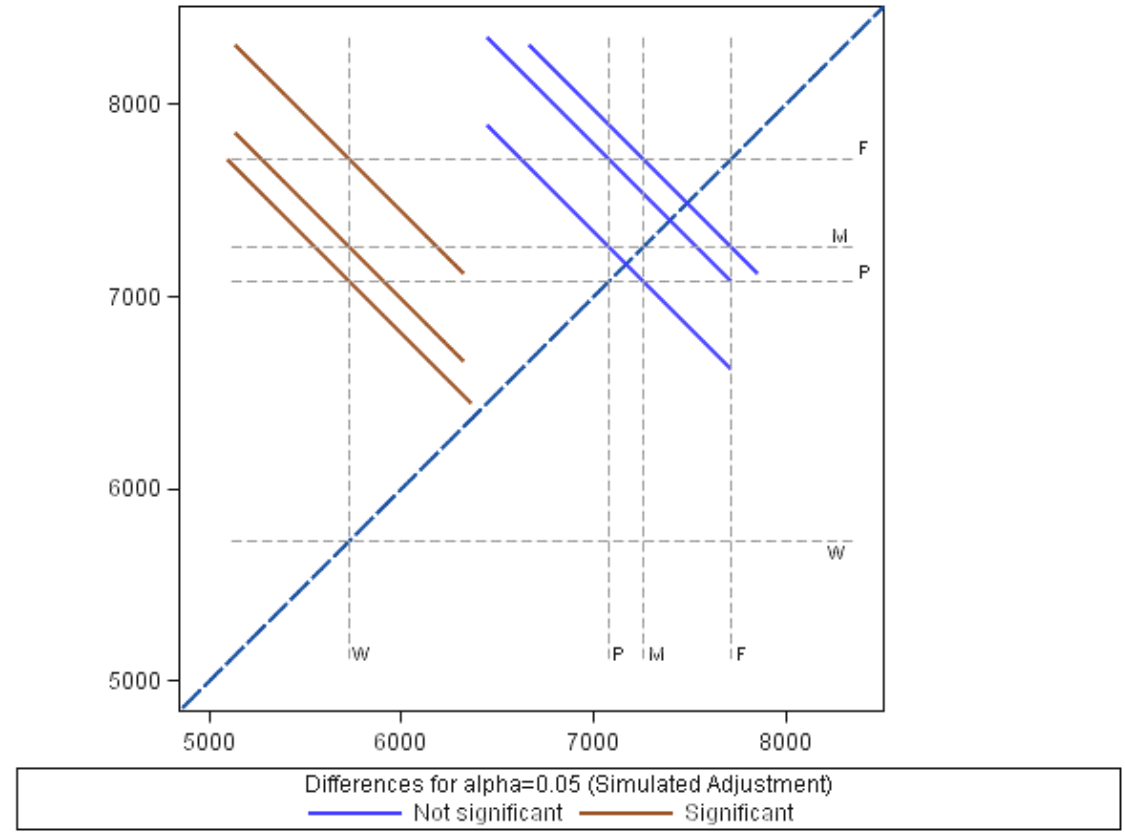
(B) Analysis of variance (ANOVA)

		Grain		Silage	
Treatment	df	F	Pr > F	F	Pr> F
Tillage (T)	1	2.5	0.21	0.14	0.73
Fertilizer (F)	1	15.59	0.03	23.3	0.02
Inoculant (I)	3	8.25	0.001	5.33	0.008
T*F	1	0.17	0.71	0.91	0.41
T*I	3	3.10	0.05	0.73	0.55
T*F*I	3	0.76	0.53	0.85	0.49

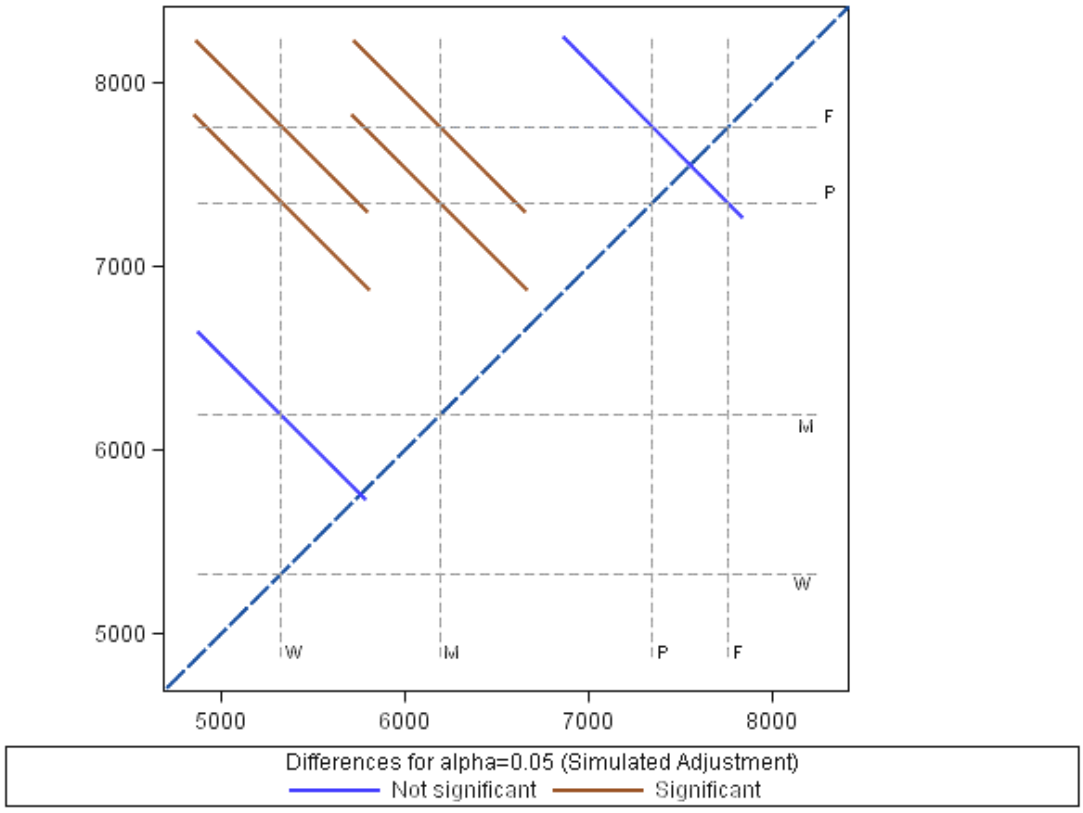
Note: Four types of inoculants and 2 types of fertilizers include the following: AMF, arbuscular mycorrhiza fungi; PGPR, plant growth promoting rhizobacteria; PGPR+AMF, co-inoculation of AMF and PGPR; W, water (no inoculation); PL, poultry litter; and NH_4; ammonium nitrate.

[a]Grain and silage are mean yield ($kg.ha^{-1}$) of corn grain and silage.

Fig. 3. Diffogram (mean-mean scatter plot) comparing the effect of inoculant on grain yield in 2007 (A) and 2006 (B). F, arbuscular mycorrhiza fungi; P, plant growth promoting rhizobacteria (PGPR); M, co-inoculation of AMF and PGPR; and W, water (no inoculation). Yield is measured in kilogram per hectare. The 45º reference line indicates whether 2 least-square means are significantly different at a significant level of 0.05. The thick lines drawn at the intersection of grid lines corresponds to (1-α) × 100% confidence interval of the difference of the 2 least-square means in each comparison. Any thick line that crosses the 45º reference line implies no significant difference for that comparison.

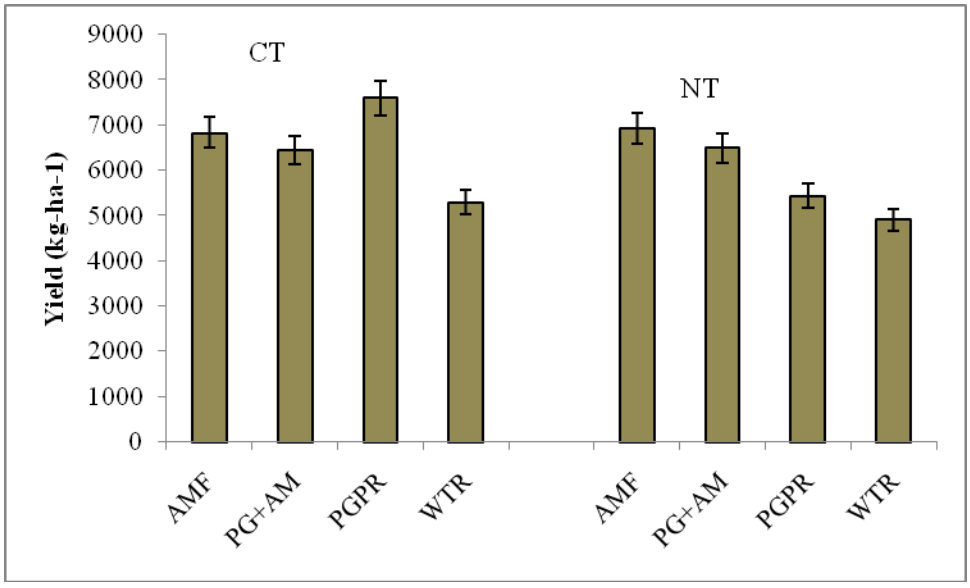


A



B

Fig. 4. Significant interactions between inoculant and tillage on grain yield in 2007. AMF, arbuscular mycorrhiza fungi; PGPR, plant growth promoting rhizobacteria; PG+AM, co-inoculation of AMF and PGPR; W, water (no inoculation); CT, conventional till; and NT, no till.



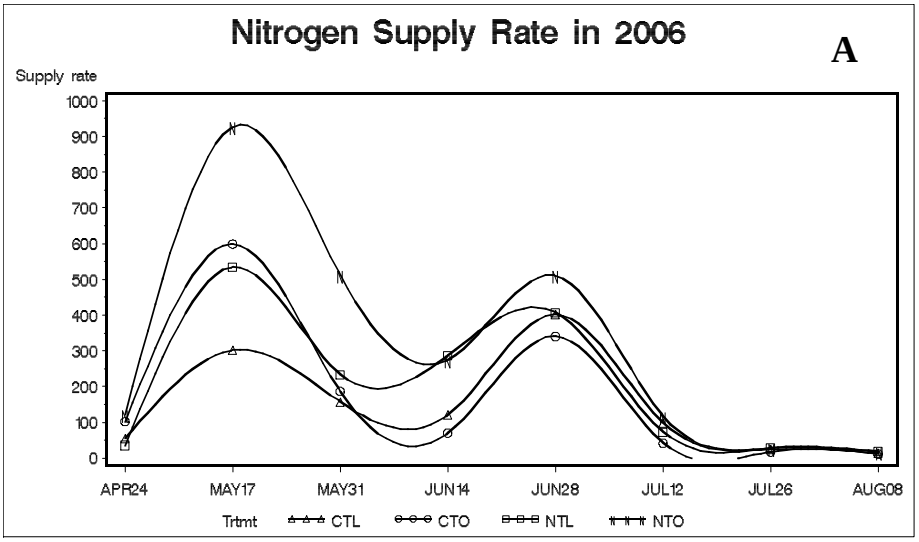
Nutrient content of PRS^{TM} probes

Figure 5 presents the fluctuations of nutrient over time in the plots in 2006. The graph was plotted only for the interaction of tillage and fertilizer without any specificity for inoculants. It was designed to measure available nutrient as a base for comparison of the effect of inoculants on plant nutrient uptake. The interactions between tillage and fertilizer types included (*i*) conventional tillage with poultry litter (CTL), (*ii*) conventional tillage with ammonium nitrate (NH_4NO_3) (CTO), (*iii*) no-till with poultry litter (NTL), and (*iv*) no-till with NH_4NO_3 (NTO). It became clear that through most of the growing season, more P was available for plant use in NTL plots. For N, higher bioavailability was more often observed in NTO plots. For potassium (K), the highest bioavailability was between CTL and NTL plots. The fluctuations in nutrient availability during the growing season and the decreases towards the end of the growing season are similar to the results observed by Galvez et al. (2001).

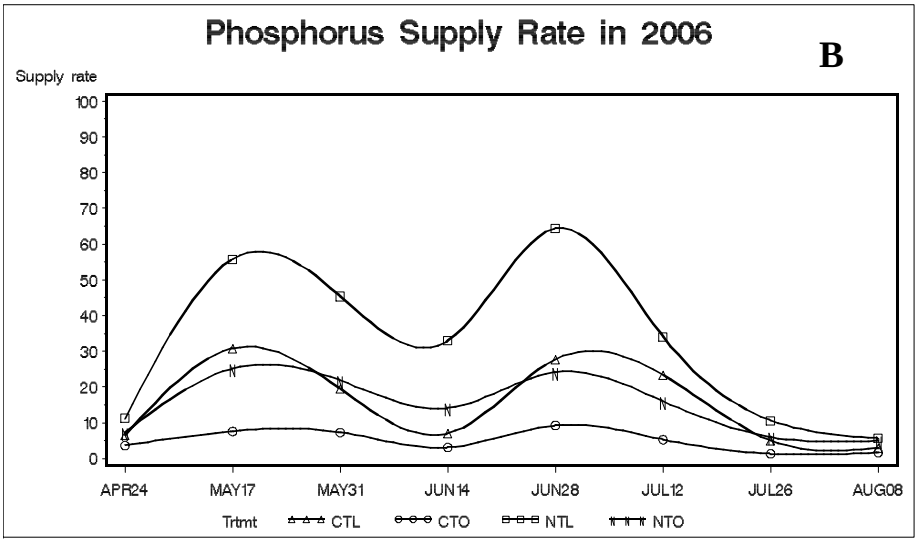
Nutrient content of soil

Soil analysis showed that the amount of nitrogen in the field increased at the end of the study in 2007 compared to 2005. The trend was the same across all treatments listed above. As the amount of nitrogen increased, the variance and standard deviation decreased (Fig. 6A). With P however, significant increases were observed in NTL and CTL plots, but not in NTO and CTO plots (Fig. 6B). For K, a significant increase was observed only in CTO plots (Fig. 6C).

Fig. 5. Supply rate (bioavailability) of N (A), P (B), and K (C) in the plots in 2006. The plots were as follows: NTO, no-till with ammonium nitrate; NTL, no-till with poultry litter; CTO, conventional till with ammonium nitrate; and CTL, conventional till with poultry litter. Horizontal axis gives the sampling date and the vertical axis is the supply rate measured in $\mu g.cm^{-2}.(\text{burial length of PRS probes})^{-1}$.



Sampling date



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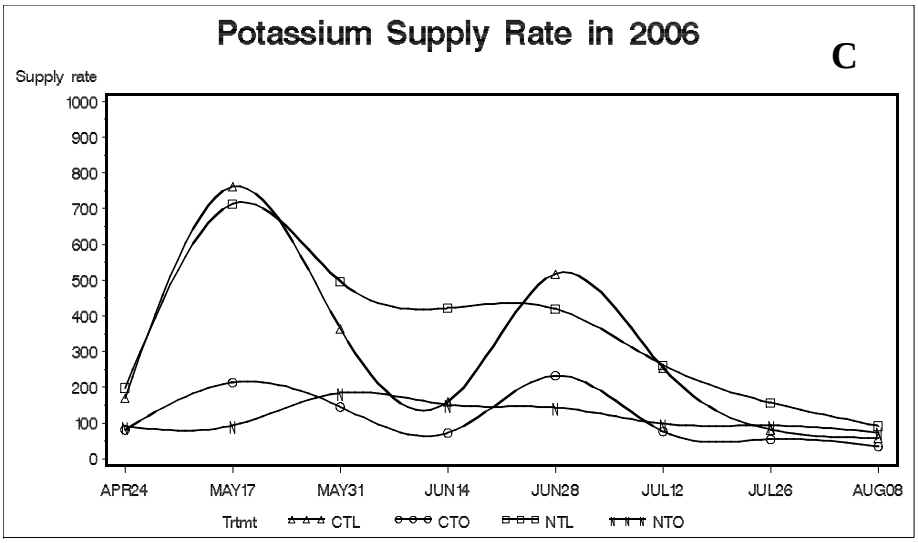
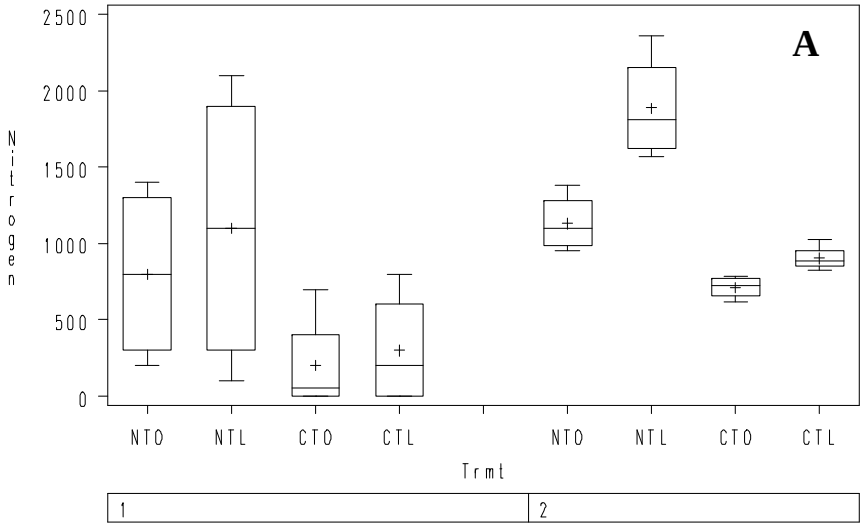
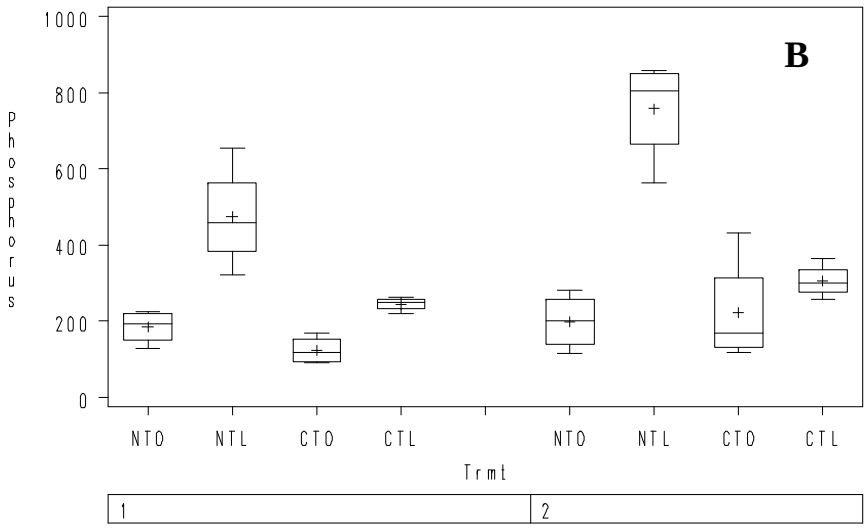
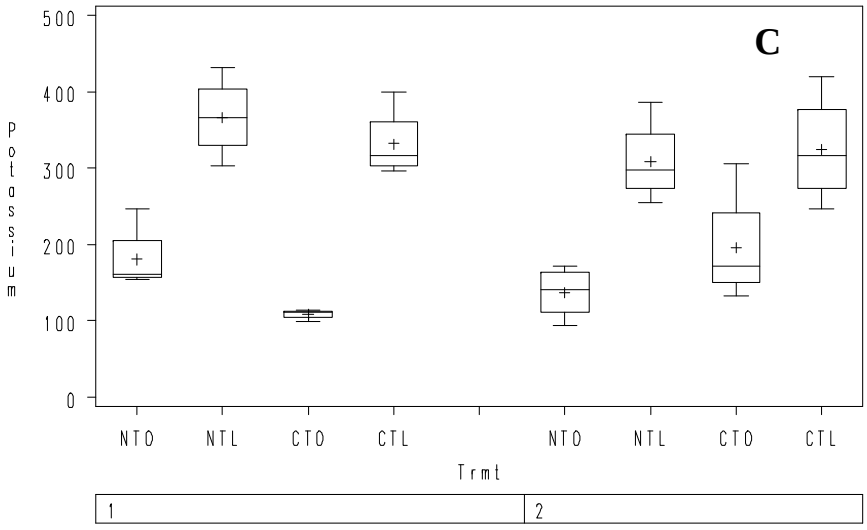


Fig. 6. Content of N (A), P (B), and K (C) in soil before and after the study. NTO, no-till with ammonium nitrate; NTL, no-till with poultry litter; CTO, conventional till with ammonium nitrate; and CTL, conventional till with poultry litter. The vertical axis is the content of the elements in the soil sample in part per million (ppm). Block number 1 represents 2005 and block number 2 represents 2007.







Nutrient contents of plant samples

Inoculant and fertilizer (Fig. 7) as well as their interaction (Fig. 8) significantly increased N content per gram of grain tissues in 2006, but not in 2007. Also, the enhancement of nutrient uptake per gram of plant tissues was not consistent across all treatments for the 2 years. Fertilizer treatment affected phosphorus uptake, but inoculant alone had no significant effect per gram of tissue. Specifically, in 2006 P value for analyzed phosphorus data were 0.003 for fertilizer, 0.2 for inoculant, 0.009 for fertilizer by inoculant

interaction in grain, but 0.03, 0.36, and 0.37, respectively, in silage. Treatment effects on nutrient uptake per gram of plant tissue could possibly be more consistent at other growth stages beside the physiological maturity stage in which samples were taken for nutrient analysis. However, we had chosen only this maturity stage for nutrient content evaluation, because that is the stage that could best reflect the amount of nutrients removed from the field through harvesting of plants.

Fig. 7. Nitrogen content per gram of grain tissues for 2006. AMF, arbuscular mycorrhiza fungi; PGPR, plant growth promoting rhizobacteria; PG+AM, co-inoculation of AMF and PGPR; W, water (no inoculation); PL, poultry litter; and NH_4, ammonium nitrate.

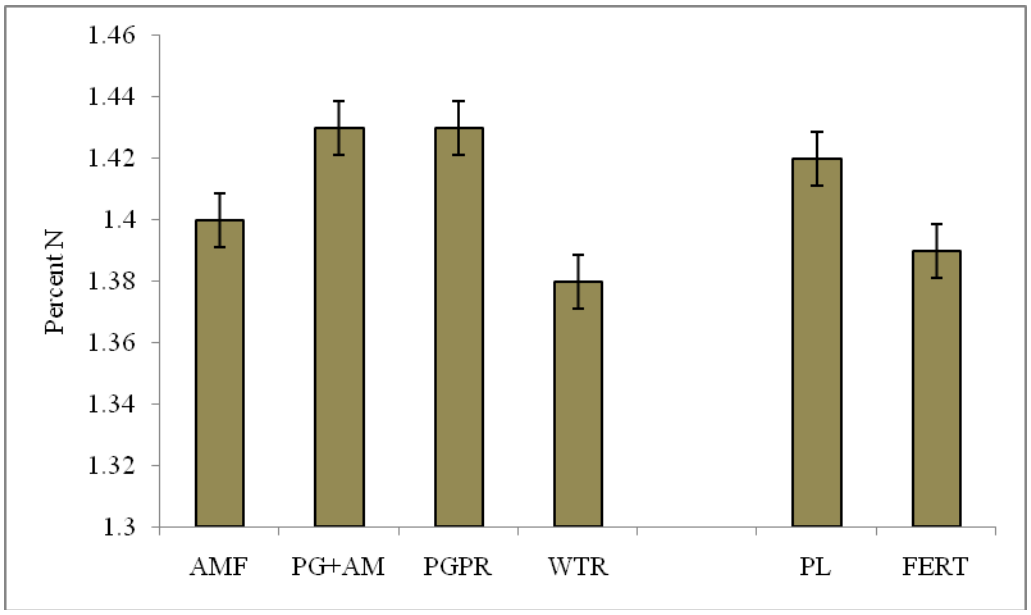
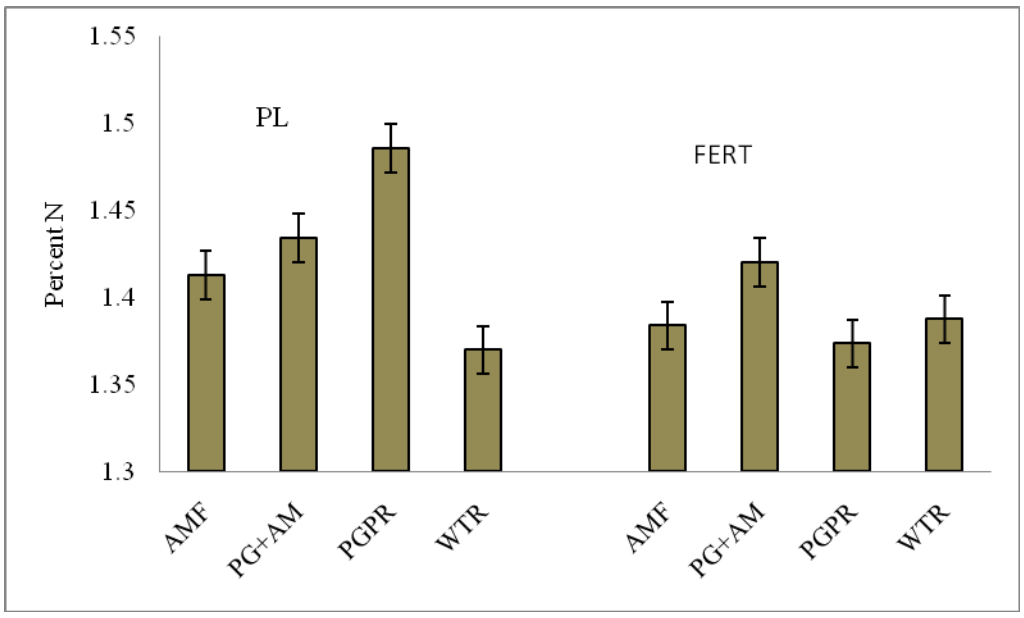


Fig. 8. Interactions of inoculant and fertilizer for nitrogen per gram of grain tissue in 2006. AMF, arbuscular mycorrhiza fungi; PGPR, plant growth promoting rhizobacteria; PG+AM, co-inoculation of AMF and PGPR; W, water (no inoculation); PL, poultry litter; and NH_4, ammonium nitrate.



The interaction effect of inoculant and fertilizer was significant on K uptake per gram of silage tissue (Fig. 9). In 2006, poultry litter interactions with inoculants significantly enhanced uptake of K in corn silage compared to ammonium nitrate interactions with inoculants. Overall, nutrient uptake (N, P, and K) in grain per plot was significantly higher for all inoculated plots (Table 2). Our focus in this report is on the 3 most important or limiting elements: N, P, and K. However, it is pertinent to mention that we also observed significant inoculant effects on some of the other elements and we have shown magnesium as an example in Table 2.

Fig. 9. Diffogram (mean-mean scatter plot) of the interaction of inoculant and fertilizer for potassium content in silage in 2006. A, ammonium nitrate; L, poultry litter; F, arbuscular mycorrhiza fungi; P, PGPR; M, AMF+PGPR; and W, water. Potassium content is measured in percent. The 45º reference line indicates whether 2 least-square means are significantly different at a significant level of 0.05. The thick lines drawn at the intersection of grid lines corresponds to (1-α) x 100% confidence interval of the difference of the 2 least square means in each comparison. Any thick line that crosses the 45º reference line implies no significant difference for that comparison.

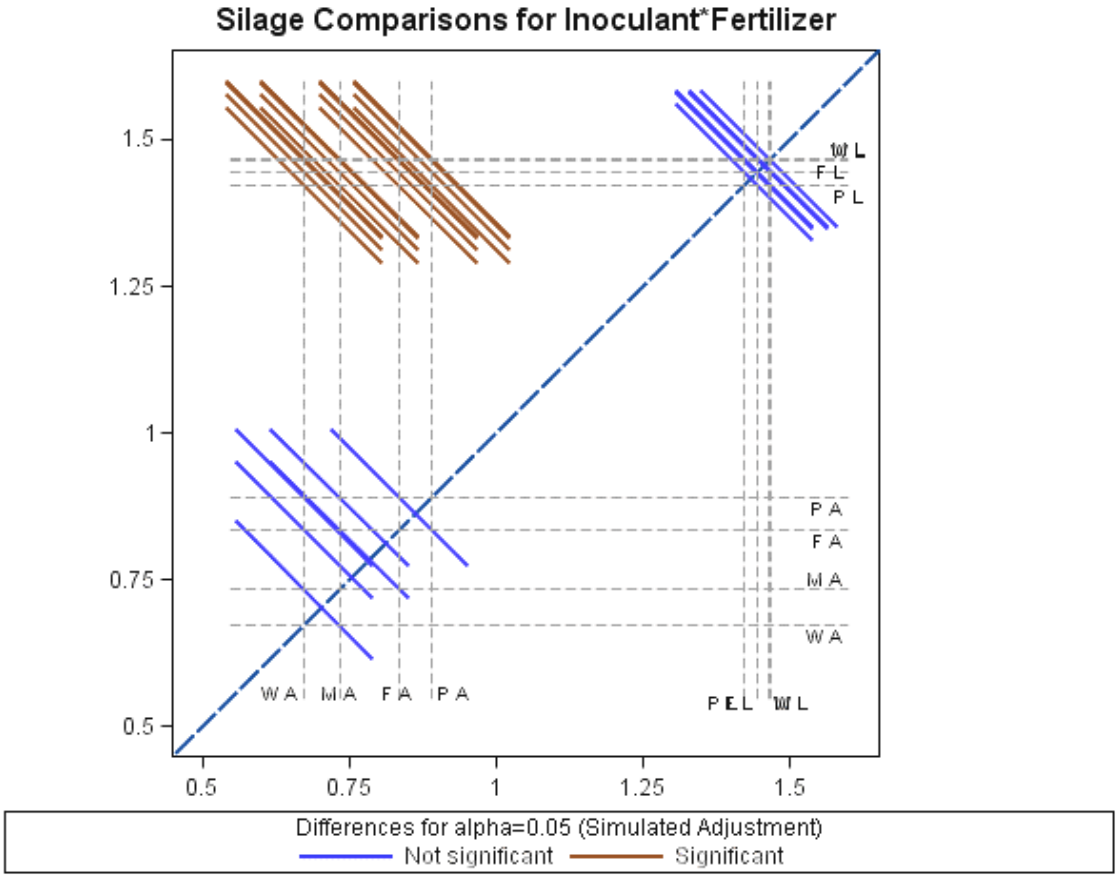


Table 2. Estimated total nutrient uptake per plot in 2007.

	Nitrogen		Phosphorus		Potassium		Magnesium	
Treatment	Grain	Silage	Grain	Silage	Grain	Silage	Grain	Silage
AMF	9929[a]	8199[a]	2424[a]	2888[a]	3310[a]	14843[a]	888[a]	2516[a]
PGPR+AMF	9002[a]	6665[ab]	2329[a]	2318[a]	3189[a]	13481[ab]	878[a]	2345[ab]
PGPR	9272[a]	6532[b]	2331[a]	2784[a]	3159[a]	13194[ab]	891[a]	2397[ab]
WTR	7401[b]	5615[b]	1948[b]	1959[a]	2646[b]	11122[b]	725[b]	1985[b]

Note: Values in each column with different letters are significantly different at p = 0.05. AMF, arbuscular mycorrhiza fungi; PGPR, plant growth promoting rhizobacteria; PGPR+AMF, co-inoculation of AMF and PGPR; W, water (no inoculation).

Discussion

Our results demonstrate that microbial inoculants can increase nutrient content of plants and overall plant growth. For example, treatment with inoculants resulted in increased N per gram of seed and N uptake per plot (Figs. 7 and 8). The use of inoculants for enhanced N uptake could therefore be applied to improve N uptake efficiency and potentially reduce nitrate leaching. Also, more P was removed from the plots which received inoculants, indicating that the uptake efficiency of P was also improved, and likewise could reduce potential losses of P to the environment (Table 2). Hence, inoculants have potential as inputs in integrated nutrient management system to help reduce build up, leaching, or run off of nutrients from fields. Treatment effects of inoculants on N and K uptake per gram of plant tissue was more strongly expressed in 2006 than in 2007 growing season and this may be related to the drought in 2007. It was obvious from the results that the soil and the general environmental conditions have impacts on the efficacy of PGPR and AMF.

Looking at the total uptake of each element on the basis of total nutrient content in grain per plot, significantly higher amounts of N, P, and K were removed from those plots

that received inoculants compared to the control (Table 2). This enhancement of nutrient uptake in plant tissues per plot due to inoculant becomes clearer by observing the effects as being dependent on plant development rather than as an uptake function (de Freitas et al. 1997; Mantelin and Touraine 2004). This finding suggests that enhanced plant growth with better root development gives the potential for greater nutrient uptake.

In our study, promotion of plant growth and yield was achieved by each inoculant and their combination in the two years reported (Fig. 2). This finding is in agreement with some previous studies (Mahaffee and Kloepper 1994; de Freitas et al. 1997; Kim et al. 1997; Kloepper et al. 2007). The results of the current study extend the previous findings by the integration of multiple factors (tillage, fertilizers, and inoculants). Contrary to previous reports (Singh and Kapoor 1998), in our study co-inoculation of PGPR and AMF did not produce synergistic effects under field conditions. Plants that received co-inoculation of PGPR and AMF showed virtually the same growth and yield compared to either inoculant alone (Fig. 1 and Table 1). Nonetheless, interactions could exist with different specific PGPR strains and AMF isolates, and the combinations of PGPR and AMF for nutrient management should be further explored.

Generally, our results supported the overall hypothesis that microbial inoculants that increase plant growth and yield can enhance nutrient uptake, and thereby remove more nutrients, especially N, P, and K from the field as part of an INM system. The explanation of Sheng and He (2006) might be the reason for the enhanced uptake of K by microbial inoculant which was observed in our study. They explained that organic acids e.g., citric, oxalic, tartaric, succinic, and α-ketogluconic, produced by PGPR, *Bacillus edaphicus* strains NBT and its mutants are able to chelate metals and mobilize K from K-containing minerals. For P, treatment effects of inoculants on uptake per gram of plant tissue were not

significant despite increased growth, yield, and P removal per plot. These results are similar to that of de Freitas et al. (1997), who reported that some of the PGPR strains significantly increased plant height or pod yield in canola but did not increase P uptake in the seed.

The variation in results for P uptake in our study is consistent with previous reports on N and P in different cropping systems. Two studies from different groups that worked with inoculants (*Azospirillum* strains) reported different results with explanations. Dobbelaere et al. (2002) reported nonsignificant treatment effects on N content of straw and grain in most conditions for wheat and maize, while Saubidet et al. (2002) reported improved uptake of inorganic N in wheat. Combining three tillage types, two farming systems, and mycorrhiza resident in the field, Galvez et al. (2001) showed that treatment effects on nutrient uptake in corn (maize) depended on growth stage. For instance, N concentrations in corn shoots were greater in plants grown under low input than under conventional agriculture at the 8 leaf (V8) stage, but the opposite occurred at the dough (R4) stage. Additionally, they observed higher P concentration in shoot for conventional than for low input farming at the vegetative stages and higher in no-till than in tilled soil at all stages of growth, but that did not translate into increased growth and yield. They explained that the high P content of the soil limited the benefit on the resident mycorrhiza population, which increased the influence of what they described as yield-depressing factors.

The information on soil nutrient content at the start and the end of this study for tillage and fertilizer combination without inoculants (Fig. 6) presents a model for how nutrients could build up in a long-term fertilization, as previously explained by Sharpley et al. (2003). Following the inclusion of inoculants (PGPR and [or] AMF), more nutrient uptake per plot was observed which could lead to reduction in nutrient build up. Removal

of those crops which had enhanced capacity at the end of the growing season would be the best step in practically reducing nutrient build-up from fertilizers. Without removing the plants, plant nutrient may get back into the biogeochemical cycle through decomposition.

Findings on bio-available nutrients as indicated by the PRS probes (Fig. 5) show that with tillage and fertilizer alone (without their interaction with inoculants), there was hardly any difference towards the end of the growing season. Earlier in the season, there was more available nutrient in poultry litter plots than inorganic fertilizer, but conventional till and no-till were not significantly different. Considering the advantages observed by Wood and Edwards (1992), particularly the cost of machinery, it is expedient to choose no-till over conventional till. Although a combination of no-till with poultry litter (NTL) tends to show more bioavailability of nutrient, it is important to note that the treatment effect of the tillage by fertilizer interaction differs from one element to the other. One pertinent question is whether the difference in bioavailability of nutrients early in the season as indicated by the PRS probes is equivalent to uptake by the plants in the absence of inoculants.

Mahaffee and Kloepper (1994) expressed the need to develop technologies and methodologies that address the problems associated with sustainable agriculture while achieving increased production above current levels in order to meet the needs of the ever growing population. Based on our results, the combination of no-till, poultry litter, and inoculants (PGPR) is promising for integrated nutrient management. The contribution of a farming system, which integrates multiple factors to improve nutrient use efficiency in a sustainable way, could be viewed from two perspectives. First, integrating crop production with livestock wastes offers one way to manage the wastes and maintain high crop productivity at the same time. Second, improved nutrient utilization efficiency from

agrochemicals through PGPR and (or) AMF can contribute to the protection of water resources against agro-pollution and reduce the growing cost of fertilizers. Given the enormity of fertility issues in agricultural sustainability, more studies should focus on microbial technologies as means of managing soil nutrients and fertilizer use.

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III. PLANT GROWTH-PROMOTING RHIZOBACTERIA ALLOW REDUCED APPLICATION RATES OF CHEMICAL FERTILIZERS

Abstract

The search for microorganisms that improve soil fertility and enhance plant nutrition has continued to attract attention due to the increasing cost of fertilizers and their negative environmental impacts. The objectives of this greenhouse study with tomato were to determine (i) if reduced rates of inorganic fertilizer coupled with microbial inoculants (PGPR or PGPR plus AMF) will produce plant growth, yield, and nutrient uptake equivalent to that obtained with full rates of the fertilizer and (ii) the minimum level that fertilizer could be reduced when inoculants were used. The microbial inoculants used in the study were a mixture of plant growth-promoting rhizobacteria (PGPR) strains *Bacillus amyloliquefaciens* IN937a and *Bacillus pumilus* T4, a formulated PGPR product, and the arbuscular mycorrhiza fungus (AMF), *Glomus intraradices*. Results showed that supplementing 75% of the recommended fertilizer rate with inoculants produced plant growth, yield, and nutrient (nitrogen and phosphorus) uptake that were statistically equivalent to full fertilizer rate without inoculants. When inoculants were used with lower rates of fertilizer, the beneficial effects were usually not noted; however, inoculation with the mixture of PGPR and AMF at 70% fertility consistently produced the same yield as the full fertility rate without inoculants. Without inoculants, use of fertilizer rates lower than the recommended resulted in significantly less plant growth, yield, and nutrient uptake or

inconsistent impacts. Further studies using isotope techniques that will reveal more specifics on the interactions and impacts of the inoculants and plant uptake of N is being conducted.

Introduction

The search for microorganisms that have capacities to improve soil fertility and enhance plant nutrition has continued to attract attention due to the increasing cost of fertilizers and their negative environmental impacts. A specific example of the negative impacts of fertilizer 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). One potential way to decrease negative environmental impacts of the continued use of chemical fertilizers is inoculation with plant growth-promoting rhizobacteria (PGPR). These bacteria exert beneficial effects on plant growth and development (Bakker et al. 2007), and many different genera have been commercialized for use in agriculture. One of the important mechanisms for these beneficial effects is PGPR-elicited enhanced nutrient availability and nutrient use efficiency. In a recent review, Glick et al. (2007) observed that some PGPR may influence plant growth by synthesizing plant hormones or facilitating uptake of nutrients from the soil through different direct mechanisms such as atmospheric nitrogen (N) fixation, solubilization of phosphorus (P), and synthesis of siderophores for iron sequestration making nutrients more available to plants.

Chemical fertilizers often have low use efficiency, meaning that only a portion of the applied nutrients are taken up by plants (Gyaneshwar et al. 2002). For example, P is precipitated (i.e., it is reactive with calcium, iron, or aluminum to form complexes) after

addition to soil, thus becoming less available to plants (Gyaneshwar et al. 2002). Another growth limiting nutrient, N, can be lost through nitrate leaching and pollution of groundwater (Biswas et al. 2000). Microbial inoculants have shown some promise in increasing nutrient availability. For example, previous reports have suggested positive impacts of microbes on N uptake involving non-legume biological fixation (Kennedy et al. 1997; Dobbelaere et al. 2001; Saubidet et al. 2002; Wu et al. 2005; Aseri et al. 2008). Also, inoculation with some microbes, including arbuscular mycorrhiza fungi (AMF), resulted in P solubilization or enhanced plant uptake of fixed soil P and applied phosphate resulting in higher crop yield (Altomare et al. 1999; Barea et al. 2002; Amir et al. 2005; Canbolat et al. 2006; Aseri et al. 2008). The main mechanism for increased availability of inorganic P appears to be through the action of organic acids synthesized by inoculants (Rodriguez and Fraga 1999).

The root system plays an essential role in plant productivity because roots are responsible for absorption of essential nutrients from the soil (Mills and Jones 1996). Therefore, better root growth is considered a prerequisite for better plant development. Many PGPR systems cause stimulation of root growth (Biswas et al. 2000; Lucy et al. 2004), sometimes via production of phytohormones by the plant or the bacteria (Lucy et al. 2004; Shaharoona et al. 2008). If root promotion by PGPR could be achieved with high frequency in the field, PGPR would be better potential tools for increasing nutrient uptake.

Two key questions arise from some of the past studies: Is it possible to reverse the current trend of applying large amounts of fertilizers by supplementing reduced fertilizer with inoculants? Can the potential benefits of PGPR and/or AMF in plant nutrient uptake be utilized by combining them with reduced levels of fertilizers? Our overall hypothesis is that

PGPR or combinations of PGPR and AMF with fertilizers will improve the use efficiency of fertilizers and lead to a reduction in the amount of fertilizer usage.

Our objectives in this study were to determine (i) if reduced rates of inorganic fertilizer coupled with microbial inoculants (PGPR or PGPR plus AMF) will produce plant growth, yield, and nutrient uptake equivalent to that obtained with full rates of the fertilizer and (ii) the minimum level that fertilizer could be reduced when inoculants were used. To achieve these objectives, we designed experiments using single strains as well as formulated PGPR products with or without AMF coupled with different fertilizer regimes. For the PGPR strains, we used a two-strain mixture, which included *Bacillus amyloliquefaciens* IN937a and *Bacillus pumilus* T4. The strains were previously reported to elicit significant effects on root development, plant growth, biocontrol, and/or induced systemic resistance (Raupach and Kloepper 2000; Kloepper et al. 2007; Ryu et al. 2007; Zhang et al. 2001).

Results from some past studies have suggested ineffectiveness of PGPR using single-strain inoculations (Egamberdiyeva and Höflich 2004; Lucy et al. 2004), but mixtures of strains provided more consistency (Belimov et al. 1995; Han and Lee 2005; Ryu et al. 2007). Some levels of interactions have been reported by co-inoculating PGPR with AMF (Barea et al. 1998; Probanza et al. 2001; Barea et al. 2002; Tatmatsiodu et al. 2006; Aseri et al. 2008). Some studies, mostly with single elements, have suggested that PGPR are more effective when nutrients become limiting (de Freitas and Germida 1990; Shaharooma et al. 2008). Here, we present the result of a study that included single elements (N and P) as well as conventional water soluble NPK fertilizer and the interaction of a two strain mixture of PGPR with AMF.

Materials and methods

Sources of inoculants and test for nitrogen fixation

Plant growth-promoting rhizobacteria (PGPR) used included two single strains that have been used in previous studies (Mahaffee and Kloepper 1997; Zhang et al. 2001; Ryu et al. 2007). The two PGPR strains, *Bacillus amyloliquefaciens* IN937a and *Bacillus pumilus* T4, were obtained from the culture collection of the Department of Entomology and Plant Pathology, Auburn University, Auburn, Alabama, and used as spore preparations. The strains were tested for ability to fix N using JNFB medium (Olivares et al. 1996). Another inoculant used was a commercial PGPR formulation, which consisted of many PGPR *Bacillus* strains with the trade name Plant Growth Activator (PGA) (Organica, Norristown, PA). In addition, the arbuscular mycorrhiza fungi (AMF) used was *Glomus intraradices* obtained from Becker Underwood (Ames, IA).

Experimental design and preliminary studies

All assays reported in this paper were conducted in the greenhouse at the Plant Science Research Center, Auburn University. The overall experimental design was a randomized complete block (RCB) with variations in the number of blocks depending on each test. The main blocks were inoculant types, while fertilizer rate was the sub-factor. Inoculant type in the preliminary studies included PGPR alone, PGPR+AMF, AMF alone, and no inoculants. Fertilizer treatments included 100%, 90%, 80%, 75%, 70%, 60%, 50%, and 0%. The 100% fertilizer rate was 1.25 g L^{-1} calculated based on the manufacturer's recommended rate of 1 level teaspoon gal^{-1}. Other rates were prepared from that; for example, 90% was obtained as 90% of 1.25 g L^{-1}.

Eight preliminary experiments were conducted with four test plants, which included tomato (*Solanum lycopersicum,* formerly *Lycopersicon esculentum*) cultivar Juliet, sunflower (*Helianthus annuus*) cultivar Valentine, Bell pepper (*Capsicum anuum*) cultivar California Wonder (Park Seed, Anderson, South Carolina), and Bermuda grass (*Cynodon dactylon*). Sunshine Professional Peat-Lite Mix (Sun Gro Horticulture, Vancouver, Canada), field soil, sand, or a mixture of 1 part field soil and 3 parts sand soil were tested as growth media. After seeding, water was applied regularly according to the greenhouse water schedule, and appropriate fertilizer treatments were applied. Greenhouse temperature was maintained at 21 - 25ºC. Based on results from the first set of preliminary tests, a fertility rate of 75% was introduced, the 0% rate was deleted, the 50% rate was used as the negative control, and nutrient analysis and microbial biomass estimation were done for all growth media.

Choice of the model growth medium: microbial biomass N and other nutrients

In order to understand N pool and fluxes (Horwath and Paul 1994) in the growth media, microbial biomass was determined for each of the media. We used chloroform fumigation-extraction methods for microbial biomass determination (Horwath and Paul 1994; Runion et al. 2004). Each growth medium had two sets of three replicates. One set was chloroform-fumigated, the other set was not fumigated, and both sets were incubated. After K_2SO_4 extraction of the samples, N content was determined by Kjeldahl method using Bran+Luebbe Flow Analyzer (Seal Analytical, Mequon, WI, USA). Microbial biomass N was calculated as μg N g^{-1} sample (fumigated) minus μg N g^{-1} sample (non-fumigated control) and expressed as μg N g^{-1} of dry sample weight (Runion et al. 2004). Additionally, total N and carbon (C) were determined using TruSpec CN (LECO, St. Joseph, MI).

Inductively coupled plasma-atomic emission spectroscopy (ICP-AES) (Varian, Victoria, Australia) was used for the determination of P, K, Mg, and Ca in the samples after extracts had been prepared through Melich I extraction method (Mills and Jones 1996; Adesemoye et al. 2008).

Sunshine Mix has high water retention capacity, which presented a problem during the dilution for fumigation and digestion process. To solve that, 1/5 dilution rate relative to others was used for Sunshine Mix and correction was made for the dilution. Based on the results of the preliminary tests, the microbial biomass N and the nutrient analysis, the 1:3 field:sand soil mixture was selected as the model growth medium. All the results presented here are from the model system of tomato in 1:3 field:sand soil mixtures.

Establishing growth curve with different rates of fertilizer without inoculation

The experiment for the growth curve was designed to give an idea of the response of tomato to different fertilizer rates without any inoculation. Experiments were set up by planting tomato seeds directly into 10-cm (4-inch) pots consisting of a mixture of 1:3 field:sand soil as the growth medium. Fertilizer was applied but no microbial inoculation. The fertilizer used was water-soluble Peters Professional® 20:10:20 Peat-Lite Special (Buddies Plant Food, Ballinger, Texas). Fertilizer treatments included 100%, 80%, 70%, 60%, and 50%. The 100% fertilizer rate was 1.25 g L^{-1} calculated based on the manufacturer's recommended rate of 1 level teaspoon gal^{-1}. Other rates were then prepared based on that; for example, 80% was obtained by calculating 80% of 1.25 g L^{-1}. Fertilization was done by applying 25 ml of solution of the appropriate treatment per plant. Each treatment had 20 replicates to allow two-time destructive sampling of ten replicates each. The first sampling was done at 4 weeks after planting (WAP) and the second at 6

WAP. Plants were removed from the pot. Roots were washed in slow-running water to remove adhering soil and laid on paper towels to drain. Plant height, stem caliper (taken at the oldest leaf position), and wet weight were recorded. Samples were dried for 7 days in the dryer at 70ºC and dry weights were taken. Growth index was estimated by multiplying height by width, and the growth index was plotted against fertilizer rates**.**

Tests with inoculants and water soluble fertilizer

In this experiment the different rates of fertilizer combined with PGPR or PGPR plus AMF were compared to the full rate of fertilizer (100%) without inoculants (positive control). The design was a 5 × 3 factorial randomized complete block. The five fertilizer treatments included 100%, 80%, 70%, 60%, and 50%. At a later stage of the study this was changed to 100%, 80%, 75%, 70%, and 50%. This change was made because it became clear that 60% could not produce a result that would compare significantly to the positive control, regardless of whether it was supplemented with inoculants. We retained 50% fertilizer treatment as the negative control and introduced 75% treatment because there were some variations in the results for 70% treatment. The three inoculant treatments were no inoculation, PGPR, and PGPR plus AMF. The fertilizer used was water-soluble Peters Professional® 20:10:20 Peat-Lite Special (Buddies Plant Food, Ballinger, Texas). Fertilizer rates were prepared as explained above. Apart from the addition of inoculants (PGPR or PGPR plus AMF), methods were similar to those used in establishing the growth curve. The PGPR formulation used in this study (PGA) was prepared at the rate of 3.78 g L^{-1} based on the label rate of 1 tablespoon gal^{-1}. In assays where a two-strain PGPR mixture was used, inoculation was done as explained in the next section below. In assays that involved AMF, *Glomus intraradices* was applied on seed at planting before filling the holes.

Tests with a two-strain mixture, AMF, and Hoagland solution

The spore suspension of the two PGPR strains (*Bacillus amyloliquefaciens* IN937a and *Bacillus pumilus* T4) were diluted appropriately and mixed together. The concentration was adjusted to log 5 cfu/ml and used for inoculation. At planting, 1 ml of the bacterial suspension was applied onto each seed in a 10-cm pot containing a 1:3 mixture of field:sand soil. A follow-up inoculation was done at one week after planting by applying 1 ml of PGPR drench per pot around the base of each plant.

The study, with preceding assays, left some questions unanswered. Were there specific effects on uptake of any of the two growth limiting nutrients (N and P) in the plants? Was the effect, if any, related to growth promotion? To answer these questions, we used Hoagland solution (Maynard and Hochmuth 2007) as the fertilizer, which enabled us to vary each element and adequately track changes that occurred. Experiments were done for N and P, and assays on each element were repeated. The design was a randomized complete block with inoculant type as the main block and fertilizer rate as the sub-factor because, in this study, fertilizer rate was more important in the statistical interaction between fertilizer rate and inoculant type. There were 3 inoculant types: (i) no inoculant, (ii) a mixture of two *Bacilli* PGPR strains, and (iii) two *Bacilli* PGPR strain mixture plus AMF, *Glomus intraradices*. Fertilizer rates reported here include 100% (full strength Hoagland solution), 80%, 75%, 70%, and 50% (negative control). The different fertilizer rates were made by appropriately varying the amount of N and P in Hoagland solution (Hershley 1994; Maynard and Hochmuth 2007). More details about preparing and applying Hoagland solution as used in this study are shown below.

Preparation of Hoagland solution and application

The content of Hoagland solution prepared for the study on P was different from that for N. For P, Hoagland solution (Formulation I) was prepared with the components as originally formulated in 1933 by Hoagland and Snyder (Maynard and Hochmuth 2007). One liter of 100% P solution was made by using 1 ml of 1 M potassium dihydrogen phosphate (KH_2PO_4), 5 ml of 1 M potassium nitrate (KNO_3), 5 ml of 1 M calcium nitrate tetrahydrate ($Ca[NO_3]_2.4H_2O$), 2 ml of 1 M magnessium sulfate heptahydrate ($MgSO_4.7H_2O$), 1 to 2 ml of Fe-EDTA, and 1 ml of micronutrient stock. The Fe stock solution was covered with aluminum foil to prevent light degradation. The micronutrient stock solution was made of 2.86 g L^{-1} boric acid (H_3BO_3), 1.82 g L^{-1} manganese chloride tetrahydrate ($MnCl_2.4H_2O$), 0.22 g L^{-1} zinc sulfate heptahydrate ($ZnSO_4.7H_2O$), 0.02 g/L molybdic acid (85% of $Na_2MoO_4.2H_2O$), and 0.08 g/L copper (II) sulfate pentahydrate ($CuSO_4.5H_2O$). The percentage of P was varied by changing only the volume of KH_2PO_4 as appropriate.

For N, Hoagland solution was prepared using a slightly different composition [20] that had only one nitrogen source (Formulation II). One liter of 100% N solution was prepared by using 7.5 ml of 1 M calcium nitrate tetrahydrate ($Ca[NO_3]_2.4H_2O$), 10 ml of 0.05 M monocalcium phosphate ($Ca[HPO_4]_2$), 20 ml of 0.1 M calcium sulfate dihydrate ($CaSO_4.2H_2O$), 5 ml of 0.5 M potassium sulfate (K_2SO_4), 2 ml of 1 M magnesium sulfate heptahydrate ($MgSO_4.7H_2O$), 2 ml of Fe-EDTA, and 1 ml of micronutrient stock. The percentage of N was varied by changing only the volume of $Ca(NO_3)_2.4H_2O$ as appropriate. After preparing the solutions, we autoclaved them and adjusted the pH to 5.8 with NaOH before use. Approximately 25 ml of each solution with different percentage of nutrient content were then applied per pot according to the experimental plan. The first

fertilizer application was done on the day of seeding. Using the volume of 25 ml maintained a low salt index level to avoid complications with germination.

Measurement of plant growth and nutrient content of plant tissues and soil

Destructive sampling was done at 4 WAP. This time was chosen for nutrient analysis because in preliminary tests, we observed that concentration of nutrients decreased with age of tissue. In each test, the height of tomato, fresh weight, and dry weight of tissue were measured. Also, in four experiments, root development or architecture was analyzed for each root before drying. Root architecture was measured with a scanner model LA 1600+ and WinRhizo software version 2004a (Regent Instruments, Inc, Sainte-Foy, Quebec, Canada). Parameters analyzed in the root system included total root length, surface area, volume, projected area, number of tips, mean diameter, and numbers of roots with diameters of 0 - 0.5 mm and 0.5 - 1 mm. Dry plant samples were analyzed for N and P contents (two growth limiting nutrients). The methods that we used for nutrient analysis were the same as was previously reported (Adesemoye et al. 2008). Nutrient (N and P) uptake of plants per treatment was estimated through uptake per gram of plant tissue multiplied by total yield per treatment (i.e., yield × percent nutrient per gram of plant tissue).

Data analysis

Data were analyzed using GLM procedure, and Fisher's protected LSD was used to separate treatment differences (Littell et al. 2006). Statistical significance was considered at $\alpha = 0.05$. Regression fitting was carried out for relationships among variables. These

analyses were done using Statistical Analysis System 9.1 (SAS Institute, Cary, North Carolina).

Results

Preliminary tests

Some results of the first set of preliminary tests either did not follow any pattern or were not consistent, especially with Sunshine Mix. A specific example was an assay with Bermuda grass in Sunshine Mix, where there was no significant difference in plant growth between 100% fertilizer without PGPR and 50% fertilizer with PGPR (data not shown). These results could not be ascribed to PGPR because the result was not the same when the assay was repeated. The analysis of growth medium used in the experiment (Sunshine Mix) revealed high amounts of N, P, and other nutrients (Table 1).

Table 1. Nutrient content of the growth media

Growth medium	N	C	P	K	Ca	Mg
Field soil	0.02	0.56	23.4	16.8	56.3	8.6
Field/Sand	0	0.19	7.3	8.8	15.4	3.2
Sunshine Mix	3.0	91.9	49.8	254.8	1245	684.8
Sand	0	0.13	0.5	5.7	5.5	2.5

Nitrogen (N) and C are in percentage while P, K, Ca, and Mg are in $\mu g\ g^{-1}$ (or ppm).Values showing (0) are below detectable limit of the equipment. The amount considered high for soil in this analysis for N is 0.29% and C is 3.62%.

Growth media and the response curve

The results obtained in tests to develop a standard response curve of tomato plants to different fertilizer rates showed that the growth of tomato was significantly greater with 100% fertility than with any other lower rates across all parameters (plant height, shoot and root, fresh and dry weights) (Table 2). Figure 1 shows the model growth curve of tomato

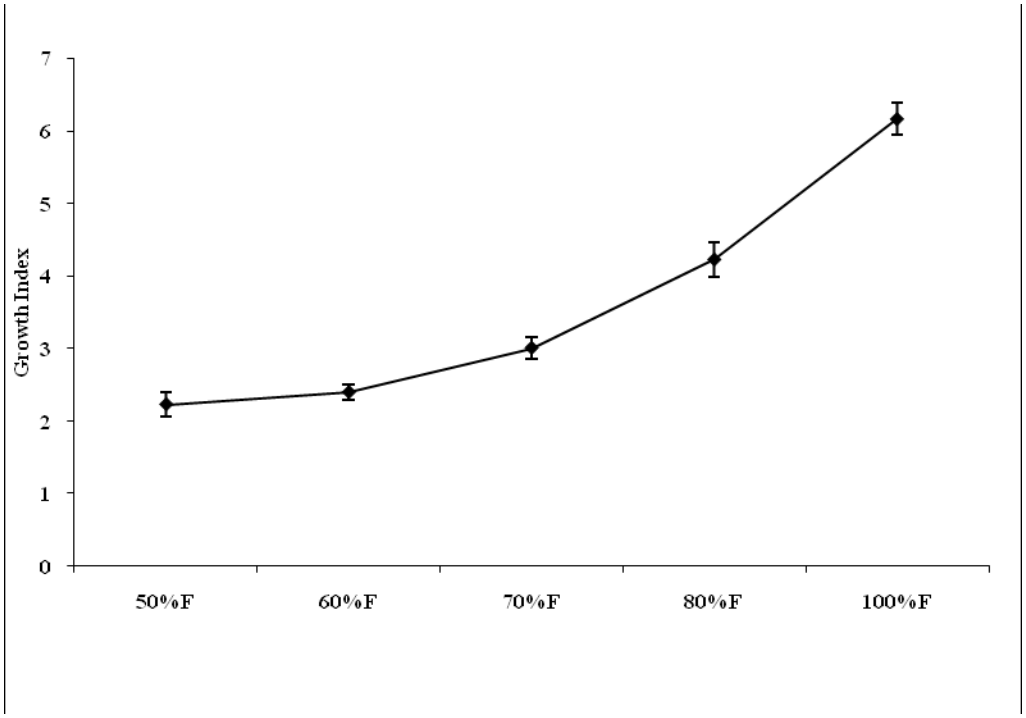
plant under the different rates of fertilizer, which is a plot of growth index against fertilizer rates at 4 weeks after planting (WAP).

Table 2. Some growth parameters for response of tomato plant to fertilizer treatments

Treatments	Fresh weight		Dry weight	
Percent fertilizer	Fresh shoot	Fresh root	Dry shoot	Dry root
100	9.13a	4.07a	2.09a	0.53a
90	7.37b	3.09b	1.31b	0.31b
80	6.43c	2.82b	1.16b	0.22c
70	5.39d	2.29c	0.89c	0.20c
60	4.28e	2.04c	0.78c	0.17c
50	1.03f	0.59d	0.18d	0.06d
$LSD_{(0.05)}$	0.89	0.69	0.29	0.06

Values in each column with different letter(s) are significantly different at p = 0.05.

Fig. 1. Growth response curve of tomato to different fertilizer rates at 4 WAP.
F, Fertilizer; and WAP, Weeks after planting.



Growth, yield, and nutrient content for tests with water soluble fertilizer

Results indicated that plant heights resulting from treatment with PGPR plus 80% or 70% of fertilizer were statistically equivalent to the heights with 100% fertility without PGPR. The effects were slightly different for co-inoculation of PGPR and AMF. Although 100% fertilizer without microbial inoculants produced statistically similar plant height as 80% or 70% of fertilizer plus PGPR and AMF, plants that receive 80% of fertilizer with PGPR and AMF grew significantly taller than those with 70% fertilizer with PGPR and AMF (Table 3). After multiplying height by width to arrive at growth index, the comparison between non-inoculated and inoculated plants showed that the inoculants significantly enhanced the growth of the plants, even at suboptimal fertilizer rates. Also, there were no differences among the growth index for plants that received 70% fertilizer plus PGPR, 80% fertilizer plus PGPR, or 100% fertilizer without PGPR. However, 100% fertility without PGPR was significantly greater than plants that received 70% fertilizer plus co-inoculation of PGPR and AMF (Fig. 2).

Table 3. Plant height of tomato at different fertilizer treatments with inoculation

Percent fertilizer	Fertilizer	Fertilizer+PGPR	Fertilizer+PGPR+AMF
100	19.9a	21.5a	20.4ab
80	17.8b	22.2a	21.2a
70	16.5c	21.3a	19.4b
60	14.9d	18.9b	17.8c
50	14.6d	19.0b	15.8d
$LSD_{(0..05)}$	1.09	1.09	1.19

Values in each column with different letter(s) are significantly different at p = 0.05. AMF = arbuscular mycorrhiza fungi, and PGPR = plant growth-promoting rhizobacteria.

There was a high correlation between the growth index and the treatments (Fig. 2) with $y = 1.218x + 2.592$, $R^2 = 0.874$; $y = 0.707x + 6.751$, $R^2 = 0.749$; and $y = 0.975x +$

5.665, $R^2 = 0.8333$ for fertilizer, fertilizer plus PGA, and fertilizer plus PGA and AMF, respectively. Comparison of the yield in tomato fruits showed that 70% or 80% fertilizer plus PGPR and AMF were comparable to 100% fertilizer without inoculants (Fig. 3). For the treatment of fertilizer plus PGPR, only inoculant-supplemented 80% fertilizer produced the same yield as 100%. The inoculants-supplemented 70% fertilizer treatment produced significantly lower yield. The results indicated that 80% of fertilizer plus inoculants produced comparable results with 100%, but similar treatment with 70% fertilizer was not consistent.

Fig. 2. Growth index of tomato at different fertilizer rates with or without inoculants.
F, Fertilizer; P, plant growth-promoting rhizobacteria; and A, arbuscular mycorrhiza fungi. Growth index is height of plant multiplied by width.

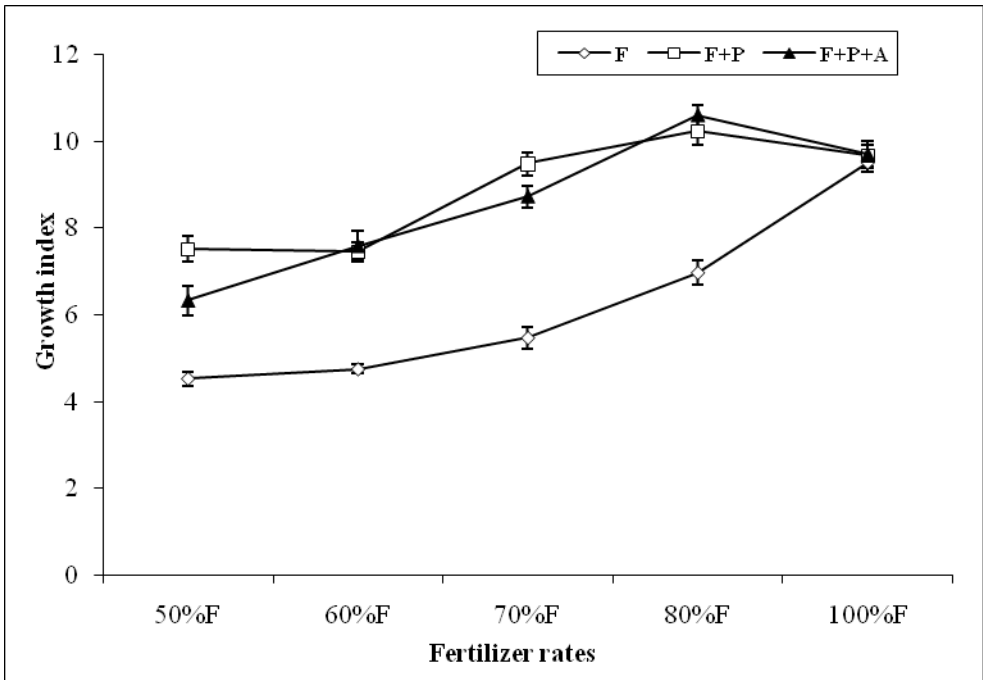


Fig. 3. Yield of tomato with or without inoculant.
F, Fertilizer; P, plant growth-promoting rhizobacteria; and A, arbuscular mycorrhiza fungi.

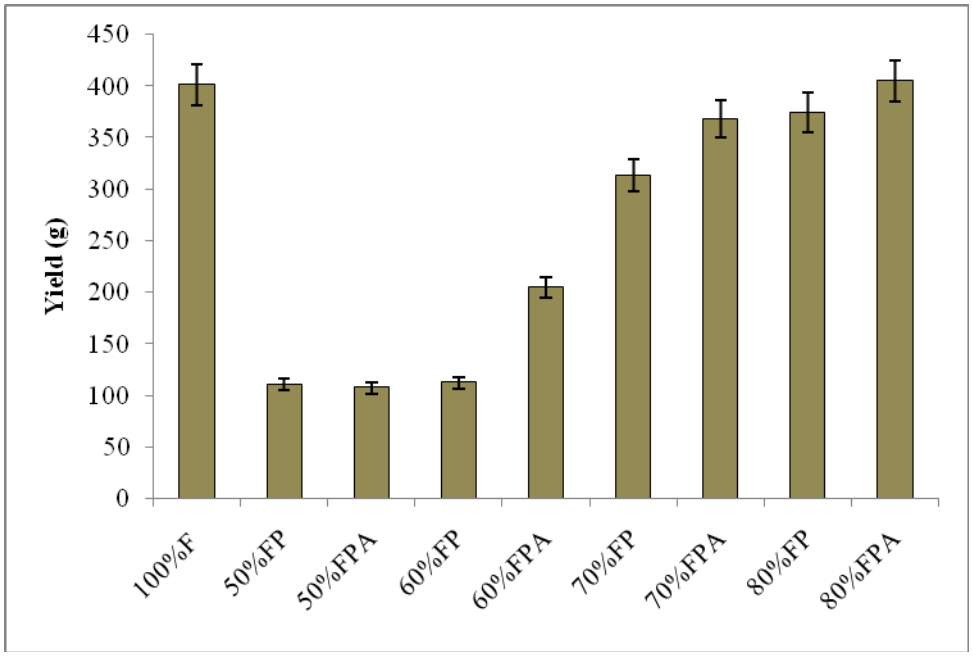


Fig. 4. Dry biomass of plants with or without inoculants.
F, Fertilizer; P, plant growth-promoting rhizobacteria; and A, arbuscular mycorrhiza fungi.

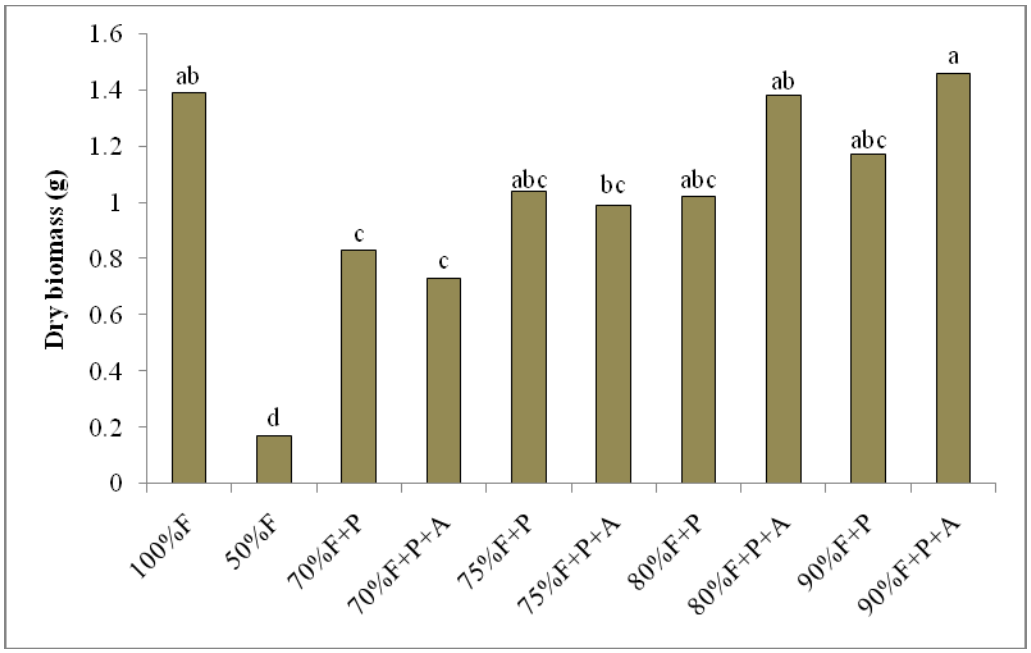
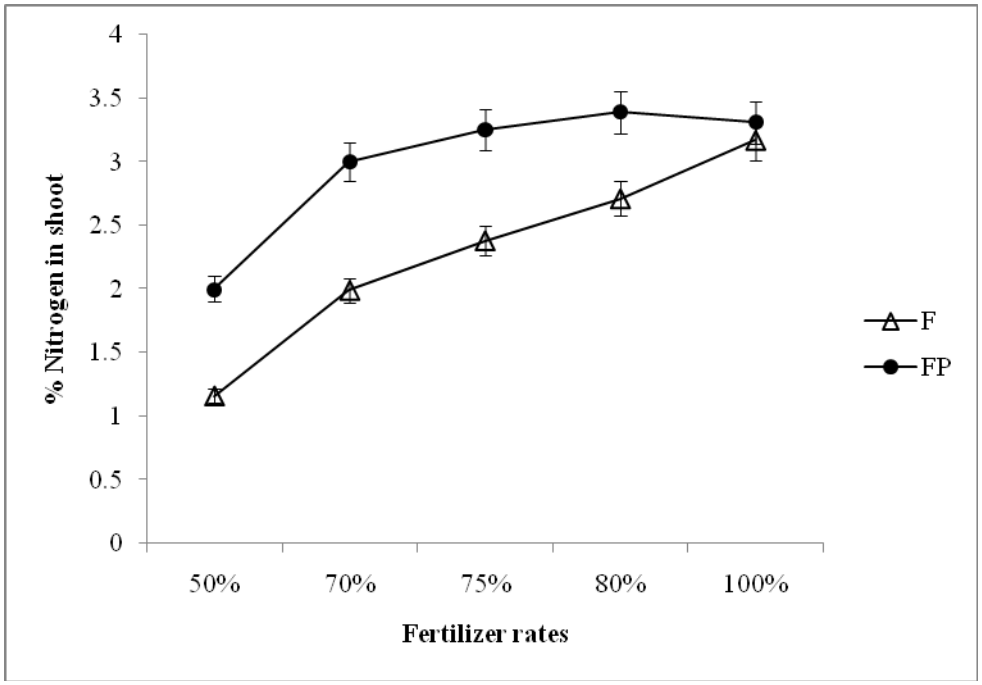


Fig. 5. Nitrogen uptake per gram of tomato shoot with or without PGPR.
F, Fertilizer and P, plant growth-promoting rhizobacteria.



Growth and nutrient content for tests with a two-strain mixture

With Hoagland solution, it was possible to track changes that occurred in growth and N and P uptake. The growth of plants that received 75% to 90% of fertilizer plus inoculation of PGPR or PGPR and AMF was comparable to the full fertilizer rate without inoculants. Also, the inoculation of PGPR or co-inoculation of PGPR and AMF produced similar effects (Fig. 4). The amount of N per gram of tomato shoot and root tissues were statistically the same for 100% fertilizer without inoculants and 75% fertilizer supplemented with PGPR (Figs. 5 and 6 for shoot and root, respectively). Also, plants that received 70% fertilizer with inoculants produced comparable amount of N in shoot as those with 100% fertilizer without inoculants (Fig. 5**).** On a whole tissue basis, 75%, 80%, or 90% fertilizer plus inoculants gave results that were equivalent to 100% fertilizer (Fig. 7). The fluctuation that occurred in the previous test using water soluble fertilizer for results on

70% fertilizer plus inoculants was also seen for the two-strain mixture on Hoagland solution for N uptake. Results for 70% treatment were not consistent. For P where AMF was one of the treatments, P uptake was significantly the same on total plant basis, but not on a per gram of tissue basis (Fig. 8). Co-inoculation of PGPR and AMF with 70% fertilizer gave the best result, resulting in P uptake equivalent to that with 100% fertility without inoculant. Compared to the positive control, significantly more P was taken up by plants treated with 90% fertilizer and inoculants (PGPR plus AMF) (Fig. 8).

Fig. 6. Nitrogen uptake per gram of root tissue with or without PGPR.

F, Fertilizer and P, plant growth-promoting rhizobacteria.

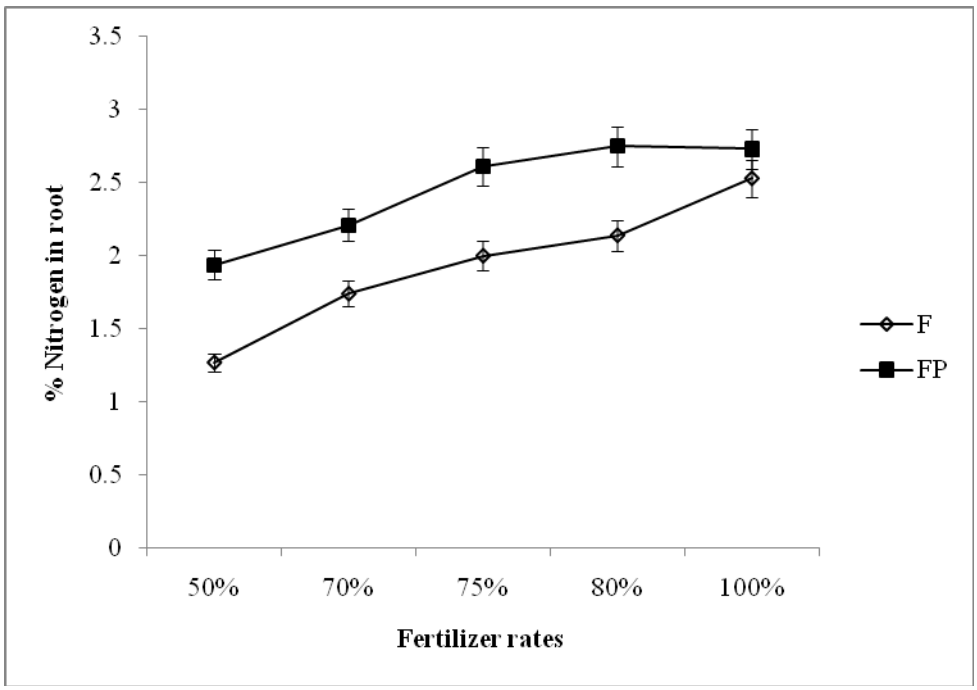


Fig. 7. Nitrogen uptake on dry whole plant basis at 4 WAP with PGPR.
Uptake was estimated by multiplying plant dry weight by % N per gram of tissue. F, Fertilizer and P, plant growth-promoting rhizobacteria.

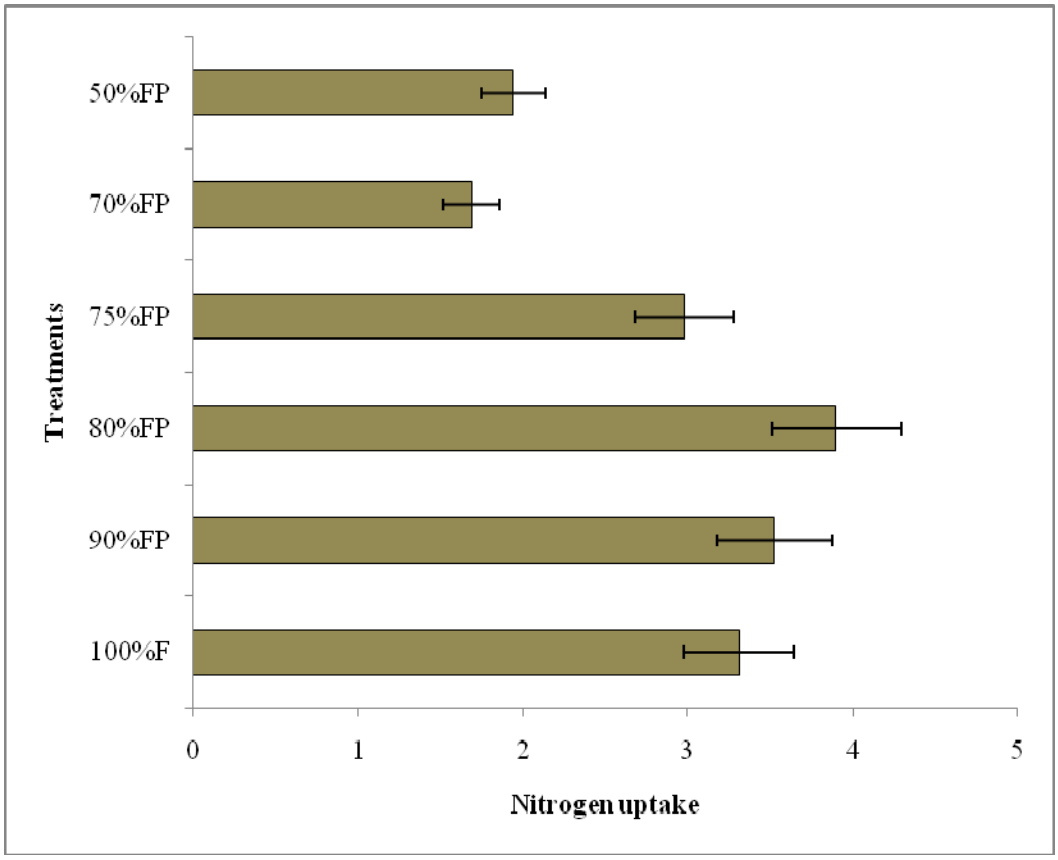
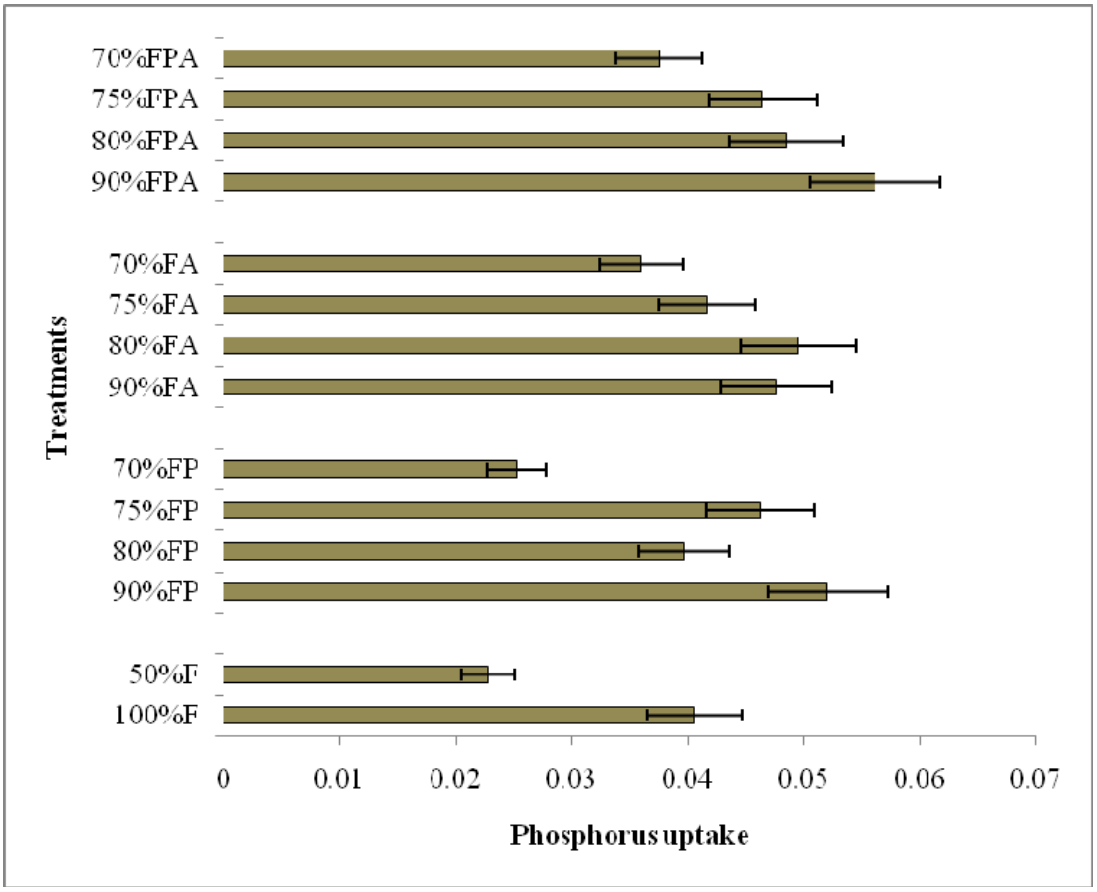


Fig. 8. Phosphorus uptake on dry whole plant basis with PGPR and AMF inoculation.
Uptake was estimated by multiplying plant dry weight by % P per gram of tissue. F, Fertilizer; P, plant growth-promoting rhizobacteria; and A, arbuscular mycorrhiza fungi.



Discussion

The results presented here support the hypothesis that PGPR or combinations of PGPR and AMF can improve the nutrient use efficiency of fertilizers. When the rate of fertilizer was reduced and inoculants were used, plant height, shoot dry weight, root dry weight, yield, and nutrient uptake were comparable to those with the full rate of fertilizer without inoculants (Table 3 and Fig. 2). After testing different reduced fertilizer rates, under our experimental conditions, 75% fertilizer was the stable minimum to which fertilizer could be reduced if supplemented with PGPR to achieve growth equivalent to 100% fertilizer without PGPR. Our results also show that 100% fertilizer produced plant growth

that was greater than all other lower rates if inoculants were not added (Table 2 and Fig. 1). This agrees with Biswas et al. (2000) who suggested an interdependence of fertilizer N inputs and inoculants for optimal gain in rice productivity.

When 70% fertilizer rate or lower was supplemented with PGPR or co-inoculation of PGPR and AMF, we observed lower growth of tomato, our model plant, or inconsistent growth that compared inconsistently to the 100% fertilizer control. This is similar to most of the results in our preliminary studies with pepper, Bermuda grass, and sunflower. In some instances, inoculant-supplemented 70% fertilizer gave growth that was comparable to 100% fertilizer without PGPR (Fig. 2 and Table 3) or comparable yield (Fig. 3, PGPR plus AMF bar). In our system the results support reduced fertilizer rates down to 75% if PGPR was added because that is the minimum at which results were consistent. This is different from the observations of Elkosa et al. (2008) and Canbolat et al. (2006) who reported no significant difference in root and shoot biomass of barley or seed yield and biomass of roots and shoots of chickpea respectively, when inoculant alone or fertilizer alone was used. Based on these results, it was suggested that inoculants could be an alternative to fertilizer (Elkosa et al. 2008). In contrast, our results demonstrate that inoculants may allow reduced rates of fertilizer, but that they will not replace fertilizer.

There were similarities in our results and those of Hernandez and Chailloux (2004) who reported that the dry weight of tomato transplants grown in the greenhouse with 75% fertilizer plus two co-inoculated PGPR was significantly greater than those with full fertilizer rate without PGPR. In our study, at reduced fertilizer rates (down to 75%) inoculants consistently enhanced dry biomass (Fig. 4). Also, N uptake per gram of tissue and N uptake on a whole plant basis were significantly better than the corresponding non-inoculated controls (Figs. 5 to 7). However, in the case of P, significant impacts resulted on

a whole plant basis but not per gram of plant tissue (Fig. 8). Hence, enhanced N use efficiency in response to inoculation was greater overall than that of P.

Our results indicate that the time of data collection for nutrient analysis as well as type and nutrient content of the growth medium are essential factors to consider before making reliable conclusions about the impact of inoculants on plant nutrient uptake. In the experiment with Hoagland solution, plants treated with 75% fertilizer plus inoculants consistently had comparable amounts of N at 4 weeks after planting (WAP) to those with 100% fertilizer without inoculants. However, results were highly variable when samples were taken at 6 WAP. A possible explanation for this could be based on previous reports that the concentration of nutrients, particularly N, P, K, S, Cu, and Zn, decreases with age of plant tissues (Mills and Jones 1996; Maynard and Hochmuth 2007). In Sunshine Mix (growth medium), we observed that total N and other nutrients were very high (Table 1) as well as the organic matter content. Common to many commercial growing mixes, the starter nutrient supplements contained in them may last for 4-6 weeks (Mills and Jones 1996), thus the impacts of microbes on nutrient uptake, especially, for short time trials will be difficult to discern using such mixes. Based on the results from preliminary studies, 1:3 field:sand soil was chosen as the model growth medium in this study and with its consistency through the study, we are recommending it for future fertility studies with inoculants.

Timing of data collection, microbial biomass/structure, and nutrient content of the growth medium may account for some variability in results of different authors (Saubidet et al. 2002; Shaharooma et al. 2008). Saubidet et al. (2002) reported that N content in wheat plants inoculated with *Azospirillum brasilense* decreased as N supply rate increased, and at the maximum N supply, the content of total N was the same between inoculated and

noninoculated wheat plants. On the other hand, Shaharoona et al. (2008) reported that N use efficiency increased in response to inoculation with *Pseudomonas fluorescens* at all fertilizer levels in wheat, causing 115%, 52%, 26%, and 27% increase over the noninoculated control at N, P, and K application rates of 25, 50, 75, and 100% recommended doses respectively. Also, other explanations for those differences in results could be the different effects of specific PGPR strains or other experimental factors.

Could there be a synergistic interaction between PGPR and AMF to improve the uptake of P and N? In our study, there appears to be some level of interaction with uptake of P, though little (Fig. 8). Co-inoculation of AMF and PGPR with 90% fertilizer resulted in plant uptake of P that was significantly higher than with full fertilizer rates, though its improvement over the inoculation of PGPR with 90% fertilizer was not significant. However, 70% fertilizer plus AMF and PGPR resulted in more P uptake than the corresponding treatment with PGPR alone (Fig. 8). Aseri et al. (2008) reported significant interaction of *Azotobacter chroococcum* and *Glomus mosseae* in pomegranate leading to better leaf area, shoot dry weight, and uptake of N, P, and K compared to either PGPR or AMF alone. This is different from a previous 3-year field study with corn (Adesemoye et al. 2008), where we did not observe consistent significant interaction between PGPR and AMF. Also, we did not observe any detrimental interaction in this study, which is in agreement with results of Barea et al. (1998).

One way that some previous studies have enhanced the performance of PGPR is co-inoculation of multiple PGPR strains (Belimov et al. 1995; Raupach and Kloepper 2000; Kloepper et al. 2007; Elcosa et al. 2008). For example, 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 *Azospirillum lipoferum* 137 and

Agrobacterium radiobacter 10 than single inoculation of any of the three organisms. We used a two-strain mixture for this study and it proved to be effective in both growth promotion and N and P uptake.

The enhancement of N uptake by plants inoculated with the PGPR strains (*Bacillus amyloliquefaciens* IN937a and *Bacillus pumilus* T4) used in our study was not via associative N fixation because no N-fixing strains of *Bacillus amyloliquefaciens* or *Bacillus pumilus* has been so reported. Also, the strains did not show blue to green coloration when grown on JNFb medium (Olivares et al. 1996), which allows associative N fixing bacteria to growth and display a blue to green color. Therefore, the enhancement of N uptake noted in our study must be due to alternative pathways. We propose a combination of the activities of the plant and the inoculants (Clarholm 1985; Saubidet et al. 2002; Vassey and Buss 2002; Raynaud et al. 2006; Kloepper et al. 2007), as a model for PGPR-enhanced N uptake in plants, according to the following scenario. The PGPR promote the growth of the plant and increase the root surface area or the general root architecture. Plants growing better in turn release higher amounts of C in root exudates. The release of more C prompts increase in microbial activity, and this process continues in a cycle. The whole process makes more N available from the soil pool, influencing N flux into plant roots, and the plant is able to take up more available N. Overall, our results suggest that inoculants could be used to allow reductions in the current high rates of fertilizer and the resulting environmental problems (Malakoff 1998; Gyaneshwar et al. 2002; Shaharooma et al. 2008), without compromising plant productivity. Further studies both in the greenhouse and on the field will provide more definitive information about the movement and uptake of N and P to plants with the impacts of inoculants (PGPR and/or AMF). One of such studies is

currently being conducted using ^{15}N isotope techniques, which will possibly reveal more specifics on the interactions and impacts of the inoculants and plant uptake of N.

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IV. IMPROVED PLANT UPTAKE OF NITROGEN WITH PGPR DEMONSTRATED WITH DIFFERENCES IN $\delta^{15}N$ IN TOMATO USING ^{15}N-DEPLETED FERTILIZER

Abstract

The techniques of ^{15}N isotope have proven useful for determining the behavior and fate of N in soil, including plant the use efficiency of applied N fertilizers. Our objective in this study was to use ^{15}N isotope techniques to demonstrate that a model plant growth-promoting rhizobacteria (PGPR) system, a two-strain mixture of *Bacillus amyloliquefaciens* IN937a and *Bacillus pumilus* T4, can enhance plant uptake of N using different rates of ^{15}N-depleted ammonium sulphate ($[^{15}NH_4]_2SO_4$). Results of the two different factorial experiments that were done showed that the dry biomass of plants which received 70% to 90% of recommended N fertilizer with PGPR inoculation was comparable to plants that received full rates of fertilizer without PGPR. Also, atom % ^{15}N per gram of tomato tissues decreased as the amount of fertilizer increased, and PGPR inoculation had significant impacts on the values. For example, the atom % ^{15}N abundance in plants that received 80% fertilizer plus PGPR was 0.1146, which was significantly lower than 0.1441 for plants that received 80% fertilizer without PGPR and equivalent to 0.1184 for plants that received 100% fertilizer without PGPR. This study further confirms that PGPR can enhance plant uptake of N from fertilizer as indicated by the differences in $\delta^{15}N$ and total N in addition to plant growth promotion. Hence, more evaluations of PGPR as components of integrated nutrient management systems are needed.

Introduction

It has been demonstrated that plant growth-promoting rhizobacteria (PGPR) can promote plant growth (Kloepper et al. 1989) and one of the mechanisms that has been proposed is increase in nutrient content of plants. There are reports showing that PGPR can increase plant growth by stimulating N uptake by plant roots (Hernandez and Chailloux 2004; Adesemoye et al. 2008). Specifically, it was suggested that the increase in plant N content might be as a result of increased fertilizer N utilization efficiency. However, there is need for more definite proof of the impacts of PGPR on fertilizer N use efficiency in plants.

Isotope tracer techniques are increasingly being used in studying the different parts of the nitrogen (N) cycle. Specifically, the use efficiency of N fertilizers by plants as affected by microbial inoculations is being studied using isotope techniques (Belimov et al. 1995; Biswas et al. 2000). Among the six known isotopes of N, ^{15}N and ^{14}N are the only isotopes that occur naturally and are stable, unlike isotopes ^{12}N, ^{13}N, ^{16}N, and ^{17}N, which are unstable with half lives of 0.0125 sec, 10.05 min, 7.36 sec, and 4.14 sec respectively. Isotope ^{15}N, which is commonly used in N tracer studies was first reported in 1930. Isotopes ^{15}N and ^{14}N coexist in nature in every substance containing N, with an almost constant abundance of 0.3663% for ^{15}N and 99.6337% for ^{14}N (Hauck and Bremner 1976).

Any fertilizer having a percentage of ^{15}N below the 0.3663% natural occurrence is referred to as depleted, but if the percentage is higher than the natural occurrence, it is referred to as enriched. Both enriched and depleted ^{15}N materials have been used in tracer studies to study N recovery (Edwards and Hauck 1974; Ditsch et al. 1992; Hauck et al. 1994). For example, both have been used to study plant use efficiency of N fertilizer or plant recovery of applied N in the laboratory (Azam et al. 1988), greenhouse (Bronson et al. 2000; Wanek and Arndt 2002), and field studies (Stevens and Laughlin 1989; Torbert et al.

1992; Zhou et al. 1998). These isotopic techniques have proven useful in estimating crop N uptake from inorganic fertilizers (Bacon et al. 1988; Bird et al. 2003). In addition, plant materials labeled with ^{15}N have been used as organic N inputs to monitor the uptake of N from organic sources (Vanlauwe et al. 1998; Hood et al. 1999). Also, ^{15}N isotope techniques have been used to study N fixation in kallar grass (Malik et al. 1987) and in legumes intercropped with cereals (wheat-bengal gram [chickpea] and maize-cowpea), by monitoring the fixed N in the legumes and the effects on the N content of the cereals (Patra et al. 1986; Torbert et al. 1996).

An underlying premise of using isotopic techniques is that the chemical identities of the isotopes are maintained in biological systems and the systems can not distinguish them (Hauck et al. 1994). Although the behavior of ^{14}N and ^{15}N are similar, they can be differentiated with specialized equipment on the basis that some of their compounds behave differently in exchange or distillation columns (Hauck et al. 1994; Mulvaney et al. 1997). Hence, ^{15}N has continued to attract interest in studies tracking the recovery of applied N fertilizer or following some aspects of the N cycle (Hauck et al. 1994; Barea et al. 2002).

We previously used non-tracer ^{14}N fertilizer to study the impacts of plant growth-promoting rhizobacteria (PGPR) on uptake and use efficiency of N fertilizer in tomato in the greenhouse (Adesemoye et al. submitted) and corn in the field (Adesemoye et al. 2008). In this study, we used ^{15}N-depleted material for the determination of percent recovery of applied N by plants, with the anticipation of obtaining a more accurate and reliable effect of applied microbes than a non-tracer method. Tracer N-fertilizer provides a definite result for studying both the behavior and fate of applied N because identification of labeled N is possible as it enters, is transformed, or leaves the system under study (Hauck and Bremner 1976; Saoud et al. 1992). However, it is important to emphasize that the use of ^{15}N in itself

does not constitute accuracy of data, but the accuracy is directly related to how exact the amount of labeled material added to the experimental system and the $\delta^{15}N$ (i.e., the relative deviation from the ratio of ^{15}N:^{14}N in atmospheric nitrogen) could be determined (Edwards and Hauck 1974; Cabrera and Kissel 1989; Mulvaney et al. 1997; Yoneyama et al. 2001; Wanek and Arndt 2002). It should be noted that it is not in all situations that tracer methods will necessarily give more accurate values than the non-tracer method (Hauck and Bremner 1976).

An important rationale for this study is that PGPR, being plant growth promoters, could play key roles in nutrient cycling in soil and have positive impacts on N availability and uptake by plants (Adesemoye et al. submitted). It has been reported that uptake of N by plant is mainly in the inorganic form, but the majority of N in the soil is in the organic form, often as complex molecules (Hodge et al. 2001). Thus, plants rely on microbes to release inorganic N through decomposition of organic materials. In addition to previous studies that we conducted with non-tracer ^{14}N, more information and understanding about plant-PGPR interaction can be obtained with ^{15}N. A better understanding of how PGPR influence plant uptake of N from soil will aid attempts to improve the use efficiency of applied N fertilizer and hence sustainability. The objective of this study was to use ^{15}N isotope techniques to demonstrate that a model PGPR system can enhance plant uptake of N using different rates of ^{15}N-depleted ammonium sulphate ($[^{15}NH_4]_2SO_4$). We hypothesized that PGPR will enhance plant uptake of N from ^{15}N-labeled fertilizer.

Materials and methods

Bacterial inoculation, test plant, and growth conditions

The experimental model system developed in a previous study (Adesemoye et al. submitted), which included tomato (*Solanum lycopersicum)* cultivar Juliet grown on a 1:3 mixture of field soil:sand was used for this study. The detail of the nutrient content of this soil mixture is shown in Table 1. Inoculants include two plant growth-promoting rhizobacteria (PGPR) strains, *Bacillus amyloliquefaciens* IN937a and *Bacillus pumilus* T4, which were used as a mixture. The PGPR strains were obtained from the culture collection of the Department of Entomology and Plant Pathology, Auburn University, Auburn, Alabama and used as spore preparations. The spore suspensions of the two PGPR strains were diluted and the concentration was adjusted to log 5 cfu/ml and used for inoculation. At planting, 1 ml of the bacterial suspension was applied onto each seed in a 10-cm (4-inch) pot containing the mixed soil before filling holes. A follow-up inoculation was done at one week after planting by applying 1 ml of PGPR drench per pot around the base of each plant.

Hydroponic solution of ^{15}N

We prepared a hydroponic solution with slight modifications of Hoagland solution (Maynard and Hochmuth 2007), producing an N-free solution (Hershley 1994). One liter of 100% N solution was prepared by using 1 M of ^{15}N-depleted ammonium sulphate ($^{15}[NH_4]_2SO_4$) (ISOTEC^{TM}, Miamisburg, OH) as the only N source. Other constituents of the fertilizer solution was 10 ml of 0.05 M monocalcium phosphate (Ca $[HPO_4]_2$), 20 ml of 0.1 M calcium sulfate dihydrate ($CaSO_4.2H_2O$), 5 ml of 0.5 M potassium sulfate (K_2SO_4), 2 ml of 1 M magnesium sulfate heptahydrate ($MgSO_4.7H_2O$), 2 ml of Fe-EDTA, and 1 ml of micronutrient stock (Adesemoye et al. submitted).The percentage of ^{15}N was varied by

changing the volume of $^{15}(NH_4)_2SO_4$ appropriately. The background atom % ^{15}N abundance in the $^{15}(NH_4)_2SO_4$ was 0.01%.

Experimental design

This report included two experiments conducted in the greenhouse at the Plant Science Research Center, Auburn University, Alabama, where temperature was maintained at 21 - 25ºC. The design for the first experiment was a 2 × 6 factorial in a randomized complete block (RCB), with ten replications. The 2 factors were with or without PGPR inoculation while the 6 factors were six rates of hydroponic fertilizer. Each fertilizer treatment had 10 replications. The six fertilizer treatments included 210, 189, 168, 157.5, 147, and 105 parts per million (ppm) N. The 210 ppm N was referred to as 100% in this paper, from which other rates (90%, 80%, 75%, 70%, and 50%) were calculated. Our second experiment was a 2 × 3 factorial in a RCB with ten replications that included with or without PGPR inoculation and 100%, 80% and 75% N rates. The amount of N in solution was varied by changing only the content of $(^{15}NH_4)_2SO_4$ appropriately. After seeding, 25 ml of hydroponic solution was applied per pot twice per week for each of the treatments (i.e., the different fertilizer rates). The plants were allowed to grow for 4 weeks before destructive sampling.

Plant sample preparation and isotope analysis

Plant heights were recorded at 4 weeks after planting (WAP). Whole plant fresh weights were taken, samples were dried at 70ºC for 7 days, and dry weights were recorded. Samples were analyzed for total N and ^{15}N contents at the Department of Natural Resources and Environmental Sciences, University of Illinois, Urbana. To estimate the impact of ^{15}N

uptake correctly requires that a portion of the tracer be recovered in a chemically definable state. The samples were digested according to standard protocols (Mulvaney 1993; Bremner 1996) and diffusion of digest was done using the Mason-Jar diffusion method (Mulvaney et al. 1997). An automated Rittenberg apparatus-mass spectrometer (ARA-MS) which utilizes the Rittenberg techniques for N isotope analysis was also used (Mulvaney and Liu 1991).

Calculation of ^{15}N uptake and statistical analysis

The amount of the material to apply, ^{15}N recovery, and $\delta^{15}N$ in the plant samples after isotope analysis were calculated. The calculations were done by adapting the methods used by previous authors with consideration for the base atom % abundance of the $[^{15}NH_4]_2SO_4$ used in this study (Hauck and Bremner 1976; Cabera and Kissels 1989; Zhou et al. 1998; Bronson et al. 2000; Wanek and Arndt 2002).

Analyses were done with the Statistical Analysis System 9.1 (SAS Institute, Cary, North Carolina). Data were analyzed using GLM procedure and Fisher's protected LSD was used to separate treatment differences. Prior to these, the residual term and the normal distribution assumption were evaluated using GLIMMIX procedure (Little et al. 2006). Statistical significance was considered at $\alpha = 0.05$ unless otherwise stated.

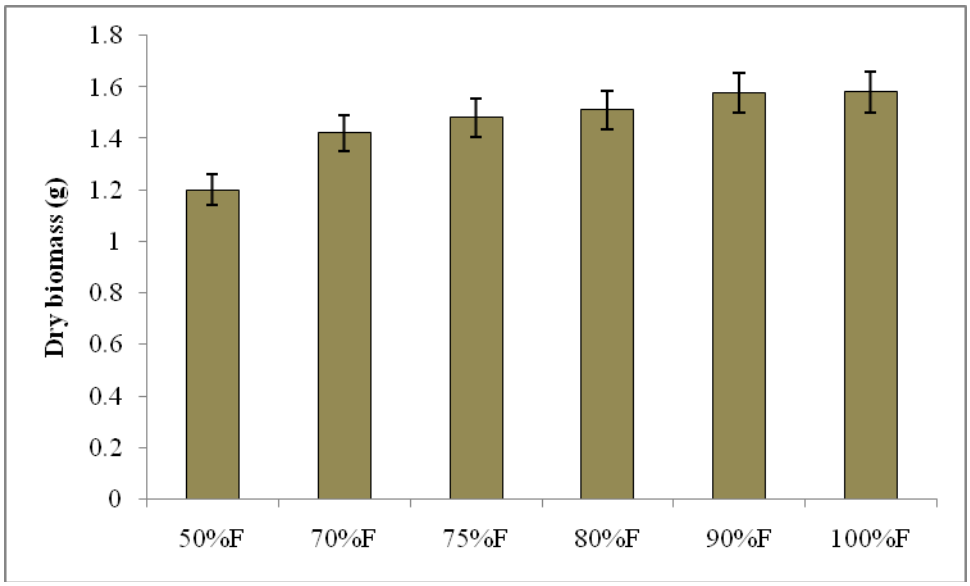
Results

Plant growth promotion

Inoculation with the tested plant growth-promoting rhizobacteria (PGPR) led to the promotion of tomato growth in terms of height and biomass. The total dry biomass of tomato samples that received PGPR inoculation together with 70%, 75%, 80%, or 90% of the full Hoagland nitrogen (N) rate was equivalent to the biomass for plants that received

the full N rates (100%) without PGPR (Fig. 1). At 50% of the N level, inoculation with PGPR resulted in biomass that was significantly less than that for the full N rate without inoculation.

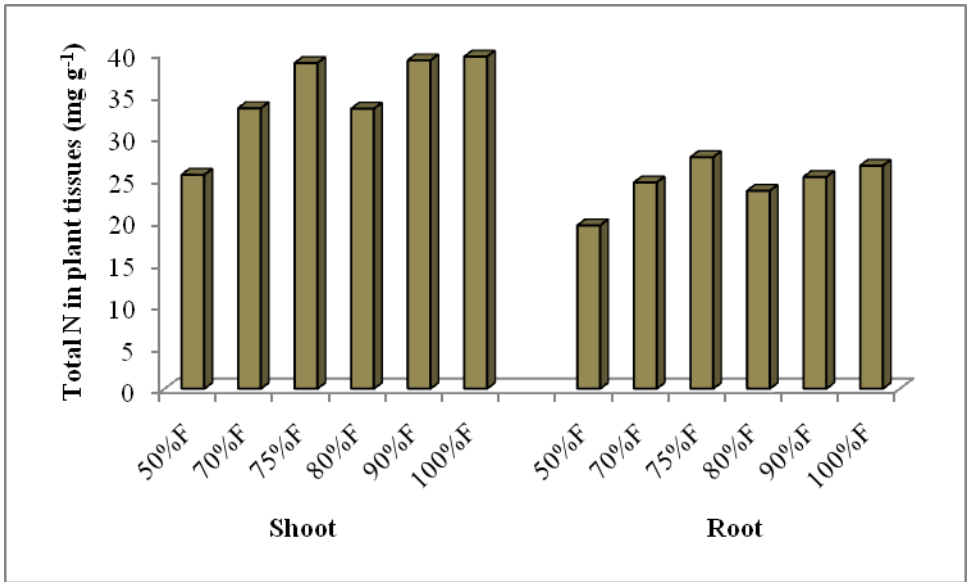
Fig. 1. Total dry biomass of samples before nutrient analysis. All (%) reflected percentage of N content. All treatments were inoculated with the PGPR mixture except the full rate (100%F).



Total nitrogen in tomato tissues

Total nitrogen (N) content (mg g^{-1}) of plant samples revealed that the responses of shoots and roots were slightly different when they were analyzed separately (Fig. 2). In shoots, the total N content in plants that received full N rates without inoculants was similar to plants that received 90% or 75% of N plus PGPR inoculation, and these three treatments were greater than the other three treatments (50%, 70%, and 80% hydroponic solution plus PGPR). Plants that received 70% or 80% of the Hoagland solution N plus PGPR contained a similar amount of N, which was greater than plants that received 50% N plus PGPR. In roots, the total N contents of all treatments were similar, except that the plants that received 50% N plus PGPR contained less N than others.

Fig. 2. Total nitrogen per gram of shoot and root tissues.
All (%) reflected percentage of N content. All treatments were inoculated with the PGPR mixture except the full rate (100%F).



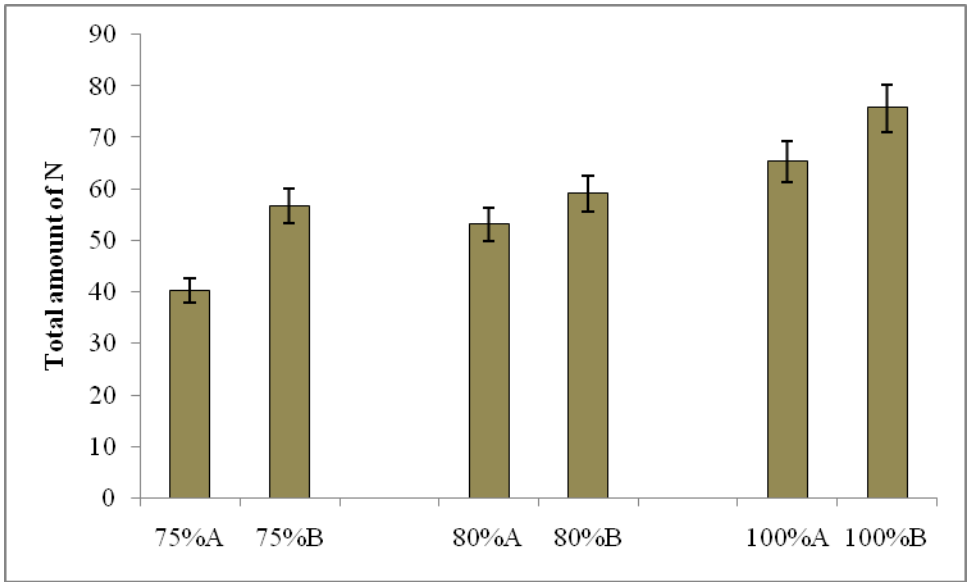
In our second experiment, in which 75%, 80%, and 100% of the Hoagland solution N with or without PGPR inoculation were tested, statistical comparison for all six treatments showed significant impact of PGPR. The addition of PGPR to each N level led to higher $\delta^{15}N$ and produced plants that contained more total N than the corresponding percent N that did not get PGPR inoculation (Fig. 3). Also, the total amount of N in plants that received 100% N rate without PGPR inoculation was not significantly greater than plants that received 75% or 80% N rate plus PGPR (Fig. 3).

Effect of plant growth-promoting rhizobacteria on $\delta^{15}N$

Generally, in both of the ^{15}N experiments, the atom % ^{15}N per gram of tissue decreased as the amount of applied total N taken up increased. This trend was the same when all rates that did not receive PGPR were compared separately from those that received

PGPR (Fig. 4). However, if those inoculated with PGPR were compared to those not inoculated, the trend was different due to higher $\delta^{15}N$. For example, the atom % ^{15}N per gram in plants that received 80% N rate plus PGPR was 0.1146, which was significantly lower ($\alpha = 0.1$) than 0.1441 of plants that received 80% N rate without PGPR, and equivalent to 0.1184 of plants that received full N (100%) without PGPR (Fig. 4). Based on total amount of ^{15}N contained in whole plant tissues, there were similarities between plants that received 100% N without PGPR treatment and those that received 75% or 80% N plus PGPR treatments. However, plants that received 100% N plus PGPR contained significantly more ^{15}N than those with 100% N without PGPR (Fig. 6).

Fig. 3. Total amount of N removed from experimental pots.
All (%) reflected percentage of N content. A, non-inoculated and B, inoculated with the PGPR mixture.



Although the amount of ^{15}N recovered per gram of plant tissues decreased with the inoculation of PGPR, the total amount per plant increased because PGPR increased the dry biomass. Shoots of plants that received 75% N with PGPR inoculation contained higher amounts of ^{15}N than the plants that received 100% N without PGPR inoculation (Fig. 5).

Additionally, plants with 80% or 90% N plus PGPR contained similar amounts of ^{15}N in their tissues compared to plants with full rate of fertilizer N without PGPR. However, plants that received 70% or 50% N plus PGPR contained less ^{15}N. In roots, all treatments had similar $\delta^{15}N$ except that plants that received 100% N without PGPR contained less amount of ^{15}N than those that received 75% N plus PGPR but more than those that received 50% N plus PGPR (Fig. 5).

Fig. 4. Atom % ^{15}N concentration in tomato tissues with the effects of PGPR.
All (%) reflected percentage of ^{15}N. A, non-inoculated and B, inoculated with the PGPR mixture. Bars with different letter(s) are significantly different at p = 0.01. The fertilizer material used was depleted in ^{15}N isotope.

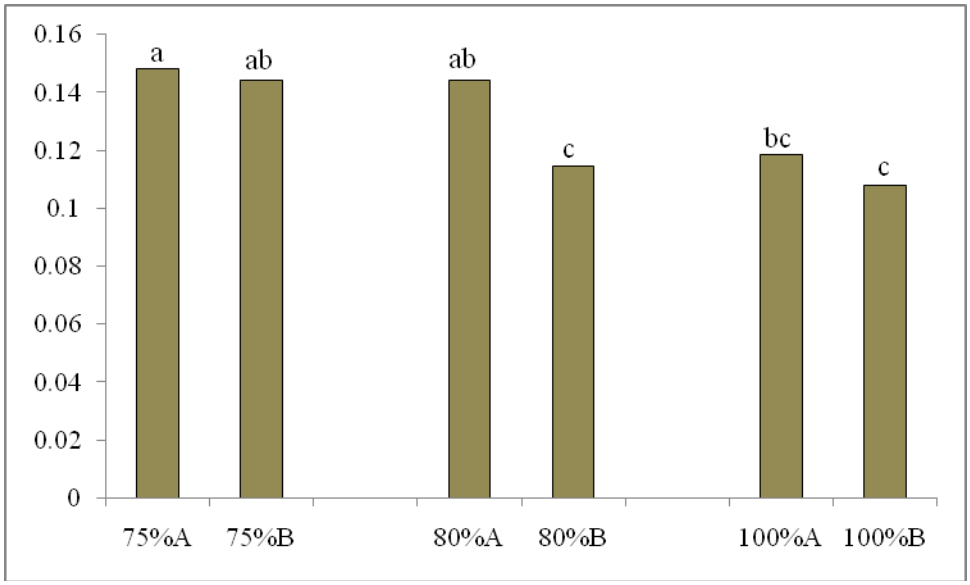


Fig. 5. Amount of ^{15}N recovered in shoot and root tissues at 4 WAP.
All (%) reflected percentage of N content. All treatments were inoculated with the PGPR mixture except the full rate (100%F).

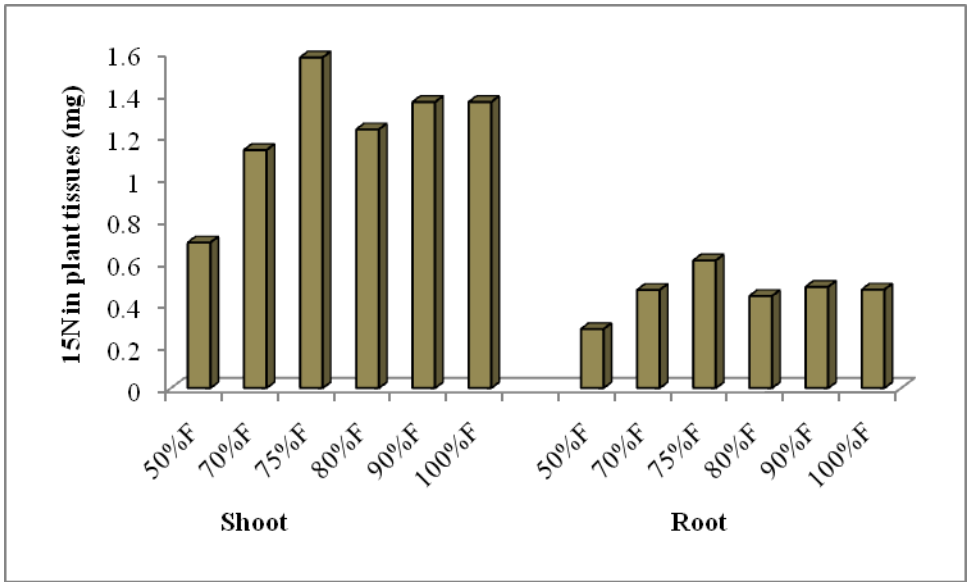
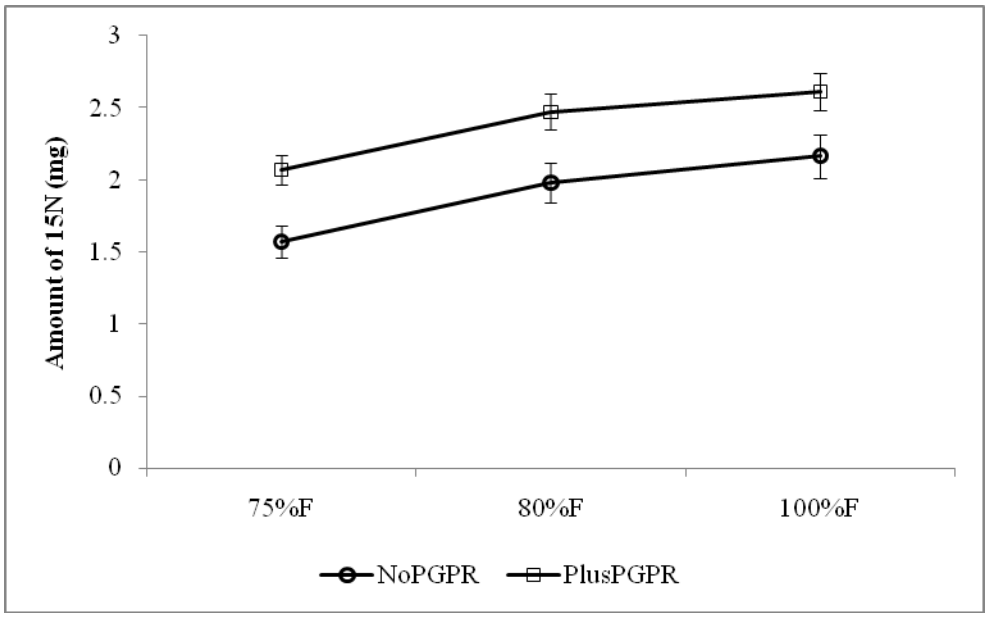


Table 1. Nutrient content of the growth media

Growth medium	N	C	P	K	Ca	Mg
Field soil	0.02	0.56	23.4	16.8	56.3	8.6
Field/Sand (1:3)	0	0.19	7.3	8.8	15.4	3.2
Sand	0	0.13	0.5	5.7	5.5	2.5

Nitrogen (N) and C are in percentage while P, K, Ca, and Mg are in µg g^{-1} (or ppm).Values showing (0) are below detectable limit of the equipment. Only the 1:3 mixture of field soil:sand was used for this study.

Fig. 6. Total amount of ^{15}N contained in whole plants grown in experimental pots.
All (%) reflected percentage of N content. NoPGPR, non-inoculated and PlusPGPR, inoculated with the PGPR mixture.



Discussion

In conclusion plant growth-promoting rhizobacteria (PGPR) have a significant impact on growth and N uptake in tomato as indicated by the differences in $\delta^{15}N$ and total N (Figs. 1-6). Using ^{15}N- depleted fertilizer, increased N uptake by PGPR treatments was observable 4 weeks after treatment. Differences in $\delta^{15}N$ alone have been previously hypothesized to indicate differences in N acquisition (Wanek and Arndt 2002). Our results agree with the work of others (Hauck and Bremner 1976; Ditch et al. 1992) showing that ^{15}N-depleted fertilizer can be reliably used in tracer studies.

There is an ongoing discussion in the literature about which methods are best for assessing fertilizer use efficiency in plants. One common method is the "difference method" which calculates difference in N uptake between fertilized and non-fertilized treatments. While the difference method provides useful data, studies that use fertilizer materials depleted or enriched in stable isotope ^{15}N offer more precision. This is because

the results of the "difference method" may be obfuscated by stimulation of N mineralization in the presence of fertilizer (referred to as added N interaction, ANI, or priming effect) (Ditch et al. 1992; Bronson et al. 2000). However, with the ^{15}N isotope approach, one can more accurately detect changes in plant tissues due to the applied treatment. The results we obtained, which are similar to Edwards and Hauck (1974) and Ditch et al. (1992) re-emphasized the usefulness of ^{15}N-depleted fertilizer.

As more of the labeled N entered the plants, there was increase in $\delta^{15}N$ and the concentration of ^{15}N in plant tissues shifted towards the 0.01 atom % ^{15}N abundance contained in the applied ^{15}N-labeled fertilizer ($[^{15}NH_4]_2SO_4$) but away from the natural 0.3363 atom % ^{15}N abundance in the soil. In this study, the concentration of ^{15}N per gram of tissue declined as applied ^{15}N fertilizer increased, indicating more plant uptake of the applied ^{15}N. With PGPR inoculation the concentration of ^{15}N further decreased (Fig. 4). This is consistent with results of previous studies. Ditsch et al. (1992) who used ^{15}N-depleted ammonium sulphate ($[^{15}NH_4]_2SO_4$) in a corn-winter rye crop rotation reported a decrease in atom % ^{15}N concentrations in rye tissue as N rate increased from zero to 336 kg N ha^{-1}.

Our observation of differences in N and ^{15}N between roots and shoots (Fig. 2 and 5) is similar to previous results (Nayak et al. 1986; Saoud et al. 1992; Wanek and Arndt 2002). For example, Saoud et al. (1992) reported less N in roots than in above- and below-ground stem in potato. Similarly, Wanek and Arndt (2002) found higher N in leaves and stems than roots with three concentrations of nitrate used in a study on soybean and ryegrass. There are many possible reasons or factors that could lead to these observations. Some of the factors are the gradient that develops between roots and shoots as a result of partial assimilation of nitrate or ammonium in the roots, the capacity to retain part of reduced N in below ground

biomass, and the pattern of export or movement of ^{15}N-labeled nitrate or ammonium to shoots (Wanek and Arndt 2002).

Better response of plants that received 75% of labeled N with PGPR inoculation as compared to full rate without PGPR inoculation demonstrates the suggestion that soil microbes are more effective at lower rates of fertilizer or when a nutrient becomes limiting (Hernandez and Chailloux 2004; Dell and Rice 2005). It should be added that in our results, the 75% rate was a threshold below which the PGPR-fertilizer interaction could not produce consistent nutrient uptake compared to full fertilizer rates that did not receive inoculation.

Overall it is clear from our studies that the tested PGPR strains promoted plant growth and enhanced plant uptake of N. These results are supported by the suggestion of Nemergut et al. (2008) that the fate of N in the ecosystem and the fraction that fuels primary production are intimately linked to the underlying soil microbial community. Also, in a northern hardwood forest in Michigan, Zogg et al. (2000) observed that much of the $^{15}NO_3$ that was applied to soil cycled through microorganisms, either directly by accumulation of N in microbial biomass or indirectly by rapid movement without retention in microbial cells, before appearing in soil organic matter and plant roots. The remaining NO_3 pool was lost to leaching. In a microplot study with ryegrass, Stevens and Laughlin (1989) reported an average total utilization of ^{15}N labeled fertilizer of 76%, and they suggested that the remaining parts might have been denitrified, leached, or removed from the microplots by soil fauna. After reviewing results from a 3-year field study (Adesemoye et al. 2008) and a greenhouse study (Adesemoye et al. submitted), we proposed a model pathway for the influence of microorganisms on the fate and flow of N, especially in relation to anthropogenic nutrient sources such as chemical fertilizers. The model is as

follows - PGPR promote the growth of the plant and increase the root surface area or the general root architecture. Plants growing better in turn release higher amounts of carbon (C) in root exudates. The release of more C prompts increase in microbial activity, and this process continues in a cycle. The whole process makes more N available from the soil pool, influencing N flux into plant roots, and the plant is able to take up more available N (Adesemoye et al. submitted).

In spite of the above mentioned observations and suggestions, many questions are yet to be answered (Nemergut et al. 2008). The warning of Nayak et al. (1986) that inferences regarding N uptake as a consequence of inoculants be made with caution is therefore understandable. To further understand PGPR activities in N uptake, it would be helpful to test, under controlled conditions, if N that accumulates within the biomass of PGPR could be transferred to plant tissues. We suggest that future experiments should use PGPR labeled with ^{15}N isotopes to test this idea.

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APPENDIX

Chapters 2 to 4 of this dissertation have either been published or submitted to the following journals.

Chapter II: Enhanced plant nutrient use efficiency with pgpr and amf in an integrated nutrient management system. (This was published, see Adesemoye et al. 2008. Can. J. Microbiol. **54:** 876-886).

Chapter III. Plant growth-promoting rhizobacteria allow reduced application rates of chemical fertilizers. (This was submitted to Microbial Ecology).

Chapter IV. Improved plant uptake of nitrogen with PGPR demonstrated with differences in $\delta^{15}N$ in tomato using ^{15}N-depleted fertilizer. (This was submitted to Journal of Environmental Quality).

The three articles have the followings as co-authors: Adesemoye, A. O. (first author), Torbert, H. O., and Kloepper, J. W. It should be noted that Adesemoye, A. O. contributed more than half of the total effort for each of the articles. Dr. H. A. Torbert, who collaborated on the work (and allowed me to use materials in his lab) and my major advisor, Dr. J. W. Kloepper, whose program funded most part of the work and who also guided me, jointly contributed less than 50% of the efforts towards the studies. I am grateful to the two of them.