THE EFFECT OF NITROGEN ON RHIZOBACTERIA ASSOCIATED WITH WHEAT SHOOT PRODUCTIVITY

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CHAPTER I

INTRODUCTION

1.1 Wheat

Of the three most essential cereal crops, including maize (*Zea mays L*), rice (*Oryza sativa L*), and wheat (*Triticum*. spp), that supply food for humans needs (Carver, 2009; Satorre and Slafer, 1999), wheat is the second most important crop in terms of production in the United States (Stine and Ball, 1922). Within the last decade wheat was considered as the third most important in terms of production in the USA after corn and soybeans (Diekmann, 2009). The annual total harvest of wheat around the world was 607 million tons in 2007 compared to 652 million tons of rice, and 785 million tons of maize in the same year (Shewry, 2009). In some states of the United State such as Kansas, Oklahoma, and Texas, wheat production is the main source of income for farmers. However, wheat cultivation has been changed due to Conservation Reserve Program (CRP) passed in the 1985 Farm Act and with additional provisions in the 1990 Farm Act, which offer farmers with other crops options instead of planting wheat. Farmers can plant about 25 % of their farms with wheat without losing their base acreage, and this is a primary factor that encourages many farmers to plant wheat (http://www.ers.usda.gov/briefing/wheat/background.htm). An average of 41.9 bushels of wheat production per acre was produced in 2000 in the United State (Lance and Garren, 2002). In 2008, the production of wheat increased to 44.9 bushel per acre and 43.7 bushel per acre in 2011 (Table 1) (http://www.ers.usda.gov/data/wheat/YBtable01.asp). In Oklahoma, a total of 74.8 million bushels of wheat were produced in

2011 (http://www.hpj.com/journal/resources/11OKWheat_smLtr.pdf).

Wheat is also very important world-wide, being cultivated in more than 100 countries throughout the world (Bushuk and Rasper, 1994). United State is one of the highest wheat producing countries after European Union, China, and India, respectively (Diekmann, 2009). The total wheat production around the world is predicted to increase by 3 % in 2011-12 and is expected to produce around 669 million tons. However, due to the climate change, the production of wheat is expected to be decreased by 7 % in the United State (to about 56 million tons), and by 1 % in European Union (to be around 134 million tons) and in China (to 115 million tons). On the other hand, production of wheat in India is expected to increase by 4 % to produce around 84 million tons (Fell, 2011).

Wheat (*Triticum* spp. L) originates from the *Poaceae* family, which includes many familiar grasses (Ten Eyck, 1914). As a monocotyledon plant with a variable numbers of ploidy levels, the diploid has 14 chromosomes such as *Triticum monococcum,* tetraploid has 28 chromosomes such as *Triticum durum,* and hexaploid has 42 chromosomes such as *Triticum aestivum* (Carver, 2009). Almost all winter wheat and most of spring wheat are hexaploid (Ten Eyck, 1914). Wheat species can be classified into many different groups, such as hard, soft spring, or winter wheat, depending on seed quality, color, and growth pattern (Bushuk and Rasper, 1994; Cornell and Hoveling, 1998).

Wheat growth is affected by many factors such as water and nutrient availability in soil, weather conditions during wheat emergence and growth (Lokhorst et al., 2009), soil fertility, pH, and air temperature (Saunders and Hettel, 1994). Overall, wheat needs irrigated environment for maximal yield (Sayre and Moreno, 1997), but is often grown under non-irrigated conditions. Irrigation is common in Asia but less common in West Asia and North Africa (Bohn and Byerlee, 1993). In addition, Climate may affect wheat variety characteristics. For instant, hard wheat that

is grown in dry areas turns into soft wheat when grown in moist environments. In general, wheat grows better in heavy clayey soils than sandy soils (Nevo et al., 2002). Wheat is very adaptable and has shown to survive a wide temperature range in the environment (Shewry, 2009). Many morphological, biochemical, and physiological characteristic of wheat could be changed by low temperature stress. However, the high temperature stress could negatively affect the yield of wheat (Narendra et al., 2010). In addition to abiotic environmental factors, wheat is also affected by biotic environmental factors such as insects, fungi, viruses, and pathogenic bacteria that have negative impact on the production of wheat (Bajaj, 1990). For example, Smuts and rusts (fungus diseases) are contributing in less of wheat yield. Where they might found in separate wheat farm or together in the same farm (Charles and Willis, 1898). However, The genetic background of wheat can have a clear impact on wheat resistance against diseases and harmful insects (Cornell and Hoveling, 1998).

Hexaploid and tetraploid wheat were reported as the most common cultivated wheat types in modern agriculture (Nevo et al., 2002). On the other hand, it was found that the most consumed species of wheat is *Triticum aestivum* (bread wheat), which represents about 80% of the consumed wheat around the world, and *Triticum durum* (macaroni, couscous Wheat) that is consumed in the Mediterranean areas (Bajaj, 1990). Due to wheats high nutrition value, it has become the main source for human caloric intake compared to other cereals (Bushuk and Rasper, 1994). Wheat deserves its importance as a valuable energy source for the human body owing to extra storage protein (7-22%) which might increase to 17-28 % in the elite wild genotypes, starch (60-80 %) (Carver, 2009), vitamins, and minerals. Wheat is used to make a variety of foods including: pasta, breakfast cereal, noodles, cakes, bread, etc. (Bajaj, 1990). Recent research indicates that wheats richness in antioxidants contained in the grain seeds contribute to suppressing free radical damage and protecting humans from chronic diseases such as cancer (Yu,

2008). Consequently, much research needs to be conducted in order to increase yield, improve quality, and enhance wheat resistance against harmful diseases and insects.

1.2 Rhizosphere

In addition to plant roots, soil is a common environment for numerous living organisms such as bacteria, fungi, protozoa, nematodes, and mites. About $10^7 – 10^{12}$ bacteria, 10^4 protozoa, 10^4 nematodes, and 5-25 km length of fungal hyphae could be found per gram of soil (Hinsinger et al., 2009). Rhizosphere soil is the thin soil layer surrounding the root system that is under the influence of the plant (typically, 1-2 mm of thickness) (Girish and Ajit, 2011), so it is a very active area for increased nutrient acquisition for plant growth (Gaskins et al., 1985; Hinsinger et al., 2009). The first definition of rhizosphere was "the soil surrounds the root surface" by Hiltner in 1904 (Girish and Ajit, 2011). The Oxygen concentration in the rhizosphere soil depends on root and microbial respiration, which might cause low Oxygen tensions to create anaerobic condition. As a result, the percentages of Oxygen in the rhizosphere zone can vary considerably compared to the bulk soil. In addition, soil rhizosphere pH can be change dramatically due to the root extrusion of protons from the epidermal cell layer (Cardon and Whitbeck, 2007). In contrast to the bulk soil, rhizosphere soil contains a lot of soluble carbohydrates (Girish and Ajit, 2011). As a result, this thin layer of soil is very metabolically active with prokaryotic and eukaryotic microorganisms (Compant et al., 2010).

There are many types of rhizosphere microorganisms classified according to the physical location around or within the plant root: wither the microorganisms associate with the root surface or the thin layer of soil surrounding the root or within the roots themselves. Rhizosphere microorganisms that associated with the surface of the root are called rhizoplane microorganisms, and those that associate with the thin layer of soil are called rhizosphere microorganisms or rhizobacteria, rhizofungi etc. Those that associate with the internal roots cells are called endophytes. Some of these microorganisms are harmful to plant growth causing diseases, and others are beneficial providing plants with added nutrition or protection against disease. In addition, rhizosphere microorganisms may be able to change nutrient uptake rate (Bhromsiri and

Bhromsiri, 2010; Gaskins et al., 1985). The specific types of microorganisms in the rhizosphere soil vary from plant to another depending on plant variety, the environment surrounding the root, and many other factors. Root secretes exudates such as carbohydrates, amino acids, etc. that serve as metabolic fuel for some microorganisms living around the root (Compant et al., 2010; Girish and Ajit, 2011; Trivedi et al., 2011). Under controlled environmental growth chamber conditions, several rhizosphere bacteria showed their ability to promote plant development (Germida and Walley, 1996). However, our knowledge about microorganisms in rhizosphere soil is still very insufficient (van Overbeek et al., 2011).

A lot of microorganisms affect plant development by excreting plant growth promoting component such as auxins, gibberellins, etc. (Kumar et al., 2011). In addition, soil microorganisms can enhance the availability of soil nutrients and their mobilization. For example, much of the soil phosphorus is not very available for plant needs, but *Bacillus*, *Enterobacter* (Bacteria), *Aspergillus* and *Penicillium* (Fungi) are capable of altering phosphorus form from the insoluble form to a more soluble form. Some rhizosphere microorganisms can fix atmospheric nitrogen such as *Rhizobium*, which makes additional nitrogen available for plant uptake (Dastager et al., 2010). In addition, rhizosphere microflora can contribute to the decrease of harmful pathogenic or deleterious microbes by producing antibiotics, siderophores, and hydrogen cyanide (Zahir et al., 2004). These are just a few of the mechanisms that are associated with plant productivity and rhizosphere microbial community.

1.3 References

Bajaj Y.P.S., 1990. Wheat, Springer-Verlag, Berlin ;New York. 687.

Bhromsiri C., Bhromsiri A., 2010. Isolation, screening of growth-promoting activities and diversity of rhizobacteria from vetiver grass and rice plants. Thai Journal of Agricultural Science 43:217-230.

Bohn A., Byerlee D., 1993. The wheat breeding industry in developing countries: An analysis of invetments and impacts. Part I of 1992/93 CIMMTY world wheat facts and trend, The Wheat Breeding Industry in Developing Countries: An Analysis of Investments and Impacts, Singapore:CIMMTY. 38.

Bushuk W., Rasper V.F., 1994. Wheat: production, properties and quality.

Cardon Z.G., Whitbeck J.L., 2007. The rhizosphere : an ecological perspective, Elsevier Academic Press, Amsterdam ;Boston. 212.

Carver B.F., 2009. Wheat : science and trade, Wiley-Blackwell, Ames, Iowa. 569.

Charles O.T., Willis G.J., 1898. The hessian fly and wheat diseases Technology & Engineering.

Compant S., Clement C., Sessitsch A., 2010. Plant growth-promoting bacteria in the rhizo- and endosphere of plants: Their role, colonization, mechanisms involved and prospects for utilization. Soil Biology & Biochemistry 42:669-678.

Cornell H.J., Hoveling A.W., 1998. Wheat : chemistry and utilization, Technomic, Lancaster, Penn. 426 .

Dastager S.G., Deepa C.K., Pandey A., 2010. Isolation and characterization of novel plant growth promoting Micrococcus sp NII-0909 and its interaction with cowpea. Plant Physiology and Biochemistry 48:987-992.

Diekmann F., 2009. Commodity of the Quarter: wheat. Journal of Agricultural & Food Information 10:289-299.

Fell J., 2011. Crops: Wheat. Australian Commodities 18:26-30.

Gaskins M.H., Albrecht S.L., Hubbell D.H., 1985. Rhizosphere Bacteria and Their Use to Increase Plant Productivity - a Review. Agriculture Ecosystems & Environment 12:99-116.

Germida J.J., Walley F.L., 1996. Plant growth-promoting rhizobacteria alter rooting patterns and arbuscular mycorrhizal fungi colonization of field-grown spring wheat. Biology and Fertility of Soils 23:113-120.

Girish S., Ajit V., 2011. Soil Enzymology. 1st ed. Springer Heidelberg Dordrecht.

Hinsinger P., Bengough A.G., Vetterlein D., Young I.M., 2009. Rhizosphere: biophysics, biogeochemistry and ecological relevance. Plant and Soil 321:117-152.

Kumar K., Amaresan N., Bhagat S., Madhuri K., Srivastava R.C., 2011. Isolation and characterization of rhizobacteria associated with coastal agricultural ecosystem of rhizosphere soils of cultivated vegetable crops. World Journal of Microbiology & Biotechnology 27:1625-1632.

Lance G., Garren B., 2002. Origin, History, and Uses of Oat (*Avena sativa*) and Wheat (*Triticum aestivum*).

Lokhorst C., Huijsmans J.F.M., de Louw P.P.M., 2009. JIAC 2009: Book of abstracts Wageningen Academic The Netherlands.

Narendra T., Sarvajeet S.G., Antonio F.T., Renu T., 2010. Improving crop resistance to abiotic stress John Wiley & Sons, Germany.

Nevo E., Korol A.B., Beiles A., Fahima T., 2002. Evolution Of Wild Emmer And Wheat Improvement: Population Genetics, Genetic Resources, And Genome Organization Of Wheat's Projenitor, Triticum Dicoccoides Springer, New York.

Satorre E.H., Slafer G.A., 1999. Wheat: ecology and physiology of yield determination.

Saunders D.A., Hettel G.P., 1994. Wheat in heat-stresses environments: Irrigated, dry areas and rice-wheat farming systems D.F: CIMMYT, Mexico.

Sayre K.D., Moreno R.O.H., 1997. Applications of raised-bed planting systems to wheat Wheat Special Report, Mexico.

Shewry P.R., 2009. Wheat:Review. Journal of Experimental Botany 60, No. 6:1537–1553.

Stine O.C., Ball C.R., 1922. Wheat production and marketing Government Printing Office, Washington.

Ten Eyck A.M., 1914. Wheat; a practical discussion of the raising, marketing, handling and use of the wheat crop, relating largely to the great plains region of the United States and Canada Campbell soil culture pub. co., Lincoln, Neb.

Trivedi P., Spann T., Wang N.A., 2011. Isolation and Characterization of Beneficial Bacteria Associated with Citrus Roots in Florida. Microbial Ecology 62:324-336.

van Overbeek L.S., Franke A.C., Nijhuis E.H.M., Groeneveld R.M.W., da Rocha U.N., Lotz L.A.P., 2011. Bacterial Communities Associated with Chenopodium album and Stellaria media Seeds from Arable Soils. Microbial Ecology 62:257-264.

Yu L., 2008. Wheat antioxidants.

Zahir Z.A., Arshad M., Frankenberger W.T., 2004. Plant growth promoting rhizobacteria: Applications and perspectives in agriculture. Advances in Agronomy, Vol 81 81:97-168.

CHAPTER II

REVIEW OF LITERATURE

2.1. Microbial Diversity

Microbial communities are influenced by many bio-geographical and ecological factors such as food network associated with the microbial community and the presence of organic and inorganic nutrients. Combining the technology of molecular biology and the analysis of phylogenetic information, it is possible to assess microbial community diversity in the soil habitat (Liao et al., 2007). The total number of species in an environment is referred as species richness, which is often measured using a variety of indexes including the nonparametric indicator Chao1. However, the relation between number of species and number of individuals is called species diversity (Spellerberg and Fedor, 2003). Various ecological diversity indices have been developed to study the diversity and richness of organisms in different ecological environments such as Alpha (α), Beta (β), Shannon-Weiner, and Simpson's index etc to name a few. (Oswald, 2007). Magurran 2004 cited from Pielou that the definition of ecological diversity is "the richness and variety of natural logical communities." Alpha (α) diversity measures species diversity within particular area or habitat such as freshwater pond. Simpson's index measures the probability if two individual samples were randomly selected from the same area they would not be from the same species. However, Shannon-Weiner Index quantifies the possibility that one species sampled from a given area doesn't predict which of the following individual will be selected next from the same area. Shannon-Weiner Index, which is extensively used to compare diversity

between different environments (Khan, 2006), which could be defined as the counting of the richness of subspecies and the portion of each subspecies in the area or treatment (http://rewhc.org/biomeasures.shtml). Evenness index sensitively measures the changes of rare species number in the same area. The large index values from the Shannon-Weiner and Simpson's index indicate that the sample has heterogeneity species profusion. Overall, ecologists believe that each environment has a few abundant species and a lot of rare species. (Oswald, 2007). In current study, the bacterial diversity was measured using Chao1 and Shannon indices utilizing RDP II pyrosequencing community analysis functions (http://pyro.cme.msu.edu/).

2.2. The Importance of Rhizobacteria

Soil surrounding the root system is very rich in microorganisms fueled by the supply of root exudates ultimately originating from the shoot through photosynthesis (Compant et al., 2010). Theses microorganisms can change the soil characteristic surrounding the root to increase nitrogen and phosphorus availability and can enhance the uptake of nutrients by plants roots (Gaskins et al., 1985). Because of their positive benefits to plant growth, the rhizosphere organisms are very important for increasing agricultural yield (Hinsinger et al., 2009). Rhizobacteria are not the only organisms inhabiting close to root surfaces- others include fungi, nematodes, protozoa and microarthropods. Some of these rhizobacteria cause diseases in plants while others stimulate plant growth (Doornbos et al., 2012). PGPR can be classified into two main groups depending on their interaction with plants: symbiotic bacteria that exist inside plant cells forming nodules and free-living rhizobacteria that are found in rhizosphere soil promoting plant growth (Hayat et al., 2010). Plant growth is affected by rhizobacteria by several mechanisms, such as: nitrogen fixation, production of hormones for growth promotion, and inhibition of pathogens (Gaskins et al., 1985; Salanturi et al., 2006; Zahir et al., 2004). Moreover, rhizobacteria are responsible for the carbon cycling of many organic compounds (Kirk et al., 2004).

Useful soil bacteria are generally referred to as plant growth promoting rhizobacteria (PGPR) (Beneduzi et al., 2008; Carlier et al., 2008; Compant et al., 2010; Hayat et al., 2010). Also, plant growth promoting rhizobacteria can be described as plant health promoting rhizobacteria (PHPR) or nodule promoting rhizobacteria (NPR). PGPR can influence plant growth directly or indirectly. The indirect effect on plant growth happens when PGPR inhibits the harmful effect of one or more phytopathogenic organisms (Abbasi et al., 2011; Beneduzi et al., 2008; Cavaglieri et al., 2009; Hassen and Labuschagne, 2010) for example: by producing an enzyme that decomposes fungal cell wall components, or releasing hydrogen cyanine which has antibiotic properties (Hayat et al., 2010). The direct reinforcement of plant growth comes from the production of compounds that facilitate the uptake of certain nutrients like nitrogen or phosphorus from the environment and producing plant growth promoting substances such as vitamins, enzymes, and hormones like cytokinins, auxins, ethylene, gibberellins and abscisic acid…etc. (Abbasi et al., 2011; Beneduzi et al., 2008; Hassen and Labuschagne, 2010; Hayat et al., 2010).

Rhizosphere microorganism researches have been divided into various areas of interest. Some of them were conducted to determine and classify rhizosphere communities. For example, Cavaglieri et al 2009 pointed out that microbial communities demonstrated structural alteration over time in maize plant. Furthermore, in their previous study, they found that maize root surfaces were associated with large number and wide diversity of bacteria and fungi. Sudini et al 2011 found that *Proteobacteria, Acidobacteria, Firmicutes,* and *Bacteroidetes* were the most dominant phyla associated with peanut cropping systems. Other researches have focused on the influences of inoculating plants with plant growth promoting bacteria. Moreover, others have focused on the effects of inoculating plants with a combination of PGPR and chemical fertilizers. Shoot and root dry weight of non-nitrogen-fixing plants: Chinese cabbage, Lemongrass, Sunflower, Linseed, Common poppy, and Maize were increased as a result of inoculating with the following

Rhizobium strains: *R galegae, R. leguminosarum bv. Trifolii, R. leguminosarum bv. Viciae,* and *Sinorhizobium meliloti* (Hossain and Martensson, 2008). Combining PGPR with chemical fertilizers has significantly improved plant productivity (Hayat et al., 2010). Canbolat et al 2006 observed significant increase in the availability of phosphate in soil when barley seeds were inoculated with *Bacillus M-13* and *Bacillus RC01*. Moreover, total barley biomass weight was increased by 20.3–25.7% by using bacterial inoculation compared with 18.9 and 35.1% when only nutrients like P and NP were applied. Research showed positive effects of inoculation using *A. brasilense* Sp246 and applying nitrogen on many growth parameters in wheat and barley. When compared to control, spike number per m^2, grain number per spike, grain yield increased by 7.2, 5.9, and 14.7 in wheat and increased by 6.6, 8.1, and 17.5 in barley respectively (Ozturk et al., 2003).

Comparing un-inoculated wheat plants, the height, shoot fresh and dry weights, root length, and root fresh and dry weights of the inoculated wheat plants inoculated with PGPR were considerably increased (Abbasi et al., 2011). In addition, Abbasi et al 2011 found that the applied combination of nitrogen and PGPR increased yield and nutrition in treated treatment. Akhtar et al 2009 found that using the PGPR and compost in mixture with chemical fertilizer significantly increased the growth and yield of wheat. In contrast, Germida and Walley 1996 found that the application of certain PGPR didn't improve spring wheat growth. De Freitas 2000 observed that inoculated wheat plants with *E. cloacae, B. polymyxa* or mixed cultures did significantly increase nitrogen content in wheat tissues. Inoculating maize seeds with *P.putida* strain R-168, *P.fluorescens* strain R-93, *P.fluorescens* DSM 50090, *P.putida* DSM291, *A.lipoferum* DSM 1691, and *A.brasilense* DSM 1690 significantly improved seed germination and seedling growth and increased leaf and shoot dry weight and leaf surface area as well (Gholami et al., 2009). Using 70% of normal fertilization with PGPR mixtures and AMF produced the same yield of tomato when compared to full fertilization when applied without inoculation (Adesemoye et al., 2009). In

addition, Carlier et al 2008 got the same result when they inoculated wheat plants with *aurantiaca* strain SR1 under fertilization and no fertilization condition. Inoculating banana with PGPR and 33% N fertilizer increased the yield by 35-51% (Mia et al., 2010). It is likely that the concentration of nitrogen fertilization affects rhizobacteria to a greater degree and helps to increase plant productivity. Furthermore, plant growth promoting rhizobacteria could contribute to less chemical fertilization use, which use may have negative environmental consequences. In nitrogen for example, it was reported that 31% of the nitrogen is required in maize with PGPR inoculation and 40% for oil palm under greenhouse condition compared with 20% for rice and 70% for sugar cane under field condition (Mia et al., 2010). Single inoculation of wheat with *Bacillus simplex, Bacillus megaterium, Bacillus cereus* strains and combination of *Bacillus cereus* strain with *Paenibacillus alvei* strain resulted in the increasing of plant shoot weights and root weights. Moreover, positive influences in tomato shoot and root weights were observed when inoculated singly with *Bacillus simplex* and *Bacillus cereus* strains (Hassen and Labuschagne, 2010). The aim of this study is to investigate and phylogenetically identify specific rhizobacterial groups associated with wheat productivity, with and without nitrogen fertilization.

2.3. The Importance of Nitrogen

Nitrogen (N) is considered the most essential nutrient for plant growth and development. Nitrogen contributes to the structure of many organic molecules such as DNA, RNA, NAD, and ATP (Canfield et al., 2005; Hasan, 2004), so it is very necessary for metabolic system in all organisms. The dry mass of all organisms including plants contains an average of 6.25% nitrogen (Bothe et al., 2007). The total amount of nitrogen on the earth is about 57.4×10^{18} kg, with most of it in the atmosphere. However; only about 0.0025 percent is available for plant growth (Lewis, 1986). Nitrogen is present in various forms in the environment, which includes the inorganic forms: ammonium NH_4, nitrite NO_2^-, nitrate NO_3^-, nitrous oxide N_2O, and nitric oxide NO, inorganic forms such as nitrogen gas N_2 (Bothe et al., 2007) and organic nitrogen forms such as amino acids, amino sugar, polypeptide and proteins (Schepers and Raun, 2008). The nitrogen cycle starts when plants absorb the nitrogen from the soil by the roots and in particular through their root hair. Plants can uptake the nitrogen in the form of nitrate or ammonium. However, nitrate is the most common nitrogen form that plant can absorb, so farmers always prefer to add nitrogen as fertilizer nitrate (Lewis, 1986). If the plant absorbs the nitrate form, the nitrate will be changed to nitrite then to ammonium in the plant. As result, ammonium will be incorporated into organic molecules component through a number of enzymatic processes. Moreover, plant can directly absorb ammonium from the soil, or incorporate it from the atmosphere based upon the process of nitrogen fixation that commonly occurs with a number of leguminous plants in symbiotic association with *Rhizobium* bacteria. The process of absorbing ammonia is called ammonia assimilation, while the process of fixing it from the atmosphere is call nitrogen fixation. Nitrogen cycle continues with the mineralizing of nitrogen compounds from dead organisms: plant, animal, bacteria …etc. Some bacteria species and fungi are capable of changing organic nitrogen to ammonium NH_4. This process is called either mineralization or ammonification. Other kinds of bacteria such as *Nitrosomonas* alter ammonium to nitrite NO_2^- then other such as

Nitrobacter changes the nitrite to nitrate NO_3^- in a process termed nitrification. The last step, changing nitrite to nitrate, is very important process because accrued nitrite in the soil is toxic for plant growth. To complete the nitrogen cycle, *Pseudomonas* alters nitrate to form nitrogen gas with a process called de-nitrification. (Bothe et al., 2007; Lewis, 1986).

Modern agriculture relies on the use of fertilization as an important resource to supply plant nutrients to increase crop production. However, plants cannot efficiently utilize all the applied nitrogen fertilizer leaving large amounts to enter our water supplies. Run off from chemical fertilizers have negative environmental consequences known as eutrophication of water bodies which has lead to environmental disasters such as the infamous "dead zone" in the Gulf of Mexico (Adesemoye et al., 2009). In addition, not all nitrogen applications enhance plant growth. For example, It has been reported that nitrogen application has not shown significant effect on chickpea and Sudan grass shoot dry weights; whereas, it has had significant effect on rape (Marschner et al., 2001). As the result of increasing costs of mineral fertilizers and their probable harms to the environment (Hassen and Labuschagne, 2010; Salanturi et al., 2006), more research needs to be conducted to better understand the natural mechanisms whereby rhizobacteria interacts with plants to increase or decrease plant productivity through providing nutrients (Hossain and Martensson, 2008).

2.4. Pyrosequencing and Rhizosphere Community Characterization

A large number of methods are available to analyze the microbial community. Many of these are based on the sequencing of microbial community DNA (Colella et al., 2003). Pyrosequencing is a relatively new technique that is capable of sequencing a complex mixture of DNA fragments thereby making it a very attractive technique to characterize the microbial community (Ahmadian et al., 2000; Marsh, 2007). Pyrosequencing depends on the luminescent detection of released pyrophosphate through a process involving several enzymatic steps

(Ronaghi, 2001). Hyman is the first person that introduced the pyrosequencing of DNA in 1988 (Michael, 2005). In addition to single sequence base and whole genome sequence, the technology of pyrosequencing can sequence thousands of specific genes in mixed samples of DNA. This system of sequencing is used in most areas of research including: those involving animals, plants, and soils (Marsh, 2007). There are two kind of pyrosequencing: Solid phase pyrosequencing and liquid phase pyrosequencing.

The basic process of pyrosequencing includes DNA templates, primers for PCR amplification, substrates including: adenosine 5'–phosphosulfate and luciferin, and enzymes which includes DNA polymerase I, ATP sulfurylase, luciferase, and apyrase. Pyrosequencing enzymatic process starts with DNA polymerization by nucleotide incorporation enhanced by a DNA polymerase enzyme. PPi is released as result of nucleotide association. ATP sulfurylase converts released PPi to ATP by luciferase which then oxidizes luciferin resulting in the production of light. All of previous enzymatic reactions take from 3-4 seconds at room temperature. The solid phase pyrosequencing uses the previous three enzymes to sequence the DNA, but the liquid phase pyrosequencing adds the forth enzyme (apyrase) (Ronaghi, 2001) which degrades the added nucleotide that failed to incorporate in the DNA template (Salk et al., 2006).

The advantages of pyrosequencing is that it consumes less time and is much cheaper than Sanger sequencing and results in a huge number of sequences, up to 40 mega bases in a single experiment. Furthermore there is no need for bacterial colonies for cloning or restriction enzymes in order to obtain sequences from mixed samples. The results produce an average of 450 bp of sequence with relatively few errors. However, as any technique, pyrosequencing has some weaknesses, such as: a short reading frame sequences (450 base pairs) and an increasing error rate with greater sequence extension. With continued development, it is believed that this technique will continue to improve where reads of over 800 base pairs may be possible (Julia et al., 2007).

2.5. References

Abbasi M.K., Sharif S., Kazmi M., Sultan T., Aslam M., 2011. Isolation of plant growth promoting rhizobacteria from wheat rhizosphere and their effect on improving growth, yield and nutrient uptake of plants. Plant Biosystems 145:159-168.

Adesemoye A.O., Torbert H.A., Kloepper J.W., 2009. Plant Growth-Promoting Rhizobacteria Allow Reduced Application Rates of Chemical Fertilizers. Microbial Ecology 58:921-929.

Ahmadian A., Gharizadeh B., Gustafsson A.C., Sterky F., Nyren P., Uhlen M., Lundeberg J., 2000. Single-nucleotide polymorphism analysis by pyrosequencing. Analytical Biochemistry 280:103-110.

Akhtar M.J., Asghar H.N., Shahzad K., Arshad M., 2009. Role of plant growth promoting rhizobacteria applied in combination with compost and mineral fertilizers to improve growth and yield of wheat (*Triticum Aestivum L.*). Pakistan Journal of Botany 41:381-390.

Beneduzi A., Peres D., da Costa P.B., Zanettini M.H.B., Passaglia L.M.P., 2008. Genetic and phenotypic diversity of plant-growth-promoting *Bacilli* isolated from wheat fields in southern Brazil. Research in Microbiology 159:244-250.

Bothe H., Ferguson S.J., Newton W.E., 2007. Biology of the nitrogen cycle, Elsevier, Amsterdam ;Oxford. 427.

Canbolat M.Y., Bilen S., Cakmakci R., Sahin F., Aydin A., 2006. Effect of plant growth-promoting bacteria and soil compaction on barley seedling growth, nutrient uptake, soil properties and rhizosphere microflora. Biology and Fertility of Soils 42:350-357.

Canfield D., Kristensen E., Thamdrup B., 2005. The nitrogen cycle, Aquatic Geomicrobiology. 205-267.

Carlier E., Rovera M., Jaume A.R., Rosas S.B., 2008. Improvement of growth, under field conditions, of wheat inoculated with *Pseudomonas* chlororaphis subsp *aurantiaca* SR1. World Journal of Microbiology & Biotechnology 24:2653-2658.

Cavaglieri L., Orlando J., Etcheverry M., 2009. Rhizosphere microbial community structure at different maize plant growth stages and root locations. Microbiological Research 164:391-399.

Colella S., Shen L., Baggerly K.A., Issa J.P.J., Krahe R., 2003. Sensitive and quantitative universal Pyrosequencing (TM) methylation analysis of CpG sites. Biotechniques 35:146.

Compant S., Clement C., Sessitsch A., 2010. Plant growth-promoting bacteria in the rhizo- and endosphere of plants: Their role, colonization, mechanisms involved and prospects for utilization. Soil Biology & Biochemistry 42:669-678.

de Freitas J.R., 2000. Yield and N assimilation of winter wheat (*Triticum aestivum L., var. Norstar*) inoculated with rhizobacteria. Pedobiologia 44:97-104.

Doornbos R.F., van Loon L.C., Bakker P., 2012. Impact of root exudates and plant defense signaling on bacterial communities in the rhizosphere. A review. Agronomy for Sustainable Development 32:227-243.

Gaskins M.H., Albrecht S.L., Hubbell D.H., 1985. Rhizosphere bacteria and their use to increase plant productivity: a review. Agriculture Ecosystems & Environment 12:99-116.

Germida J.J., Walley F.L., 1996. Plant growth-promoting rhizobacteria alter rooting patterns and arbuscular mycorrhizal fungi colonization of field-grown spring wheat. Biology and Fertility of Soils 23:113-120.

Gholami A., Shahsavani S., Nezara S., 2009. The Effect of Plant Growth Promoting Rhizobacteria (PGPR) on Germination, Seedling Growth and Yield of Maize. . World Academy of Science, Engineering and Technolog 49.

Hasan H., 2004. Nitrogen Rosen Pub. Group, New York.

Hassen A.I., Labuschagne N., 2010. Root colonization and growth enhancement in wheat and tomato by rhizobacteria isolated from the rhizoplane of grasses. World Journal of Microbiology & Biotechnology 26:1837-1846.

Hayat R., Ali S., Amara U., Khalid R., Ahmed I., 2010. Soil beneficial bacteria and their role in plant growth promotion: a review. Annals of Microbiology 60:579-598.

Hinsinger P., Bengough A.G., Vetterlein D., Young I.M., 2009. Rhizosphere: biophysics, biogeochemistry and ecological relevance. Plant and Soil 321:117-152.

Hossain M.S., Martensson A., 2008. Potential use of Rhizobium spp. to improve fitness of non-nitrogen-fixing plants. Acta Agriculturae Scandinavica Section B-Soil and Plant Science 58:352-358.

Julia L., Peter A.L., Steve M., 2007. Gene cloning; principles and applications, Scitech Book News, Portland, United States, Portland.

Khan A.S., 2006. Methodology for Assessing Biodiversity.

Kirk J.L., Beaudette L.A., Hart M., Moutoglis P., Khironomos J.N., Lee H., Trevors J.T., 2004. Methods of studying soil microbial diversity. Journal of Microbiological Methods 58:169-188.

Lewis O.A.M., 1986. Plants and nitrogen, Edward Arnold, London. 104.

Liao P.C., Huang B.H., Huang S., 2007. Microbial community composition of the Danshui river estuary of northern Taiwan and the practicality of the phylogenetic method in microbial barcoding. Microbial Ecology 54:497-507.

Magurran A.E., 2004. Measuring biological diversity, Blackwell Pub., Malden, Ma. 256

Marschner P., Yang C.H., Lieberei R., Crowley D.E., 2001. Soil and plant specific effects on bacterial community composition in the rhizosphere. Soil Biology & Biochemistry 33:1437-1445.

Marsh S., 2007. Pyrosequencing protocols, Scitech Book News, Book News, Inc., Portland, United States, Portland.

Mia M.A.B., Shamsuddin Z.H., Wahab Z., Marziah M., 2010. Rhizobacteria as bioenhancer and biofertilizer for growth and yield of banana (*Musa spp. cv. 'Berangan'*). Scientia Horticulturae 126:80-87.

Michael L.M., 2005. Emerging technologies in DNA sequencing Genome Research 15:1767–1776.

Oswald J.S., 2007. Ecology and ecosystem conservation, Scitech Book News, Book News, Inc., Portland, United States, Portland.

Ozturk A., Caglar O., Sahin F., 2003. Yield response of wheat and barley to inoculation of plant growth promoting rhizobacteria at various levels of nitrogen fertilization. Journal of Plant Nutrition and Soil Science-Zeitschrift Fur Pflanzenernahrung Und Bodenkunde 166:262-266.

Ronaghi M., 2001. Pyrosequencing sheds light on DNA sequencing. Genome Research 11:3-11.

Salanturi A., Ozturk A., Akten S., 2006. Growth and yield response of spring wheat (*Triticum aestivum L.*) to inoculation with rhizobacteria. Plant Soil and Environment 52:111-118.

Salk J.J., Sanchez J.A., Pierce K.E., Rice J.E., Soares K.C., Wangh L.J., 2006. Direct amplification of single-stranded DNA for pyrosequencing using linear-after-the-exponential (LATE)-PCR. Analytical Biochemistry 353:124-132.

Schepers J.S., Raun W., 2008. Nitrogen in agricultural systems, American Society of Agronomy :Crop Science Society of America :Soil Science Society of America, Madison, Wis. 965.

Spellerberg I.F., Fedor P.J., 2003. A tribute to Claude Shannon (1916-2001) and a plea for more rigorous use of species richness, species diversity and the 'Shannon-Wiener' Index. Global Ecology and Biogeography 12:177-179.

Sudini H., Liles M.R., Arias C.R., Bowen K.L., Huettel R.N., 2011. Exploring Soil Bacterial Communities in Different Peanut-Cropping Sequences Using Multiple Molecular Approaches. Phytopathology 101:819-827.

Zahir Z.A., Arshad M., Frankenberger W.T., 2004. Plant growth promoting rhizobacteria: Applications and perspectives in agriculture. Advances in Agronomy, Vol 81 81:97-168.

CHAPTER III

THE EFFECT OF NITROGEN ON RHIZOBACTERIA ASSOCIATED WITH WHEAT SHOOT PRODUCTIVITY

ABSTRACT

Rhizobacteria are known to be closely associated with plant productivity. Our objectives in this study were to identify rhizobacteria associated with wheat shoot productivity with and without added urea nitrogen and characterize the richness and diversity for the rhizosphere community using the Shannon and Chao1 index. Wheat was grown for four cycles of planting and harvesting to condition the soil for the wheat associated microbial community. At the fourth cycle wheat plants were grown singly in homogenized and conditioned soil fertilized to 112 or 39.3-kg/ha N using urea as a nitrogen source and 96 plants per N treatment. After eight weeks of wheat growth, rhizosphere soil plus roots were extracted and grouped into two discrete categories based on shoot weights (low or high). Thus plants were categorized according to nitrogen treatment and productivity into four discrete classifications: high productivity with nitrogen, high productivity without nitrogen, low productivity with nitrogen and low productivity without nitrogen. DNA from each category was extracted directly from rhizosphere soil and pyrosequenced to quantify the community OTU (Operational Taxonomic Unit) composition based on the 16S rRNA gene. OTU classification was through the RDP II pyrosequencing pipeline. Shannon and Chao1 indices were used to analyze the diversity of the rhizobacterial community. OTUs most closely correlated both positively and negatively with productivity were

identified. A total of 49 significant OTUs were associated with wheat plants treated with nitrogen. *Gemmatimonadetes, Acidobacteria, Proteobacteria,* and *Bacteroidetes* were the most common phyla associated with wheat productivity when supplemented with nitrogen fertilizer. Of the bacterial groups evaluated, *Actinobacteria* was the most common phylum, and *Laceyella* #35239 and *Marmoricola* #46721 were the most significant OTUs associated with high biomass plant. The most significant OTUs associated with low biomass plants were *Firmicutes* #48486 *Actinobacteria* #38639, *Rhodospirillaceae* #50459, *Sporichthya* #51614, *Hyphomicrobiaceae* #50895, *Ensifer* #29687, and *Micromonosporaceae* #18302. These may represent deleterious rhizobacteria. Based on Chao1, rhizobacterial diversity was significantly greater in high biomass plants (P = 0.046) compared to low biomass plants. However, rhizobacteria diversity between nitrogen treatments was not significantly different according to Shannon and Chao1 indices. This work forms the basis of future studies to functionally characterize the rhizobacterial community with respect to nitrogen addition and shoot biomass productivity.

3.1. Introduction

Wheat is the most dominant crop in the world due to its importance for human caloric intake, nutrition, and food security (Carter, 2002; Hassen and Labuschagne, 2010). It is the main component for human nutrition in many regions around the world, and is very versatile in that wheat can be used as flour for bread, pasta, bagels, crackers, and cakes. In addition, it is rich in carbohydrates, which are important sources of energy for the human body. Due to the huge world demand for food and the increase in world population, much research has been conducted to increase food production in order to satisfy human needs around the world. Environmental, physiological, agricultural, and genetic studies have been utilized to increase crops productivity.

Chemical fertilization is one of most important agricultural practices that have significantly increased wheat productivity. However, fertilization can have negative environmental effects because a plant utilize only a portion of applied chemical fertilizer and the other portion remains as chemical residue in the soil or is leached off in the ground water (Adesemoye et al., 2009). Utilizing microbial symbiotic associations with wheat plants to increase nutrient efficiency, with minimum inputs, in order to increase wheat productivity, is a major focus of recent research.

The rhizosphere soil is one of the most microbial abundant and microbially active environments on the earth (Hayat et al., 2010; Hinsinger et al., 2009). It is fueled by root exudations (Hinsinger et al., 2009) stimulating selective microbial community whose function changes with development plant (Cavaglieri et al., 2009). Plant growth promoting rhizobacteria, which usually are found in rhizosphere soil (Bhromsiri and Bhromsiri, 2010; Hassen and Labuschagne, 2010), are bacteria that play a substantial part in increase plant productivity (Carlier et al., 2008; Upadhyay et al., 2009). These kinds of bacteria act to positively effect soil structure and increase organic matter content (Hayat et al., 2010). The effect of rhizobacteria on

plants is dependent upon soil characteristics, plant species, and plant genotypes (Hossain and Martensson, 2008).

Rhizobacteria are known to produce plant growth promoting substances. It was found that about 80% of isolated rhizosphere bacteria release indole acetic acid (IAA), which is a plant growth regulator. In addition, about 90% of rhizosphere microorganisms can produce cytokinins that contribute plant cell division (Hayat et al., 2010). Lipo-chito-oligosaccharides (LCOs), nodulation signals, that enhance seed germination, are excreted by *rhizobia* (Hossain and Martensson, 2008). Rhizobacteria can contribute to plant growth and soil fecundity by fixing atmospheric nitrogen or by producing antibiotics against pathogens to enhance plant growth (Akhtar et al., 2009; Hassen and Labuschagne, 2010; Hayat et al., 2010). Much research revealed clear positive effect of PGPR on growth and yield of many different crops (Abbasi et al., 2011) such as *Azospirillum* sp., which has the ability to fix atmospheric nitrogen and produce plant growth promoting hormones like gibberellins and auxins (Ozturk et al., 2003).

Plant growth promoting rhizobacteria have been used to increase the yield of crops in many situations. Several morphological and physiological traits such as nutrient uptake, plant biomass, leaf size, and N tissue content of crops could be enhanced by inoculating plants with *Azospirillum* sp. (Gholami et al., 2009). Plant growth promoting bacteria when inoculated into an agricultural system have been termed "biofertilizers". When phosphorus is added to soil as a soluble fertilizer, it quickly become immobilized. Whereas inoculating plants or soil with phosphate-solubilizing bacteria (PSB) such as *Bacillus* spp. was found very helpful in solubilizing fixed phosphorus increasing its plant availability. Thus, the abundance of rhizobacteria in the rhizosphere soil increase soil nutrient availability for plants (Canbolat et al., 2006). In this research we sought to characterize the rhizobacteria community in productive vs unproductive plants fertilized and not fertilized with nitrogen. Accordingly the specific objectives of this research were: **1-** Identify rhizobacteria associated with wheat shoot

productivity with and without added urea nitrogen. **2-** Characterize the richness and diversity for the whole rhizosphere community using the Shannon and Chao1 Index, respectively.

3.2. Materials and Methods

3.2.1. Soil and Soil Preconditioning

The experiment was conducted in a single Easpur Loam soil obtained from the Efau field with a history of wheat production located at OSU Experiment Station, in Payne County, Oklahoma. Available inorganic elements according to soil tests were P: 67 lbs/A, K: 300 lbs/A, OM: 2.06%, NO_3-N: 70 lbs/A and pH was 7.00. Plant growth was maintained in an environmentally controlled greenhouse facility on the OSU campus the spring of 2010. Hard red spring wheat seeds (*Triticum aestivum var Grandin*) were sown in homogenized soil, mixed for 20 minutes using a large cement mixer and divided into two groups with one group targeted to receive nitrogen and the other not. Prior to the experiment the soil was preconditioned with 3 cycles of wheat growth to Feekes stage 9 and harvest. Before planting each cycle, soil analysis was conducted by the Soil Water and Forage Analytical Laboratories at Oklahoma State University, Stillwater. At each cycle, 96 wheat plants were planted singly in 2.8 liter TPOT1 pots (Stuwe and Sons, Corvalis OR). Temperature in the greenhouse was set at 23 / 18 ^{0}C day/night during the growth season and varied less than 3 degrees from the set points. Water moisture was gravimetrically maintained between 15% and 36% soil water content using a Spectrum Technologies TDR 100 Field Scout soil moisture meter equipped with a 4.7 inch probes.

3.2.2. Wheat Fourth Cycle, Planting and Harvesting

Experimental wheat was planted on April 2nd 2010 for the fourth cycle at the same greenhouse facility under the same conditions described above. Nitrogen was added in the form of urea to the treated soil prior to planting before mixing. Available inorganic elements before planting for the un-fertilized treatment were NO_3-N: 39.3-kg/ha, P: 68 lbs/A, K: 297 lbs/A, and

pH was at 7.1, and for fertilized treatment NO_3-N: 94 lbs/A, P: 65 lbs/A, K: 304 lbs/A, and pH was 7.1. As a result, nitrogen level was adjusted in the nitrogen treated pots by adding Urea nitrogen to 112-kg/ha nitrogen as a solution to each planted pots.

Plants were harvested on May; 22nd 2010 when the plants were at Feekes stage 9 and both fresh and dry shoot weights were determined. Roots were carefully removed from the soil, and the non-rhizosphere soil was discarded by three consistent shakes of the root system. The roots with clinging soil were added to 25 ml of 100 mM pyrophosphate with 0.1% SDS (sodium dodecyl sulfate) and shaken at 250 rpm for 15 min. After shaking the roots were removed and the rhizosphere soil extracts were stored at -80 °C. Rhizosphere soil samples were categorized according to the fresh weights of their corresponding shoots from high biomass (upper third) to low biomass (lower third) for each nitrogen treatment. Thus, rhizosphere soil samples were divided into four categories: high biomass plants treated with nitrogen (HN), low biomass plants treated with nitrogen (LN), untreated high biomass plants (HNN), and untreated low biomass plants (LNN). Each category was composed of four replicates for a total of 16 experimental units, with 10-11 different rhizospheres per experimental unit (2 fertilizer treatments x 2 productivity levels x 4 replications= 16 experimental units). The available inorganic elements in the soil after harvesting the plants for un-fertilized treatment were NO_3-N: 6 lbs/A, P: 64lbs/A, K: 226 lbs/A, and pH was 7.3; and for fertilized treatment were NO_3-N 45 lbs/A, P: 59 lbs/A, K: 226 lbs/A, and and pH was 7.2.

3.2.3. DNA Extraction Procedure

DNA extraction from each extract for each experimental unit using a protocol developed by Dr. Anderson (personal communication). We found that the DNA extracted using this protocol was of much higher quality and quantity than DNA that extracted by commercial Mobio kit (data not shown). Rhizosphere soil was rapidly thawed in a 37 C^0 water bath. A volume of rhizosphere

soil solution containing 500 mg of suspended soil was loaded into a bead beating tube and the tube centrifuged at 11,000g for 2 minutes to pellet the soil and bacteria. The supernatant was discarded and 1 ml of TENS buffer (Tris, EDTA, NaOH, and SDS) added to the tube. The tube was bead-beat at 2,500 rpm briefly to re-suspend the pellet and the soil pelleted by centrifugation at 10,000g for 2 min. The pellet was rinsed and centrifuged with TENS buffer one more time. To the pellet, 0.74 g of a 50:50 mixture of 0.5 and 0.1 mm zirconium beads was added with 600 µl of extraction buffer (EB), and 450 µl of chloroform/isoamyl alcohol (CI, 24:1). The tube was bead-beated at 5000 rpm for 1 minute, then placed on ice and bead beated two more times with ice incubation in between. The tube was centrifuged at 10,000 g for 5 minutes and the supernatant removed to a new tube. To this tube 0.5 volumes of 7.5 M ammonium acetate was added, mixed completely, and placed on ice for 5 minutes to precipitate unwanted contaminants. After 5 minutes the tube was centrifuged at 14,000 g for 5 minutes and the supernatant removed to a new 2 ml tube. An equal volume of phenol/chloroform/isoamyl (PCI, 24:25:1) alcohol was added to the tube, the lid was tightly closed and the tube was mixed for 5 minutes end over end. The tube was centrifuged at 14,000 g for 2 minutes and the upper phase transferred to a new 2 ml tube. An equal volume of isopropyl alcohol was added to the tube, then placed on ice for 5 minutes and centrifuged at 14,000 g for 5 minutes. The supernatant was discarded and the alcohol was allowed to evaporate for 15 minutes. The pellet was re-suspended in 100 ml of 100 mM TRIS buffer pH 8.0 and loaded directly on top of a prepared Sephacryl 300 HR spin column. The column was centrifuged at 3,000 g for 20 seconds and the flow through was saved. The flow through was loaded on top of a prepared DEAE column and centrifuged at 3,000 g for 20 seconds. The column was reloaded and centrifuged again at 3,000 g. The column was washed with 500 µl of wash buffer twice with 3,000 g centrifugation in between. A total of 500 µl of elution Buffer was added to the top of the column and centrifuged at 3,000 g for 20 seconds. To the DEAE eluant, 500 µl of isopropyl alcohol was added and then placed on ice, centrifuged at 14,000 g for 5 minutes, and the supernatant was discarded. The pellet was rinsed with 70% ethanol, centrifuged at 14,000 g

for 5 min. The supernatant was discarded and drained on a paper towel for 15 minutes. The pellet was re-suspended in 50 µl of 100 µl of 100 mM TRIS pH 8.0. The procedure gave high yield and very good purity as determined by the Nano-drop spectrophotometer (Thermo Scientific, Delaware) based upon the 260nm/280nm absorbance ratios, which averaged 1.80, and above. In addition, DNA quantity averaged 48.08 ng/ µl (Appendix 6).

3.2.4. Quality Characterization of DNA

PCR was performed on DNA extracted from the rhizosphere soil to assess quality of the DNA and specificity of the PCR process using 27 F and 519R primers (Intigrated DNA technologies, IDT, Coralville Iowa), that amplify a specific fragment of the 16S rRNA gene. The content of PCR tubes was 9.2 µl water, 250 nM 27F, 250 nM 519R primer, 2 µl 10X buffer, 250 mM dNTP, 2 mM MgCl2, and one µl of extracted DNA. PCR was achieved at 94°C for 1 min, 50°C for 40 sec, and 72°C for 2 min for 35 cycles. PCR product was analyzed using an agarose electrophoresis gel to insure that the 16S rRNAs were amplified (Fig.1).

3.2.5. Pyrosequencing and Analysis of Sequences

After DNA was extracted and assessed for quality and quantity, the DNA samples were submitted to ResearchTesting Laboratories, Lubbuck TX for pyrosequencing. Returned sequences were checked for quality using the quality checking software by the ResearchTesting Laboratories and the RDP II pipeline, aligned and clustered at a 1.0% dissimilarity to define the basic phylogenetic unit for comparison using the RDP II pyrosequencing functions. Sequences were identified for their experimental unit of origin based on unique bar coded primer at the beginning of each sequence. The numbers of sequences for each OTU was determined in an Excel spreadsheet. Two factors analysis of variance were used to identify OTUs that varied statistically with respect to productivity level and nitrogen addition based on a p value less than

0.05. OTUs were ranked according to response to productivity and nitrogen based on the lowest p values.

3.2.6. Community Analyses

A total of 11667 sequences per experimental unit were selected randomly to carry out ecological indices analysis (Shannon, and Chao1) in order to assess the diversity, abundance and richness of the rhizobacterial community in each treatment. In the current study, bacterial diversity was estimated by using the Shannon index and Chao1 using the RDP II pyrosequencing community analysis functions (http://pyro.cme.msu.edu/). Two factor analysis of variance (ANOVA) using the SYSTAT version 10.2 software functions (SYSTAT, Chicago IL) were used to identify significances between the microbial diversity indexes in both treatments for each index with four replications per treatment.

3.3. Results

3.3.1. Plant Growth Parameters and Treatments

Plants were grown in homogenized field soil with and without nitrogen to Feekes stage 9. Upon harvest, plants were categorized into low biomass and high biomass categories according to their shoot fresh weights. From Table 2 results revealed that high shoot biomass plants under nitrogen treatment had greater average shoot fresh weights at 7.93 g than those under no nitrogen treatment at 6.30 g. The average of high biomass plants showed 60.5% greater shoot biomass than the low biomass plants under fertilized nitrogen condition and 43.2% greater under unfertilized nitrogen condition. The average dry weight for high biomass plants under nitrogen treatment was at 2.05 g per plant and 1.65 g under no nitrogen treatment. Similar indicators were found in total root fresh weight in both treatments. Where the average total root fresh weight of high biomass plants was 22.45 g under nitrogen treatment and it was 17.33 g under no nitrogen treatment. In addition, average number of spikes of high biomass plants was also greater in nitrogen treatment

showing a 1.09 fold increase than in no nitrogen treatment. Significant differences were observed in the shoot biomass between plants that have high shoot biomass treated with nitrogen and those untreated indicating the importance of nitrogen application to increase wheat shoot biomass. However this difference was not significant in the low shoot biomass plants between nitrogen treated and untreated wheat. A histogram of the distribution of shoot dry weight and the number of plants in both treatments is presented in Figure 2. The high productivity categorized plants treated with nitrogen had shoot dry weights greater than 2.0 g to a maximum of 2.4 g while those untreated with nitrogen were greater than 1.7 g to 2.1 g. The low productivity categorized plants treated with nitrogen ranged from 0.6 g to 1.5 g, while the untreated plants ranged from 0.5 to 1.3 g. The greatest numbers of plants were found in the medium biomass category treated with nitrogen at 1.8 g while for untreated with nitrogen was at 1.6 g shoot dry weights. The overall distribution of plant biomass was shifted towards higher productivity compared to the no nitrogen treatment resulting in an overall increased overall population variance. The lower biomasses were distributed over a wider range of biomass dry weights than are the other categories.

3.3.2. Sequence Numbers

DNA from the four respective treatments with four replications per treatment were submitted to the ResearchTesting Laboratories for pyrosequencing. Pyrosequencing yielded 276,124 original sequences, which after quality screening was reduced to 264,981 sequences. These were aligned and clustered by the RDP II pyrosequencing pipeline (http://pyro.cme.msu.edu/) resulting in 147,822 clusters. Of these clusters 554 had more than 40 sequences. Our results were based on the least square analysis of OTUs with more than 40 total overall sequences across all experimental units. Representative isolates from each cluster were determined using the RDP II dereplication tool in the RDP II classifier. Numbers of OTUs were determined in each treatment group. The highest total number of OTUs was associated with low shoot biomass plants grown under unfertilized condition (Table 2). Two factors analysis of

variance was used to identify specifically those treatments that yielded greater numbers of OTUs in our analysis. Statistical analysis indicated that nitrogen addition significantly decreased number of OTUs by an average of 3.9% overall. High productivity plants tended to have slightly fewer OTUs than low productivity plants, but the differences were not significant.

3.3.3. Significant Nitrogen Associated OTUs

Examining the effect of nitrogen application regardless of the interaction between nitrogen and productivity, 49 of significant operational taxonomic units (OTUs) were found associated with wheat plants treated with 112-kg/ha total soil nitrogen (Table 3). Most of these OTUs were from *Actinobacteria* (17), *and Proteobacteria* (17). Other phyla showing multiple OTUs when treated with nitrogen included: *Acidobacteria* (4) and *Firmicutes* (4), *Gemmatimonadetes* (3), and *Chloroflexi* (3). OTUs from *Devosia* #33420 *Gemmatimonas* #16482, *Flavisolibacter #152, Tumebacillus #46565, Solirubrobacter #37889, Catelliglobosispora #16128, Firmicutes #48486, Nannocystis #4401, Bradyrhizobiaceae #34864*, and *Promicromonosporaceae #18715* corresponded with the highest total number of sequence with over 100 sequences each. Moreover, 12 OTU of 49 OTUs showed significant relationship not only with nitrogen application but also with productivity as well, including: *Rhodospirillaceae* #50459, *Hyphomicrobiaceae* #50895, *Marmoricola* #46721, *Micromonosporaceae* #18302, to name a few. Nineteen of the OTUs listed in Table 3 were more associated with plants with high shoot biomass, such as: *Marmoricola* #46721, *Acidobacteria Gp16* #50877, *Actinomycetales* #53107, *Shinella* # 8843.*etc*. Nine of these OTUs associated with high shoot biomass were from *Actinobacteria* phylum. Thirty OTUs were significantly associated with low shoot biomass when plants were grown under fertilized nitrogen condition such as *Flavisolibacter* #152 , *Nannocystis* #4401, *Proteobacteria* #5469 , *Hyphomicrobiaceae* #50895 , *Myxococcales* #11488 , *Streptomyces* #13019 , *Devosia* #33420 , *and Rhizobium* #40165 ……etc. Most of these OTUs were tied to the phylum *Proteobacteria.* If we take the interaction

between productivity and nitrogen into consideration, some of the OTUs in this treatment showed significant association under nitrogen application interaction with low productivity, such as: *Flavisolibacter* #152, *Rhodospirillaceae* #50459, *and Hyphomicrobiaceae* #50895 while *Rhizobiales* #18245 interacted with high productivity (Table 3).

In spite of the significance of nitrogen application, four OTUs showed significantly greater number of sequences (p value < 0.05) with wheat plants when nitrogen was not applied and soil test values indicated 39.3-kg/ha soil total nitrogen. While all four showed greater numbers of sequences when nitrogen was not applied, neither of them showed significant greater sequence numbers with respect to productivity, but *Pseudonocardia #50310* and *Rhizobiales #36931* were very close in this regard. Of these four, two were from phyla *Actinobacteria* and *Proteobacteria* (Table 4).

3.3.4. Significant High Productivity OTUs

Examining OTUs based on plant shoot biomass productivity differences, 20 OTUs were significantly associated with plants that had high shoot biomass (Table 5), and 21 OTUs were significantly associated with plants that had low shoot biomass (Table 6). From Table 5, it is observed that *Actinobacteria* (11) was the most common phylum associated with high productivity in wheat plants. The most significant OTUs in this category are *Laceyella* #35239 and *Marmoricola* #46721**.** It was found that OTUs, *Marmoricola* #46721, *Acidobacteria Gp16* #50877, *Shinella* #8843, *Actinomycetales* #53107, and *Rhizobium* #48780, were significantly associated with wheat plants that have high shoot biomass regardless if nitrogen was applied or not. *Actinobacteria*, *Planctomycetes* and *Proteobacteria* phyla have shown the highest abundance of sequences in among all the other phyla in the high productivity category. The interaction between nitrogen and productivity showed that *Rubrobacter* #5983 has the significant associate with high productivity and nitrogen interaction.

3.3.5. Significant Low Productivity OTUs

When the OTUs associated with low shoot biomass were classified, it was found that the majority of the phyla in this group were from *Alphaproteobacteria* (9). The most significant OTUs within this classification were *Actinobacteria* #38639, *Rhodospirillaceae* #50459, *Sporichthya* #51614, *Hyphomicrobiaceae* #50895, *Ensifer* #29687, and *Micromonosporaceae* #18302. *OTUs Devosia* #33420, *Rhodospirillaceae* #50459, *Bradyrhizobiaceae* #34684, and *Hyphomicrobiaceae* #50895 showed significant association with both nitrogen and productivity. Despite nitrogen application, it was found that phyla of *Actinobacteria, Proteobacteria, Firmicutes,* and *Chloroflexi* were associated with low wheat shoot biomass. *Firmicutes,* and *Proteobacteria* phyla represented the highest number of total sequence among the entire group. The OTU *Rhodospirillaceae* #50459 was significant with nitrogen and productivity interaction (Table 6).

3.3.6. Nitrogen and Productivity Interaction

Based on the interaction between productivity and nitrogen, 35 OTUs were found significantly associated with wheat plants responding to nitrogen application regardless of wheat biomass productivity. Equal numbers of OTUs (14) were associated with high and low shoot biomass regardless of nitrogen application. A total number of 6 OTUs corresponded with low wheat shoot biomass whereas 5 OTUs corresponded with high wheat shoot biomass when nitrogen was applied (Fig: 3). Four OTUs associated with wheat when nitrogen did not applied.

3.3.7. Community Analysis

There was no significant difference in the microbial diversity as determined by the Shannon Index among plants grown under fertilized and unfertilized condition (P = 0.147). However, the interaction between nitrogen application and productivity was significant (0.02). Same as nitrogen treatment, productivity had no significant affect on microbial diversity among

all treatments: although low and high productivity showed a nearly significant P value of 0.064. Nevertheless, plants that have low shoot biomass under unfertilized condition had the lowest associated OTUs diversity with an average of 8.30 (fig: 4) as measured by the Shannon Index in the exponential form. In addition, plants that have high shoot biomass grown under unfertilized condition showed the highest level of diversity with an average of 4675.

Nitrogen application has no significant affect on the microbial diversity associated with wheat plants ($P = 0.21$) as measure by the Chao1 index. With Chao1 index, plants that have high shoot biomass have the highest microbial richness than those have low shoot biomass. On the other hand, there was a significant effect of productivity ($P = 0.046$) on the ecological diversity as measured by the Chao1 index. In addition, the interaction between nitrogen and productivity was significant (0.01). Plants that have high shoot biomass grown under unfertilized condition have the greatest number of bacterial species (fig: 4), while plants with low shoot biomass grown under unfertilized conditions have the lowest ecological diversity as measured by the Chao1 index.

3.4. Discussion

The community structure of microbial population in rhizosphere soil is strongly affected by both environmental factors surrounding the root, such as: temperature, water, nutrient availability, as well as the kind of soil, and plant species (Wu et al., 2011). Similar to what Ozturk et al 2003 findings, our results showed that the average of both shoot fresh and dry weight and root fresh weight of wheat plants grown under fertilized condition were higher than those of plants grown under unfertilized condition by 9.2%, 6.9%, and 9.1 respectively (Table 2). In addition, the average number of stems and spikes in current study positively affected by urea nitrogen application were higher by 7.1% and 4.3%, respectively, than those under unfertilized condition (Table 2). Similarly Ten Eyck 1914 indicated that fertilized soil positively increased wheat yield than unfertilized soil. The previous results indicate that nitrogen is a very important nutrient and has clear positive effect on increasing crop growth and production (Adesemoye et al., 2009; Akhtar et al., 2009).

Many techniques are available to isolate and classify bacteria from the soil (Durbin, 1961) such as: screening bacterial isolates cultured on growth medium for particular characteristics, DGGE (Denaturing Gradient Gel Electrophoresis), FRACS (full ribosomal amplification, cloning and Sanger sequencing), and PRADS (Sanger sequencing) (Dowd et al., 2008). However, it has been known for some time that culture-based techniques are not able to identify the vast majority of the microbial community (Duineveld et al., 2001; Durbin, 1961; Maria, 2007). Estimates of the fraction available to cultural techniques vary from 0.1 to 10 % of total soil bacteria (Duineveld et al., 2001). Often those that are able to be cultured are the fast growers that respond to high nutrient concentrations in the typical laboratory media (Durbin, 1961). In contrast to cultural techniques, microbial diversity and the relative abundance of species can be much more broadly quantified using molecular methods. Molecular methods are considered to be very effective in identifying and characterize microorganisms under a range of environmental conditions (Maria,

2007). DGGE as one molecular based method, has been used to examine the effect of seasonal variations and environmental perturbations on the microbial community (Pintado et al., 2003). However, DGGE has some problems, as well, where uneven temperature distribution within DGGE gels has been shown to cause multiple heteroduplexbands. Furthermore DGGE is known to reveal only the most abundant organisms within the microbial community population (Ward et al., 1998). Another challenge with using DGGE molecular method is that multiple species can be represented in the same band (Temmerman et al., 2004). While DGGE and other electrophoretic methods are effective in certain situations further advances in technology have provided additional methodologies for microbial community analysis. Pyrosequencing is another relatively new technique that is capable of sequencing a complex mixture of DNA fragments (Ahmadian et al., 2000; Marsh, 2007). Pyrosequencing has been effective in whole genome sequencing of small microbial genomes of *Mycoplasma gemialium* (580,069 bases) with a 99.96% accuracy. Furthermore, pyrosequencing saves time and expense compared to the traditional Sanger sequencing (Domnita, 2010; Jonathan, 2009). Compare to DGGE pyrosequencing is capable of sampling the microbial community to a much greater depth. Pyrosequencing of microbial communities is rapidly becoming the method of choice for microbial ecologists. In the current study, pyrosequencing was used to identify rhizobacteria associated with wheat productivity. In this study pyrosequencing technique was effective in identifying rhizobacteria associated with nitrogen fertilization associated with plant productivity.

Higher number of significant OTUs associated with wheat plants grown under fertilized condition were observed corresponding with higher productivity similar to what Ozturk et al 2003 were found when they combined *A. brasilense* Sp246 with different levels of nitrogen fertilizers, suggesting the great impact of fertilization practices on soil bacterial community abundance and composition. In current study, most of the OTUs associated with wheat plants treated with nitrogen were from *Actinobacteria* (17) and *Proteobacteria* (17) phyla. In addition, *Gemmatimonadetes, Acidobacteria, Proteobacteria,* and *Bacteroidetes* were found the most

common phyla that have the highest significant relationship. Shen et al 2010 found the same common phyla in addition to others when they investigated the effect of long-term applied fertilizations (inorganic N, organic manure, and half of mixed inorganic N and organic manure in addition to control) on the native soil bacterial community indicating the great effect of nitrogen fertilization on bacterial community. The same results were found by Zhang et al 2011 concerning the phyla *Actinobacteria, Proteobacteria Gemmatimonadetes, Acidobacteria,* and *Bacteroidetes* in addition to other phyla in cotton rhizosphere that leads them to conclude that these phyla are common to the rhizosphere regardless if nitrogen fertilizers where applied or not. Our results showed that most of the OTUs that associated with high shoot biomass wheat plants were contained within the *Actinobacteria* phyla, and that those associated with low shoot biomass wheat plants were contained within the *Alphaproteobacteria* phyla.

Firmicutes were found only in soil surrounding diseased cotton plants (Zhang et al., 2011). In the current study, *Firmicutes* #48486 was found significantly associated with plants that have low shoot biomass, and also had a significant relationship with productivity and nitrogen at the same time, indicating that *Firmicutes* phylum may contain many deleterious rhizobacteria in wheat plants. In addition, other OTUs such as *Nannocystis* #4401, *Proteobacteria* #40787, *Hyphomicrobiaceae* #10116, *Devosia* #33420, *and Rhizobium* #40165 were significantly associated with low shoot biomass under fertilized condition in present study. *Flavisolibacter* #152 was found to be one of the most significant OTUs associated with low biomass wheat grown under fertilized condition indicating that *Flavisolibacter* could be deleterious bacteria for wheat growth. In addition the interaction between productivity and nitrogen for *Flavisolibacter* was significant (0.026) concluding that nitrogen fertilizer might be an enhancer of *Flavisolibacter* effect on low biomass wheat. Although there is no mention in the literature of the deleterious role of *Flavisolibacter*, this genera was found in the rhizosphere soil of Ephrates poplar (Wang et al., 2011). *Acidobacteria Gp10* #20779, which belongs to *Acidobacteria* phyla, was found as one of

the most significant OTUs associated with low biomass wheat plants treated with nitrogen fertilizer. In contrast, Lee et al 2008 found that more than 50 % of the sequences isolated from chestnut tree rhizosphere were related to *Acidobacteria* phyla concluding that *Acidobacteria* phylum might positively contribute in some biochemical pathways in rhizosphere soil. Both OTUs: *Rhodospirillaceae* #50459 and *Hyphomicrobiaceae* #50895, which belong to *Proteobacteria* phylum, were some of the most significant OTUs that associated with low biomass wheat plants suggesting that these phyla contain a number of deleterious bacteria. Similarly, Lu et al 2006 found that some of the abundant bacteria (*Rhodospirillaceae* and *Hyphomicrobiaceae)* that inhibited the growth of root rice belonged to *Alphaproteobacteria* phylum. It is concluded that many OTUs from this phyla characterized in this study might have deleterious rhizobacteria with respect to wheat growth.

Our results indicated that *Bacillus* #11320 showed significant association with wheat plants having high shoot biomass under fertilized condition. Similarly, Mia et al 2010 found that the shoot and root growth of Bananas plant (*Musa* spp. cv. Berangan) increased when inoculated with *Bacillus.* sp with the addition of 33% N, which indicates that the inoculation with *Bacillus* is an enhancer of plant growth. In addition, Hassen and Labuschagne (2010) got the same positive result (increasing shoot and root weight) after single inoculating wheat and tomato plants with *Bacillus simplex, Bacillus megaterium,* and *Bacillus cereus. sp*. Thus our results in addition to those of others suggest that *Bacillus sp* are associated with plant growth promotion. OTUs: *Marmoricola* #46721, *Acidobacteria Gp16* #50877, *Shinella* #8843, *Actinomycetales* #53107, and *Rhizobium* #48780 were associated with high biomass wheat plants and significant with both treatments (nitrogen and productivity), which suggest that these also are plant growth promoters. For example, *Rhizobium* can fix atmospheric nitrogen, which makes additional nitrogen available for plant uptake (Dastager et al., 2010). In addition, *Planctomyces* was significantly associated with high biomass wheat plants in the absence of nitrogen, indicating that this OTU might also be

a beneficial rhizobacteria for wheat plant growth. Furthermore, *Gemmatimonas* #16482 was one of the most significant OTUs that associated with high biomass wheat plants grown under fertilized condition, which could indicate that this particular OTU could be a significant PGPR. The same genera were found to be abundant in sludge samples taken from Dongting Lake influenced by the nitrogen-fertilizer residues. Furthermore, they found that the nitrogen and phosphorus content in polluted samples were 1.9 and 1.47 respectively times more comparing with the control samples (Yang et al., 2012). In addition to *Gemmatimonas, Rhizobiales* #18245 was one of the most significant OTUs associated with high biomass wheat plants treated with N fertilizer. Symbiotic *Rhizobiales* members were found to have a simulation effect on RpoN-dependent gene for nitrogen fixation expression (Dombrecht et al., 2002). In addition, RodriguesCoelho et al 2008 found that 26 % of the *nifH* sequences, which isolated from sorghum rhizosphere treated with different levels of N fertilization, was related to *Rhizobiales* order. From our data and from the literature it appears that that many *Rhizobiales* could be classified as PGPR. *Pseudonocardia* #50310 was another significant OTU that associated with high biomass wheat plants untreated with N fertilizer. Espana et al 2011 found that *Pseudonocardia* was one of the dominant bacteria in the soil treated with maize residues, concluding that *Pseudonocardia* can contribute decomposing organic matter and increase nutrient availability in the soil. *Laceyella* #35239 is another significant OTUs associated with high biomass wheat plants (could be PGPR). Carrillo et al 2009 found that *Laceyella* was the most abundant Alkalithermophilic actinomycetes among other bacteria when it was isolated from living sugar cane plants, renewal rhizospheres, and residual leaves concluding that *Laceyella* could play an important role decomposing the plant residues and increasing nutrients availability in soil rhizosphere. Applying Shannon and Chao1 indices has shown non-significant differences in bacterial diversity among all treatments and their experimental units except the significance for the Chao1 index for productivity (P = 0.046). Dissimilarly Crecchio et al 2007 found that there was significant effect of N fertilization on bacterial community associated with wheat plant compared to crop residue management which

had no effect on the diversity of bacteria. Similar to Lupwayi et al 2010 when they indicated that the application of chemical fertilization has no influence on microbial communities. However the interaction between productivity and nitrogen was significant with Shannon and Chao1 indices. Over all, both indices indicated that low biomass plants grown under unfertilized condition have the lowest bacterial diversity, which might indicate that N fertilizer application increases the bacterial diversity. On the other hand, both indices showed that high biomass plants grown under unfertilized condition have the highest bacterial diversity that indicates the essential effect of bacterial diversity to enhance wheat growth. It is likely that under unfertilized conditions the rhizosphere community is enriched with rhizobacteria associated with nitrogen nutrition. In spite of the lack of observed effect on productivity, nitrogen urea application was shown to positively influence the diversity of the bacterial community associated with wheat plants. The fact that this effect appeared with the Chao1 index but not the Shannon index suggests that the changes in diversity are associated with the rarer members of the community. Similarly, Shen et al 2011 found that the abundance of ammonia-oxidizing bacteria (AOB) in semiarid temperate grassland was increased by N fertilizer application.

3.5. Conclusion

In conclusion, the present study reveals the effect of nitrogen fertilization on rhizobacteria associated with wheat shoot productivity. Our study showed greater wheat biomass production under fertilized than unfertilized conditions. In addition, this study showed that there was higher number of significant OTUs associated with wheat plants grown under fertilized condition rather than those grown under unfertilized condition, which indicates the important of nitrogen fertilization to improve rhizobacteria community structure associated with wheat productivity. At a very specific level the study identifies specific OTUs associated with high and low productivity and with and without nitrogen additions. Isolation and characterization of these specific OTUs may provide more information concerning their role in enhancing productivity under differing levels of fertility. Moreover, further experiments can be conducted on representatives of the phyla that showed significant effect including testing them after isolation for plant growth promotion capabilities. Researches need to be conducted in order to investigate the influence of a range of nitrogen concentration and its impact on wheat productivity and the corresponding changes in the rhizobacterial communities. This study was conducted under only one soil system and near optimal environment and therefore future studies under a variety of soils, environmental and cultural systems are needed to better establish generalized conclusions associated with nitrogen addition and productivity. This work also uncovered a number of OTUs that showed deleterious effects. Further research should be applied to investigate the possible harmful effects associates with these specific OTUs and members of deleterious dominated phyla. Finally much research need to be done to develop specific microbial symbiotic associations with wheat plants to increase nutrients efficiency as a supplement to chemical fertilization, in order to increase wheat productivity and economy.

Tables

Table 1. Wheat production in the United State from 2008–2011. http://www.ers.usda.gov/data/wheat/YBtable01.as

Year	Planted acreage	Harvested (million acres)	Production (million bushels)	Yield (bu/acre)
2008/09	63.19	55.7	2,499.16	44.9
2009/10	59.17	49.89	2,218.06	44.5
2010/11	53.59	47.62	2,206.92	46.3
2011/12	54.41	45.71	1,999.35	43.7

Table 2. Growth parameters and the number of OTUs per treatment.

	Treatments				*p value*	
Parameter	HN[a]	LN[b]	HNN[c]	LNN[d]	N[e]	Prod[f]
Average Shoot Biomass (fwt g)	7.93 ± 0.66	4.94 ± 0.90	6.30± 0.32	4.40± 0.70	<0.001	<0.001
Average of Shoot Dry Weight (g)	2.05 ± 0.19	1.24 ± 0.31	1.65± 0.17	1.21± 0.30	<0.001	<0.001
Average of Root fresh weight (g)	22.45 ± 9.16	14.81 ± 6.27	17.33± 6.16	13.72± 7.51	0.017	<0.001
Average Number of Stem	2.15 ± 0.57	1.52 ± 0.57	1.97±0.47	1.21±0.42	0.007	<0.001
Average Number of Spikes	1.82 ± 0.47	1.12 ± 0.33	1.67±0.48	1.03±0.17	0.071	<0.001
Number of OTUs/Treatment	1830 ± 16.0	1894 ± 21.7	1922±11.1	1954±13.9	<0.001	0.150

[a] Average + standard deviation for plants with high shoot biomass under N fertilized condition

[b] Average + standard deviation for plants with low shoot biomass under N fertilized condition

[c] Average + standard deviation for plants with high shoot biomass under unfertilized condition

[d] Average + standard deviation for plants with low shoot biomass under unfertilized condition

[e] P value for nitrogen treatment

[f] P value for productivity

Table 3. OTUs significantly increased with nitrogen application.

	Classification		*Number of Sequences*					*P Value*			*Interactions*
Cluster ID	OTU[a]	Phylum	Total	H[b]	L[c]	N[d]	NN[e]	N[f]	Prod[g]	PxN[h]	
152	*Flavisolibacter*	*Bacteroidetes*	196	95	101	137	59	<0.001	0.710	0.026	N INxLP
16482	*Gemmatimonas*	*Gemmatimonadetes*	214	110	104	147	67	<0.001	0.625	1.000	N
18245	*Rhizobiales*	*Proteobacteria*	52	29	23	41	11	<0.001	0.305	0.004	N INxHP
20779	*Gp10*	*Acidobacteria*	86	34	52	72	14	<0.001	0.081	0.081	N
19849	*Pseudonocardia*	*Actinobacteria*	42	19	23	37	5	0.001	0.596	0.430	N
27614	*Gp10*	*Acidobacteria*	70	33	37	48	22	0.001	0.497	0.187	N
14580	*Chloroflexi*	*Chloroflexi*	75	43	32	51	24	0.002	0.124	0.314	N
44304	*Gemmatimonas*	*Gemmatimonadetes*	54	19	35	45	9	0.002	0.100	0.145	N
50547	*Streptomyces*	*Actinobacteria*	59	36	23	47	12	0.002	0.161	0.576	N
34684	*Bradyrhizobiaceae*	*Proteobacteria*	119	47	72	81	38	0.003	0.049	0.931	LP x N
40226	*Acrocarpospora*	*Actinobacteria*	43	23	20	30	13	0.003	0.531	0.531	N
50895	*Hyphomicrobiaceae*	*Proteobacteria*	46	12	34	36	10	0.003	0.007	0.063	LP x N
50459	*Rhodospirillaceae*	*Proteobacteria*	79	7	72	65	14	0.004	0.001	0.005	LP x N
5469	*Proteobacteria*	*Proteobacteria*	49	23	26	40	9	0.005	0.745	0.589	N
31099	*Rhizobiales*	*Proteobacteria*	46	29	17	36	10	0.006	0.145	0.319	N
33420	*Devosia*	*Proteobacteria*	364	122	242	263	101	0.006	0.029	0.602	LP x N
46565	*Tumebacillus*	*Firmicutes*	175	81	94	109	66	0.007	0.350	0.350	N
46721	*Marmoricola*	*Actinobacteria*	48	33	15	33	15	0.007	0.007	1.000	HP x N
37076	*Solirubrobacterales*	*Actinobacteria*	49	21	28	36	13	0.009	0.367	0.251	N
11320	*Bacillus*	*Firmicutes*	42	25	17	28	14	0.011	0.114	0.410	N
13019	*Streptomyces*	*Actinobacteria*	45	20	25	32	13	0.011	0.443	0.642	N
4401	*Nannocystis*	*Proteobacteria*	150	48	102	115	35	0.014	0.075	1.000	N
5034	*Chloroflexi*	*Chloroflexi*	70	35	35	48	22	0.014	1.000	0.394	N
50877	*Gp16*	*Acidobacteria*	57	39	18	40	17	0.014	0.022	0.400	HP x N

40165	*Rhizobium*	*Proteobacteria*	40	12	28	32	8	0.016	0.086	0.186	N
18302	*Micromonosporaceae*	*Actinobacteria*	98	33	65	63	35	0.017	0.008	0.846	LP x N
14189	*Azospirillum*	*Proteobacteria*	41	23	18	26	15	0.020	0.249	0.812	N
40787	*Proteobacteria*	*Proteobacteria*	68	26	42	52	16	0.021	0.263	0.774	N
8843	*Shinella*	*Proteobacteria*	74	47	27	47	27	0.022	0.022	0.214	HP x N
10116	*Hyphomicrobiaceae*	*Proteobacteria*	41	14	27	29	12	0.022	0.067	0.879	N
16130	*Actinomycetales*	*Actinobacteria*	77	34	43	59	18	0.022	0.574	0.297	N
13394	*Actinomycetales*	*Actinobacteria*	93	45	48	58	35	0.023	0.740	0.116	N
15946	*Gemmatimonas*	*Gemmatimonadetes*	87	47	40	53	34	0.023	0.355	0.099	N
14920	*Solirubrobacter*	*Actinobacteria*	54	25	29	37	17	0.027	0.624	0.806	N
53107	*Actinomycetales*	*Actinobacteria*	42	27	15	27	15	0.029	0.029	0.686	HP x N
11488	*Myxococcales*	*Proteobacteria*	46	17	29	31	15	0.032	0.093	0.766	N
21953	*Blastococcus*	*Actinobacteria*	52	21	31	34	18	0.032	0.155	0.155	N
41740	*Firmicutes*	*Firmicutes*	58	32	26	37	21	0.032	0.380	0.555	N
33046	*Proteobacteria*	*Proteobacteria*	50	24	26	31	19	0.033	0.694	0.133	N
37889	*Solirubrobacter*	*Actinobacteria*	167	79	88	94	73	0.034	0.325	0.579	N
16128	*Catelliglobosispora*	*Actinobacteria*	154	83	71	92	62	0.035	0.361	0.139	N
48780	*Rhizobium*	*Proteobacteria*	82	51	31	50	32	0.036	0.022	0.609	HP x N
7472	*Catelliglobosispora*	*Actinobacteria*	52	32	20	34	18	0.037	0.104	0.569	N
19072	*Gp3*	*Acidobacteria*	42	15	27	31	11	0.038	0.188	0.499	N
23554	*Rhizobiales*	*Proteobacteria*	49	19	30	33	16	0.039	0.159	0.243	N
28251	*Kitasatospora*	*Actinobacteria*	88	48	40	55	33	0.041	0.422	0.319	N
18715	*Promicromonosporaceae*	*Actinobacteria*	101	58	43	64	37	0.043	0.234	0.298	N
48486	*Firmicutes*	*Firmicutes*	152	51	101	100	52	0.048	0.041	0.533	LP x N
51086	*Chloroflexi*	*Chloroflexi*	98	33	65	65	33	0.049	0.049	0.688	LP x N

[a] Operational Taxonomic Unit

[b] The number of sequences that associated with high shoot biomass plants

[c] The number of sequences that associated with low shoot biomass plants

[d] The number of sequences that associated with plants grown under N fertilized condition

[e] The number of sequences that associated with plants grown under unfertilized condition

[f] *p Values* for nitrogen treatment

[g] *P Values* for productivity treatment

[h] *P Values* for the interaction

Table 4. OTUs significantly associated with wheat plants when nitrogen was not applied.

Classification			*Number of Sequences*					*P Value*			*Interactions*
Cluster ID	OTU[a]	Phyla	Total	H[b]	L[c]	N[d]	NN[e]	N[f]	Prod.[g]	PxN[h]	
12024	*Acetobacteraceae*	*Proteobacteria*	43	20	23	13	30	0.018	0.637	0.874	NN
24652	*Blastococcus*	*Actinobacteria*	61	27	34	24	37	0.031	0.213	0.061	NN
36931	*Rhizobiales*	*Proteobacteria*	94	55	39	37	57	0.023	0.060	0.219	NN
50310	*Pseudonocardia*	*Actinobacteria*	158	91	67	65	93	0.032	0.060	0.082	NN

[a] Operational Taxonomic Unit

[b] The number of sequences that associated with high shoot biomass plants

[c] The number of sequences that associated with low shoot biomass plants

[d] The number of sequences that associated with plants grown under N fertilized condition

[e] The number of sequences that associated with plants grown under unfertilized condition

[f] *P Values* for Nitrogen treatment

[g] *P Values* for Productivity treatment

[h] *P Values* for the interaction between nitrogen and productivity

Table 5. OTUs significantly associated with high productivity wheat plants.

Classification			*Number of Sequences*					*P Value*			*Interactions*
Cluster ID	OTU[a]	Phyla	Total	H[b]	L[c]	N[d]	NN[e]	N[f]	Prod.[g]	PxN[h]	
35239	*Laceyella*	*Firmicutes*	47	35	12	29	18	0.085	0.002	0.618	HP
46721	*Marmoricola*	*Actinobacteria*	48	33	15	33	15	0.007	0.007	1.000	HP x N
45960	*Solirubrobacter*	*Actinobacteria*	55	42	13	27	28	0.919	0.010	0.759	HP
39100	*Actinobacteria*	*Actinobacteria*	40	29	11	24	16	0.226	0.014	0.358	HP
32434	*Streptomyces*	*Actinobacteria*	48	33	15	24	24	1.000	0.014	0.137	HP
25656	*Solirubrobacter*	*Actinobacteria*	171	97	74	90	81	0.300	0.017	0.558	HP
3624	*Nocardioides*	*Actinobacteria*	86	54	32	43	43	1.000	0.020	0.169	HP
50877	*Gp16*	*Acidobacteria*	57	39	18	40	17	0.014	0.022	0.400	HP x N
8843	*Shinella*	*Proteobacteria*	74	47	27	47	27	0.022	0.022	0.214	HP x N
48780	*Rhizobium*	*Proteobacteria*	82	51	31	50	32	0.036	0.022	0.609	HP x N
33666	*Gp16*	*Acidobacteria*	40	28	12	16	24	0.230	0.026	1.000	HP
21163	*OP10*	*OP10*	76	58	18	32	44	0.460	0.026	0.227	HP
53107	*Actinomycetales*	*Actinobacteria*	42	27	15	27	15	0.029	0.029	0.686	HP x N
35691	*Planctomyces*	*Planctomycetes*	84	62	22	40	44	0.811	0.031	0.811	HP

36874	*Conexibacter*	*Actinobacteria*	59	40	19	33	26	0.435	0.032	0.735	HP
29565	*Mycobacterium*	*Actinobacteria*	52	34	18	27	25	0.766	0.032	0.766	HP
18854	*Deltaproteobacteria*	*Proteobacteria*	56	37	19	30	26	0.605	0.034	0.795	HP
5983	*Rubrobacter*	*Actinobacteria*	53	32	21	29	24	0.304	0.036	0.003	INNxHP
44025	*Marmoricola*	*Actinobacteria*	42	28	14	25	17	0.211	0.039	1.000	HP
4813	*Balneimonas*	*Proteobacteria*	61	41	20	28	33	0.602	0.044	0.093	HP

[a] Operational Taxonomic Unit

[b] The number of sequences that associated with high shoot biomass plants

[c] The number of sequences that associated with low shoot biomass plants

[d] The number of sequences that associated with plants grown under N fertilized condition

[e] The number of sequences that associated with plants grown under unfertilized condition

[f] *P Values* for Nitrogen treatment

[g] *P Values* for Productivity treatment

[h] *P Values* for the interaction between nitrogen and productivity

Table 6. OTUs significantly associated with low productivity wheat plants.

Classification			*Number of Sequances*					*P V alue*			*Interactions*
Cluster ID	OTU[a]	Phyla	Total	H[b]	L[c]	N[d]	NN[e]	N[f]	Prod.[g]	PxN[h]	
38639	*Actinobacteria*	*Actinobacteria*	55	6	49	29	26	0.680	<0.001	0.890	LP
50459	*Rhodospirillaceae*	*Alphaproteobacteria*	79	7	72	65	14	0.004	0.001	0.005	N INxLP
51614	*Sporichthya*	*Actinobacteria*	67	15	52	40	27	0.229	0.004	0.304	LP
50895	*Hyphomicrobiaceae*	*Alphaproteobacteria*	46	12	34	36	10	0.003	0.007	0.063	LP x N
18302	*Micromonosporaceae*	*Actinobacteria*	98	33	65	63	35	0.017	0.008	0.846	LP x N
29687	*Ensifer*	*Alphaproteobacteria*	150	38	112	61	89	0.254	0.008	0.325	LP
30768	*Chloroflexus*	*Chloroflexi*	71	21	50	38	33	0.615	0.011	0.615	LP
10313	*Nocardioides*	*Actinobacteria*	47	15	32	29	18	0.080	0.012	0.611	LP
28372	*Phenylobacterium*	*Alphaproteobacteria*	47	16	31	21	26	0.382	0.018	0.596	LP
12636	*Rhizobiales*	*Alphaproteobacteria*	47	13	34	19	28	0.288	0.024	0.405	LP
21064	*Rhodoplanes*	*Alphaproteobacteria*	49	13	36	27	22	0.587	0.025	0.120	LP
34643	*Proteobacteria*	*Alphaproteobacteria*	92	22	70	56	36	0.318	0.028	1.000	LP
33420	*Devosia*	*Alphaproteobacteria*	364	122	242	263	101	0.006	0.029	0.602	LP x N
46198	*Rubrobacter*	*Actinobacteria*	43	14	29	24	19	0.435	0.032	0.172	LP

51468	*Mycobacterium*	*Actinobacteria*	43	9	34	16	27	0.309	0.033	0.777	LP
34607	*Bacillus*	*Bacilli*	47	16	31	26	21	0.450	0.037	0.065	LP
48486	*Firmicutes*	*Bacilli*	152	51	101	100	52	0.048	0.041	0.533	LP x N
44048	*Xanthomonadaceae*	*Gammaproteobacteria*	91	30	61	43	48	0.728	0.047	0.249	LP
49501	*Anaerolineaceae*	*Anaerolineae*	49	19	30	27	22	0.337	0.048	0.097	LP
34684	*Bradyrhizobiaceae*	*Alphaproteobacteria*	119	47	72	81	38	0.003	0.049	0.931	LP x N
51086	*Chloroflexi*	*Chloroflexi*	98	33	65	65	33	0.049	0.049	0.688	LP x N

[a] Operational Taxonomic Unit

[b] The number of sequences that associated with high shoot biomass plants

[c] The number of sequences that associated with low shoot biomass plants

[d] The number of sequences that associated with plants grown under N fertilized condition

[e] The number of sequences that associated with plants grown under unfertilized condition

[f] *P Values* for Nitrogen treatment

[g] *P Values* for Productivity treatment

[h] *P Values* for the interaction between nitrogen and productivity

FIGURES

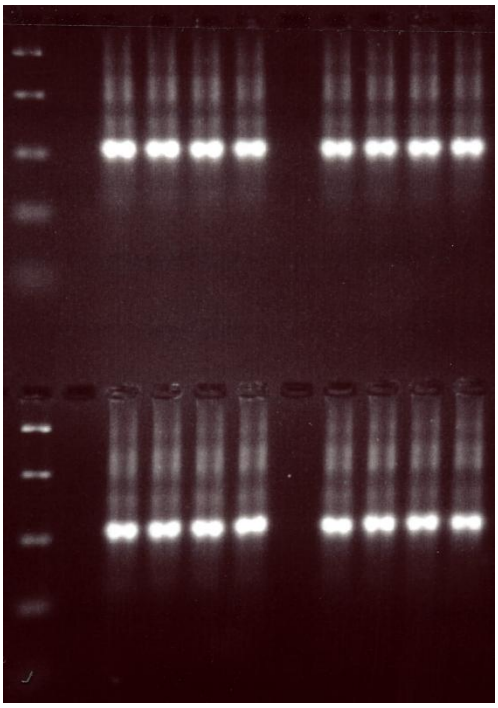


Fig. 1: Electrophoresis gel showing PCR products for the 16s rRNA gene from the four treatments and four replications: high productivity with nitrogen (upper left), low productivity with nitrogen (upper right), high productivity without nitrogen (lower left), and low productivity without nitrogen (lower right).

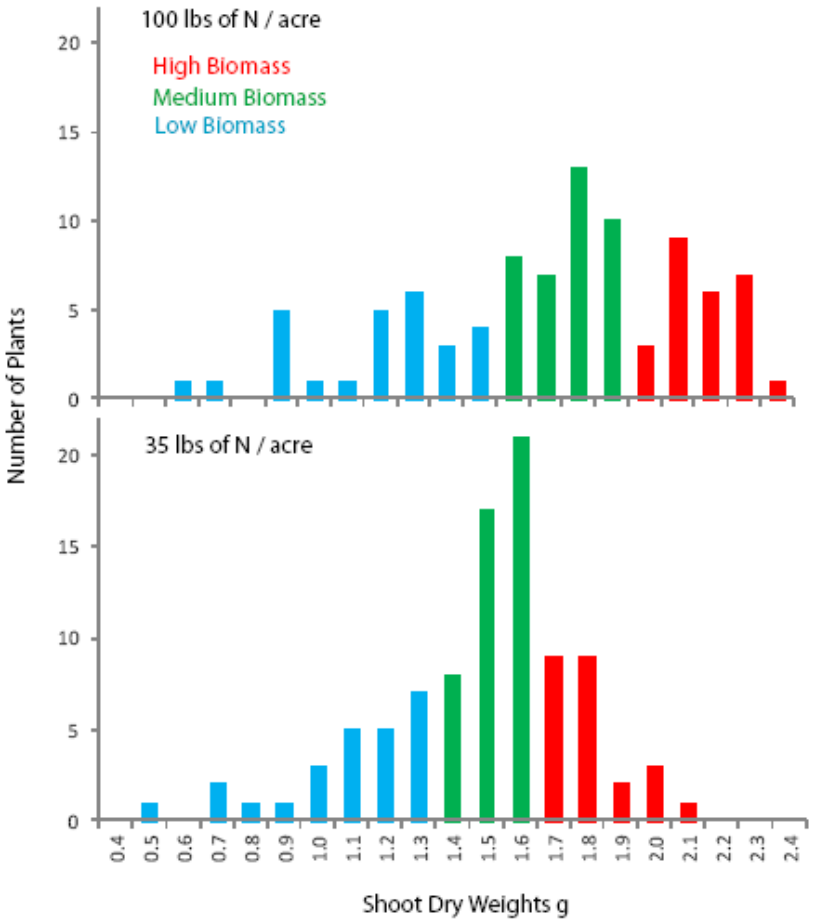


Fig 2: Distribution of shoot dry weight with plant numbers in plants treated or untreated with urea nitrogen at 0.1 g intervals from 0.4g to 2.4 g shoot dry weights.

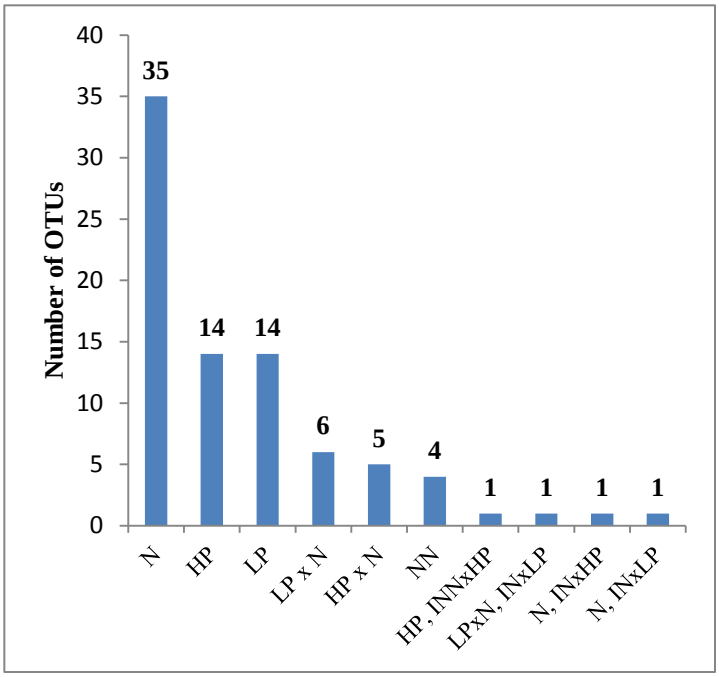


Fig. 3: Number of significant OTUs categorized according to treatment and interaction: N number of significant OTUs that associated with plants grown under N fertilized condition. HP number of significant OTUs associated with high productivity plants. LP number of significant OTUs associated with low productivity plant. LP × N number of significant OTUs associated with low productivity plant that grown under N fertilized condition. HP × N number of significant OTUs associated with high productivity plant that grown under N fertilized condition. NN number of significant OTUs associated with plant that grown under unfertilized condition.

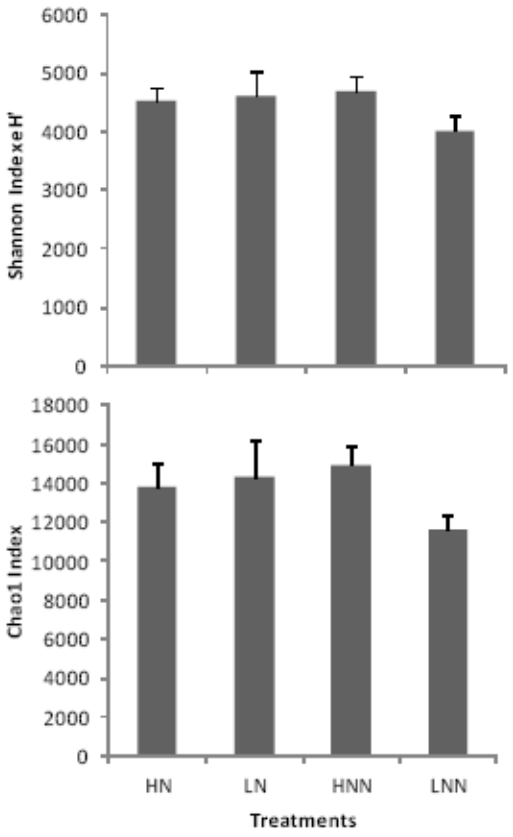


Fig. 4: Diversity indexes including the Shannon index expressed in the exponential form (upper chart) and the Chao1 index (lower chart) for the following treatments: HN high productivity plants grown under N fertilized condition. LN low productivity plants grown under N fertilized condition. HN high productivity plants grown under unfertilized condition. LN low productivity plants grown under unfertilized condition.

3.6. References

Abbasi M.K., Sharif S., Kazmi M., Sultan T., Aslam M., 2011. Isolation of plant growth promoting rhizobacteria from wheat rhizosphere and their effect on improving growth, yield and nutrient uptake of plants. Plant Biosystems 145:159-168.

Adesemoye A.O., Torbert H.A., Kloepper J.W., 2009. Plant growth-promoting rhizobacteria allow reduced application rates of chemical fertilizers. Microbial Ecology. 58:921-929.

Ahmadian A., Gharizadeh B., Gustafsson A.C., Sterky F., Nyren P., Uhlen M., Lundeberg J., 2000. Single-nucleotide polymorphism analysis by pyrosequencing. Analytical Biochemistry 280:103-110.

Akhtar M.J., Asghar H.N., Shahzad K., Arshad M., 2009. Role of plant growth promoting rhizobacteria applied in combination with compost and mineral fertilizers to improve growth and yield of wheat (*Triticum Aestivum L.*). Pakistan Journal of Botany 41:381-390.

Bhromsiri C., Bhromsiri A., 2010. Isolation, screening of growth-promoting activities and diversity of rhizobacteria from vetiver grass and rice plants. Thai Journal of Agricultural Science 43:217-230.

Canbolat M.Y., Bilen S., Cakmakci R., Sahin F., Aydin A., 2006. Effect of plant growth-promoting bacteria and soil compaction on barley seedling growth, nutrient uptake, soil properties and rhizosphere microflora. Biology and Fertility of Soils 42:350-357.

Carlier E., Rovera M., Jaume A.R., Rosas S.B., 2008. Improvement of growth, under field conditions, of wheat inoculated with *Pseudomonas chlororaphis* subsp *aurantiaca* SR1. World Journal of Microbiology & Biotechnology 24:2653-2658.

Carrillo L., Ahrendts M.R.B., Maldonado M.J., 2009. Alkalithermophilic actinomycetes in a subtropical area of Jujuy, Argentina. Revista Argentina De Microbiologia 41:112-116.

Carter B., 2002. The importance of wheat quality. Agricultural Horizons.

Cavaglieri L., Orlando J., Etcheverry M., 2009. Rhizosphere microbial community structure at different maize plant growth stages and root locations. Microbiological Research 164:391-399.

Cherobaeva A.S., Stepanov A.L., Kravchenko I.K., 2011. Comparative analysis of soil DNA extraction methods to study various ammonium-oxidizing bacteria and archaea by PCR-DGGE. Moscow University Soil Science Bulletin 66:50-57.

Crecchio C., Curci M., Pellegrino A., Ricciuti P., Tursi N., Ruggiero P., 2007. Soil microbial dynamics and genetic diversity in soil under monoculture wheat grown in different long-term management systems. Soil Biology & Biochemistry 39:1391-1400.

Dastager S.G., Deepa C.K., Pandey A., 2010. Isolation and characterization of novel plant growth promoting *Micrococcus sp* NII-0909 and its interaction with cowpea. Plant Physiology and Biochemistry 48:987-992.

Dombrecht B., Marchal K., Vanderleyden J., Michiels J., 2002. Prediction and overview of the RpoN-regulon in closely related species of the Rhizobiales. Genome Biology 3.

Domnita C., 2010. Hematopathology: genomic mechanisms of neoplastic diseases Springer Science, New York.

Dowd S.E., Sun Y., Secor P.R., Rhoads D.D., Wolcott B.M., James G.A., Wolcott R.D., 2008. Survey of bacterial diversity in chronic wounds using pyrosequencing, DGGE, and full ribosome shotgun sequencing. Bmc Microbiology. 8.

Duineveld B.M., Kowalchuk G.A., Keijzer A., van Elsas J.D., van Veen J.A., 2001. Analysis of bacterial communities in the rhizosphere of chrysanthemum via denaturing gradient gel

electrophoresis of PCR-amplified 16S rRNA as well as DNA fragments coding for 16S rRNA. Applied and Environmental Microbiology 67:172-178.

Durbin R.D., 1961. Techniques for the observation and isolation of soil microorganisms. Botanical Review 27:522-560.

Espana M., Rasche F., Kandeler E., Brune T., Rodriguez B., Bending G.D., Cadisch G., 2011. Identification of active bacteria involved in decomposition of complex *maize* and soybean residues in a tropical Vertisol using (15)N-DNA stable isotope probing. Pedobiologia 54:187-193.

Gholami A., Shahsavani S., Nezara S., 2009. The Effect of Plant Growth Promoting Rhizobacteria (PGPR) on Germination, Seedling Growth and Yield of Maize. . World Academy of Science, Engineering and Technolog. 49.

Hassen A.I., Labuschagne N., 2010. Root colonization and growth enhancement in wheat and tomato by rhizobacteria isolated from the rhizoplane of grasses. World Journal of Microbiology & Biotechnology 26:1837-1846.

Hayat R., Ali S., Amara U., Khalid R., Ahmed I., 2010. Soil beneficial bacteria and their role in plant growth promotion: a review. Annals of Microbiology 60:579-598.

Hinsinger P., Bengough A.G., Vetterlein D., Young I.M., 2009. Rhizosphere: biophysics, biogeochemistry and ecological relevance. Plant and Soil 321:117-152.

Hossain M.S., Martensson A., 2008. Potential use of Rhizobium spp. to improve fitness of non-nitrogen-fixing plants. Acta Agriculturae Scandinavica Section B-Soil and Plant Science 58:352-358.

Jonathan P., 2009. Bioinformatics and functional genomics John Wiley & Sons, Canada.

Lee S.-H., Ka J.-O., Cho J.-C., 2008. Members of the phylum Acidobacteria are dominant and metabolically active in rhizosphere soil. Fems Microbiology Letters 285:263-269.

Lu Y.H., Rosencrantz D., Liesack W., Conrad R., 2006. Structure and activity of bacterial community inhabiting rice roots and the rhizosphere. Environmental Microbiology 8:1351-1360.

Lupwayi N.Z., Grant C.A., Soon Y.K., Clayton G.W., Bittman S., Malhi S.S., Zebarth B.J., 2010. Soil microbial community response to controlled-release urea fertilizer under zero tillage and conventional tillage. Applied Soil Ecology 45:254-261.

Maria B.H., 2007. Application of PCR and DGGE to characterise the microflora of farmed fish, Fisheries and Aquaculture, University of Bergen, Seafood Processing Research.

Marsh S., 2007. Pyrosequencing protocols, Scitech Book News, Book News, Inc., Portland, United States, Portland.

Mia M.A.B., Shamsuddin Z.H., Wahab Z., Marziah M., 2010. Rhizobacteria as bioenhancer and biofertilizer for growth and yield of banana (*Musa* spp. cv. 'Berangan'). Scientia Horticulturae 126:80-87.

Ozturk A., Caglar O., Sahin F., 2003. Yield response of wheat and barley to inoculation of plant growth promoting rhizobacteria at various levels of nitrogen fertilization. Journal of Plant Nutrition and Soil Science-Zeitschrift Fur Pflanzenernahrung Und Bodenkunde 166:262-266.

Pintado J., Guyot J.P., Ampe F., 2003. Multiple competitive PCR-DGGE as a tool for quantifying and profiling defined mixed cultures of lactic acid bacteria during production of probiotics from complex polysaccharides. Journal of Applied Microbiology 95:921-933.

Rodrigues Coelho M.R., De Vos M., Carneiro N.P., Marriel I.E., Paiva E., Seldin L., 2008. Diversity of nifH gene pools in the rhizosphere of two cultivars of sorghum (*Sorghum bicolor*) treated with contrasting levels of nitrogen fertilizer. Fems Microbiology Letters 279:15-22.

Shen J.P., Zhang L.M., Guo J.F., Ray J.L., He J.Z., 2010. Impact of long-term fertilization practices on the abundance and composition of soil bacterial communities in Northeast China. Applied Soil Ecology 46:119-124.

Shen X.Y., Zhang L.M., Shen J.P., Li L.H., Yuan C.L., He J.Z., 2011. Nitrogen loading levels affect abundance and composition of soil ammonia oxidizing prokaryotes in semiarid temperate grassland. Journal of Soils and Sediments 11:1243-1252.

Temmerman R., Huys G., Swings J., 2004. Identification of lactic acid bacteria: culture-dependent and culture-independent methods. Trends in Food Science & Technology 15:348-359.

Ten Eyck A.M., 1914. Wheat; a practical discussion of the raising, marketing, handling and use of the wheat crop, relating largely to the great plains region of the United States and Canada Campbell soil culture pub. co., Lincoln, Neb.

Upadhyay S.K., Singh D.P., Saikia R., 2009. Genetic Diversity of Plant Growth Promoting Rhizobacteria Isolated from Rhizospheric Soil of Wheat Under Saline Condition. Current Microbiology 59:489-496.

Wang Y., Cai F., Tang Y.L., Dai J., Qi H., Rahman E., Peng F., Fang C.X., 2011. Flavitalea populi gen. nov., sp nov., isolated from soil of a Euphrates poplar (*Populus euphratica*) forest. International Journal of Systematic and Evolutionary Microbiology 61:1554-1560.

Ward D.M., Ferris M.J., Nold S.C., Bateson M.M., 1998. A natural view of microbial biodiversity within hot spring cyanobacterial mat communities. Microbiology and Molecular Biology Reviews 62:1353.

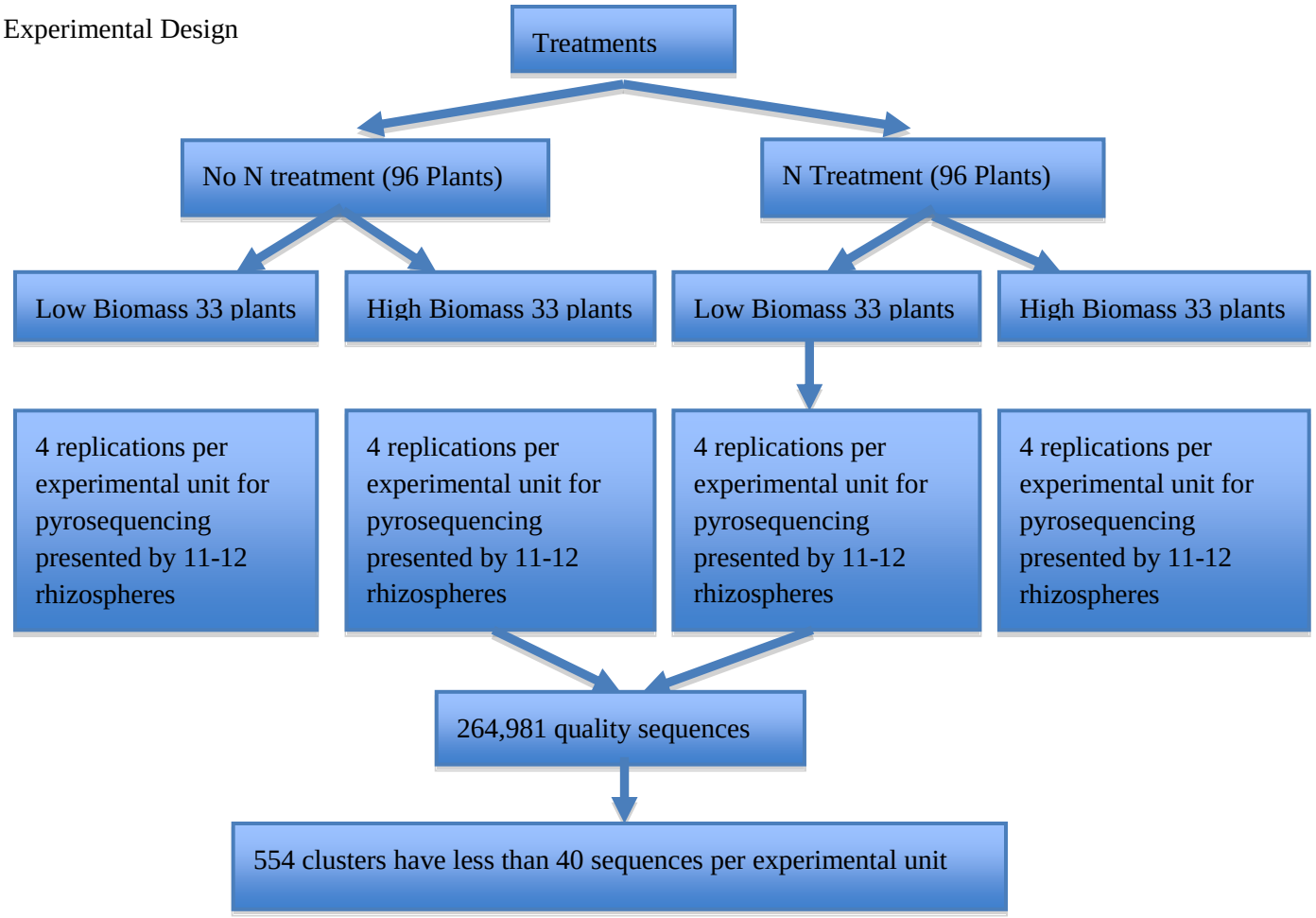
Wu M.N., Qin H.L., Chen Z., Wu J.S., Wei W.X., 2011. Effect of long-term fertilization on bacterial composition in rice paddy soil. Biology and Fertility of Soils 47:397-405.

Yang Y., Wang X., Shi J.F., Li J.J., 2012. The influence of the discharging sewage on microbial diversity in sludge from Dongting Lake. World Journal of Microbiology & Biotechnology 28:421-430.

Zhang Y., Du B.H., Jin Z.G., Li Z.H., Song H.N., Ding Y.Q., 2011. Analysis of bacterial communities in rhizosphere soil of healthy and diseased cotton (*Gossypium* sp.) at different plant growth stages. Plant and Soil 339:447-455.

APPENDICES

1- Experimental Design



2- Growth parameters for high biomass plants grown under no nitrogen treatment

Number of Spikes	Number of Stems	Shoot Fresh Weight	Shoot Dry Weight	Total Root Fresh Weight
1	2	05.86	01.40	17.49
2	2	06.80	01.42	26.61
1	2	05.84	01.42	10.80
1	1	06.47	01.43	11.32
2	2	06.41	01.43	20.87
1	1	06.21	01.43	13.44
1	2	06.37	01.45	26.99
2	2	06.20	01.47	25.68
1	2	06.17	01.54	14.67
2	2	05.94	01.54	10.87
2	2	06.22	01.55	10.27
2	2	06.12	01.55	18.62
2	2	06.07	01.56	07.72
2	3	06.66	01.58	27.31
1	1	06.16	01.58	11.57
1	2	06.53	01.60	12.08
2	3	06.48	01.65	16.00
2	2	06.48	01.66	19.29
2	2	06.30	01.67	22.20
2	2	06.05	01.67	27.33
2	2	06.25	01.69	09.96
2	2	06.36	01.71	14.90
1	2	05.97	01.76	13.61

2	2	05.90	01.76	14.15
2	2	06.75	01.78	18.62
2	2	06.38	01.79	15.15
2	2	06.61	01.80	25.81
1	1	05.99	01.80	17.95
2	2	07.02	01.83	13.73
2	2	05.83	01.83	10.43
2	3	06.08	01.92	15.53
1	2	06.94	01.94	22.21
2	2	06.58	02.07	28.58

3- Growth parameters for low biomass plants grown under no nitrogen treatment

Number of Spikes	Number of Stems	Shoot Fresh Weight	Shoot Dry Weight	Total Root Fresh Weight
1	1	02.60	00.41	05.76
1	1	02.76	00.61	03.64
1	1	02.74	00.64	08.94
1	1	03.45	00.75	11.62
1	1	04.02	00.88	12.41
1	1	04.82	00.97	10.05
1	1	04.94	01.00	24.49
1	1	03.80	01.07	12.30
1	1	03.58	01.07	04.46
1	1	04.80	01.08	31.27
1	1	04.94	01.09	09.74
1	2	04.86	01.10	15.75
1	1	04.04	01.13	26.85
1	1	04.89	01.17	13.47
1	1	04.34	01.18	09.16
1	1	04.09	01.26	07.76
1	1	04.35	01.31	22.40
1	1	04.68	01.32	27.90
1	1	04.88	01.34	10.48
1	1	04.27	01.37	09.84
1	2	04.98	01.38	12.25
1	1	04.72	01.42	05.47
1	2	04.54	01.42	12.55

1	1	04.37	01.44	07.07
1	2	05.06	01.46	06.20
2	2	05.02	01.49	12.61
1	1	04.69	01.49	10.10
1	2	05.01	01.51	11.14
1	1	05.00	01.51	13.27
1	1	05.00	01.51	24.93
1	1	04.67	01.51	21.72
1	1	04.88	01.56	11.31
1	2	04.44	01.56	25.82

4- Growth parameters for high biomass plants grown under nitrogen treatment

Number of Spikes	Number of Stems	Shoot Fresh Weight	Shoot Dry Weight	Total Root Fresh Weight
1	1	09.05	01.46	25.08
2	3	07.44	01.69	11.90
2	2	07.25	01.75	19.00
2	2	07.17	01.80	23.75
1	2	07.77	01.86	20.59
2	2	07.61	01.86	09.99
2	3	07.17	01.86	24.60
2	3	07.81	01.88	37.36
2	2	07.22	01.98	23.34
1	2	07.14	01.98	13.95
2	2	07.09	01.99	17.33
1	1	07.74	02.02	15.05
1	1	07.31	02.02	18.31
3	3	07.61	02.03	15.26
2	2	07.86	02.05	15.67
2	3	08.25	02.06	39.36
2	2	07.11	02.07	17.07
2	2	07.83	02.09	15.66
2	2	07.37	02.09	20.50
2	3	08.27	02.11	25.12
1	2	08.34	02.15	23.79
2	3	07.55	02.15	26.13
2	2	07.96	02.16	16.85

2	2	08.34	02.17	22.83
2	2	08.18	02.17	12.98
2	2	08.41	02.21	20.46
2	2	07.63	02.21	11.03
2	2	09.18	02.23	23.70
2	2	08.67	02.23	32.18
2	3	08.44	02.24	37.68
2	2	08.30	02.27	17.50
2	2	08.84	02.28	47.22
1	2	09.67	02.35	39.49

5- Growth parameters for low biomass plants grown under no nitrogen treatment

Number of Spikes	Number of Stems	Shoot Fresh Weight	Shoot Dry Weight	Total Root Fresh Weight
1	1	02.84	00.56	08.02
1	1	03.32	00.69	06.44
1	1	03.77	00.81	08.75
1	1	03.62	00.83	07.10
1	1	04.23	00.84	10.78
1	1	04.43	00.88	15.82
1	1	04.22	00.88	24.82
1	2	04.50	00.92	14.98
1	1	03.49	01.09	11.38
1	1	04.90	01.11	12.37
1	1	04.57	01.17	10.08
1	2	04.37	01.17	14.73
1	2	05.64	01.18	17.44
1	2	04.08	01.19	06.66
1	1	05.77	01.21	29.24
1	1	05.47	01.21	15.23
1	1	05.74	01.22	21.33
1	2	05.69	01.27	12.53
1	2	04.74	01.29	12.12
1	2	04.70	01.30	18.51
1	2	05.83	01.35	06.71
1	1	04.47	01.39	20.57
1	2	05.99	01.44	15.27

1	2	05.48	01.49	09.97
2	2	05.79	01.50	23.31
1	2	05.61	01.52	10.08
1	1	05.89	01.54	23.84
1	1	05.42	01.54	25.09
2	2	05.73	01.59	21.55
1	1	05.18	01.60	20.14
1	2	06.08	01.63	12.16
2	2	05.43	01.63	07.62
2	3	06.11	01.76	14.20

6- Nano-drop spectrophotometer result for extracted DNA

Experimental Unit	ng/µl	260/280	260/230
1	13.90	01.63	01.78
2	51.30	01.95	01.99
3	42.60	01.80	01.92
4	43.40	01.80	01.90
5	50.10	01.76	01.82
6	48.40	01.86	01.97
7	67.20	01.87	01.95
8	55.00	01.80	01.91
9	44.80	01.76	01.90
10	55.50	01.77	01.99
11	51.80	01.81	01.94
12	43.10	01.77	01.92
13	50.40	01.74	01.90
14	51.10	01.79	01.91
15	48.20	01.87	01.95
16	52.40	01.78	01.88
Averages	48.08	01.80	01.91

VITA

Samia Ahmed Ali Elghair

Candidate for the Degree of

Master of Science

Thesis: THE EFFECT OF NITROGEN ON RHIZOBACTERIA ASSOCIATED WITH WHEAT SHOOT PRODUCTIVITY

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Biographical:

Education:

Completed the requirements for the Master of Science in Plant and Soil Sciences at Oklahoma State University, Stillwater, Oklahoma in May, 2012.

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Experience: Collaborated with Agriculture Research Center, Tripoli, Libya in olive improvement using *in vitro* culture from 2007 to 2008. Demonstrator in Botany Department, Al-Mergeb University, Khoms, Libya from 2005-2008.

Name: Samia Ahmed Ali ELghair Date of Degree: May, 2012

Institution: Oklahoma State University Location: Stillwater, Oklahoma

Title of Study: THE EFFECT OF NITROGEN ON RHIZOBACTERIA ASSOCIATED WITH WHEAT SHOOT PRODUCTIVITY

Pages in Study: 73 Candidate for the Degree of Master of Science

Major Field: Plant and Soil Sciences

Scope and Method of Study: The effect of nitrogen fertilizer on rhizobacteria associated with wheat (*Triticum aestivum)* shoot productivity was determined by using pyrosequencing technique. Easpur Loam soils was treated with 112-kg/ha N or left untreated at 39.3-kg/ha N using urea as a nitrogen source and 96 plants per N treatment. Plant rhizospheres were categorized according to corresponding shoot weights resulting in four treatment combinations based upon with and without nitrogen and high and low productivity classifications. Two factor ANOVA was used to identify operational taxonomic units (OTUs) with significant association with either nitrogen or productivity.

Findings and Conclusions: A total of 94 OTUs were significantly associated with productivity and/or nitrogen treatment. Of these, 49 were associated with wheat grown under nitrogen application, 4 without nitrogen application, 20 with high shoot biomass, and 21 with low shoot biomass. *Gemmatimonadetes, Acidobacteria, Proteobacteria,* and *Bacteroidetes* were the most common phyla associated with wheat productivity when supplemented with nitrogen fertilizer. Of the bacterial groups evaluated, *Actinobacteria* was the most common phylum, and *Laceyella* #35239 and *Marmoricola* #46721 were the most significant OTUs associated with high biomass plant. The most significant OTUs associated with low biomass plants were *Firmicutes* #48486 *Actinobacteria* #38639, *Rhodospirillaceae* #50459, *Sporichthya* #51614, *Hyphomicrobiaceae* #50895, *Ensifer* #29687, and *Micromonosporaceae* #18302. Based on Chao1 rhizobacterial diversity was significantly greater in high biomass plants (P = 0.046) compared to low biomass plants. However, rhizobacteria diversity between nitrogen treatments was not significantly different according to Shannon and Chao1 indices. Data obtained from this study suggested that significant associations exist among rhizobacteria with respect to plant productivity and nitrogen addition. In the future, a better understanding of the relationships between the rhizobacteria community and wheat productivity may afford us opportunities to maximize wheat production by minimizing fertilizer inputs, resulting in increased economic returns for the producer.

ADVISER'S APPROVAL: Mike P. Anderson