ISOLATION AND ASSESSMENT OF NITROGEN-FIXING AND PHOSPHATE-SOLUBILIZING BACTERIA FOR USE AS BIOFERTILIZERS

by

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ABSTRACT

Nitrogen and phosphorus are essential macronutrients for plant growth. The major sources of N and P in agriculture are chemical fertilizers. Nitrogen in the soil can be lost due to denitrification, leaching, and volatilization. The only phosphate source in the world, rock phosphate, is being depleted. When it is applied to soil, phosphate is quickly immobilized and cannot be absorbed by the plants. In addition, chemical fertilizers are not affordable for many of the world's farmers. Thus, there is a growing opportunity for efficient biofertilizers. N-fixing and P-solubilizing microorganisms are the two major groups of biofertilizers. The overall aims of this study were i) to conduct comprehensive analyses of N-fixing and P-solubilizing bacteria in specific crop situations in Alabama; and ii) to characterize and select effective strains for use as biofertilizers. Two main strategies for isolating strains were conducted. One was to isolate them from selected perennial Gramineae (giant reed and switchgrass); the other was to isolate from specific long-term rotations where nutrient application had been controlled (the Old Rotation and Cullars Rotation experiments). A total of 354 N-fixing and P-solubilizing bacterial strains were isolated. The N-fixing activity of all the putative N-fixing strains was confirmed using acetylene reduction assay, and the P-solubilizing activity was confirmed by both qualitative and quantitative evaluations. Auxin and siderophore production were also evaluated *in vitro*, and 16S rDNA sequencing was used for molecular identification. The results showed a wide diversity of bacterial genera. Five new genera were first reported to have N-fixing activity. Selected N-fixing and P-solubilizing strains that had shown outstanding performance during *in vitro* tests were inoculated on corn, wheat, and pepper under greenhouse conditions. Interestingly, the results showed that the selected N-fixing and P-solubilizing bacteria could promote corn, wheat, and pepper growth by increasing

plant growth parameters, such as root/shoot dry weight, height, and SPAD, and the root morphology parameters. Among corn and wheat inoculated with selected strains, an improvement of nutrient uptake rate was found in the analysis of aboveground tissues. But the results showed plant growth promotion and nutrient uptake are not linked.

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LIST OF ABBREVIATIONS

PGPR, Plant Growth- Promoting Rhizobacteria

ARA, Acetelyene Reduction Assay

MPIK, Modified Pikovskaya's Medium

CAS, Chrome azurol S

PGP, Plant Growth-Promotion

ACC, 1-Aminocyclopropan-1-Carboxylate

KSB, Potassium-Solubilizing Bacteria

PSB, Phosphate-Solubilizing Bacteria

TSA, Tryticase Soy Agar

CHAPTER 1 LITERATURE REVIEW

1.1 OVERVIEW OF CHEMICAL FERTILIZERS

Many external factors, such as soil conditions and climate, can affect plant growth and yield. Compared to factors related to climate, soil conditions are easier to manage to achieve minimal soil-related losses. Low yield from poor soil is often due to low availability of soil nutrients that are required for plant growth. One approach to increase plant yields and plant available nutrients is to add chemical fertilizers. Chemical fertilizers can provide macro nutrients, such as nitrogen (N), phosphorus (P), and potassium (K), and micro nutrients, such as copper (Cu) and iron (Fe), that are essential for the crop growth and yield. N, P, and K are the three major essential macronutrients for biological growth and development.

1.1.1 Nitrogen Fertilizer

Since the invention in the early 20th century of the Haber-Bosch process, which produces ammonia by using a catalyst on nitrogen and hydrogen gas, artificial nitrogen fertilizer has been widely used in agriculture. Due to the fast growing world population and requirement of high level of crop yields, the consumption of N fertilizer in agriculture has increased over 20-fold in the last 50 years (Heffer & Prud'homme, 2013)

The most common artificial nitrogen fertilizers are anhydrous ammonia (82% N), urea (46% N), nitrogen solutions (UAN solution, 28-32% N), ammonium sulfate (21% N), and ammonium nitrate (33-34% N). Anhydrous ammonia is a gas at normal atmospheric temperature and has the slowest pressure of all N fertilizers to convert to

nitrate (plant available form). The application is very important, since it must be injected into a moist soil. If not, N loss by volatilization can occur. Anhydrous ammonia is hazardous to handle and toxic to living organisms (Vitosh & Johnson, 1995). Urea is a favorable N fertilizer to farmers since it is easy to handle and store, and it converts to nitrate fairly quickly, usually in less than two weeks in the spring. Denitrification and leaching can be serious if the soil is wet and coarse. Surface volatilization can be a problem if the urea is not placed in contact with the soil and the weather is dry for several days after spreading. Because of problems with leaching and denitrification of urea, ammonium nitrate is a good choice for surface application where ammonia volatilization is expected, but it is still not preferred since it is very hygroscopic and can be explosive when mixed with carbonaceous materials (Calvo, 2013). Calcium ammonium nitrate (CAN) (25-26% N) is also nitrate and ammonium fertilizers, was developed as non-explosive alternative to pure ammonium nitrate. The storage of CAN is crucial since it, should be kept absolutely dry; fire should be avoided to prevent decomposition.

Artificial N fertilizers are essential to the crop yield and are widely used in agriculture systems; but their use also has some negative effects in the environment. They are subject to leaching, denitrification, and volatilization. Some of the N fertilizers have the potential to be hazardous to humans in concentrated form.

1.1.2 Phosphorus fertilizer

Global sources of phosphorus fertilizers include rock phosphate, manure, human excreta, and guano. The last two make up only a tiny part of phosphorus fertilizer. Rock phosphate is the main phosphate source used in the manufacturing of most commercial

phosphate fertilizers on the market. Thirty-five countries currently produce rock phosphate, and 15 others have potentially exploitable resources (IFA, 2009). The value of rock phosphate depends on physical accessibility, purity, and phosphate content. The world's known rock phosphate supply is limited, while the demand continues to increase. Due to low availability of P in native material, high transportation costs, and small crop response, rock phosphate is in a limited supply worldwide, and very little rock phosphate is currently used in agriculture. In addition to the shortage of rock phosphate, another problem is that phosphorus can only be absorbed by the plants in two forms, $H_2PO_4^-$ and HPO_4^{2-}. When phosphate fertilizer is applied to the soil, it can be quickly immobilized and made unavailable to plants. Most agricultural soils contain large reserves of phosphorus, a considerable part of which has accumulated as a consequence of regular applications of P fertilizers. However, a large portion of soluble inorganic phosphate applied to soil as chemical fertilizers is rapidly immobilized soon after application and becomes unavailable to plants due to phosphate fixation and precipitation by aluminum, calcium, iron, magnesium, soil type, and soil pH. In acid soil, phosphorus is fixed by free oxides and hydroxides of aluminum and iron, while in alkaline soils it is fixed by calcium, causing a low efficiency of soluble P fertilizers, such as super calcium phosphate (Rodr ́guez & Fraga, 1999).

1.1.3 Potassium fertilizer

Potassium is the third major plant and crop nutrient after nitrogen and phosphorus. Potash is any of various mined and manufactured salts that contain potassium in water-soluble form, which is important for agriculture because it stimulates

early growth, increases protein production, water retention, yield, nutrient value, color, texture, and disease resistance of food crops. Potassium occurs in the soil in three forms: readily available potassium (K^+ ion); slowly available potassium (fixed by certain mineral); and unavailable potassium (most of the potassium in the soil). The common source of fertilizer potassium is muriate of potash (60% K_2O), which chemically is potassium chloride (KCl). Potassium chloride is highly water soluble. At excessive rates, muriate of potash can cause salt damage to plants.

In summary, there are many advantages to using chemical fertilizers. They work immediately and contain all necessary nutrients that are ready to use. Further, they are relatively inexpensive and convenient to buy and use. However, along with the rapid growth of world population and the tremendous increase in use of chemical fertilizers, the environmental issues such as leaching, runoff, emission, and eutrophication of waterways have become significant due to low absorption rate of chemical fertilizers by plants, which is only about 50% (Vessey, 2003, Miransari, 2010). Chemical fertilization can also accumulate the toxic waste in the soil and decrease the enzyme activities of soil microbes, soil pH, and soil structure (Böhme & Böhme, 2006). With regard to the cost, chemical fertilizers are unaffordable for many of the world's farmers who rely on soil organic matter as a nutrient source in subsistence agriculture. Problems associated with chemical N/P/K fertilizers increase the need to develop economically viable and environmentally sustainable fertilizers to replace or partially replace chemical fertilizers.

There are many alternative methods of soil fertilization, such as organic fertilizer (plant residues containing organic compounds that directly, or by their decay, increase

soil fertility), green manure, manure intercrop or organic-supplemented chemical fertilizers, and use of beneficial living microorganisms (biofertilizer). There are disadvantages to the use of organic fertilizers, such as the use plant residues as alternative fertilization and the concern about their efficiency. For example, compared to chemical fertilization (kg ha^{-1}), much higher amounts of organic fertilization are needed to supply adequate amounts of plant nutrients (Miransari, 2010). Furthermore, long-term or heavy application of organic fertilizers to agricultural soils may result in salt, nutrient, or heavy metal accumulation, which may adversely affect plant growth, soil organisms, water quality, and animal and human health (Chen, 2006). The use of beneficial living microorganisms as an alternative method for improving nutrient uptake, promoting plant growth, and increasing yield production has proved to be advantageous (Miransari, 2010, Adesemoye & Kloepper, 2009). As a result, there is a growing opportunity for the use of beneficial living microorganisms as biofertilizers.

1.2 BIOFERTILIZERS

Biofertilizers, by definition, are substances that contain living microorganisms that, when applied to seed, plant surfaces, or soil, colonize the rhizosphere or the interior of the plant and promote growth by increasing the supply or availability of primary nutrients to the host plant. This definition separates biofertilizers from organic fertilizers, green manure, manure, intercrop, or organic-supplemented chemical fertilizer (Vessey, 2003). The living microorganisms, which promote plant growth by improving the nutrient status of the plant, include rhizospheric fungi, rhizospheric bacteria, symbiotic bacteria, and non-symbiotic endophytic bacteria. Rhizospheric fungi, such as arbuscular

mycorrhizae (Bethlenfalvay & Barea, 1994) and *Penicillium bilaii* (Vessey & Heisinger, 2001), are known to have plant growth-promoting effects by increasing the nutrient status of host plants.

1.2.1 Plant growth-promoting rhizobacteria

Research and agricultural implementation of plant growth-promoting rhizobacteria (PGPR) have grown considerably since the term was first used by Kloepper and Schroth in the late 1970s (Kloepper & Schroth, 1978). Some PGPR can be considered biofertilizers, while others that promote plant growth by control of deleterious organisms, are biopesticides. Strains of PGPR are found in a wide variety of rhizosphere and endophytic bacteria that, when grown in association with a host plant, result in stimulation of growth of their host. Rhizobacteria are also called rhizospheric bacteria, which are isolated from the rhizosphere of the plant. Some PGPR appear to promote growth by acting as both biofertilizer and biopesticide. For example, strains of *Burkholderia cepacia*, which were isolated from maize rhizosphere, have shown biocontrol characteristics to *Fusarium spp.* and growth-promoting capabilities in maize (Vessey, 2003, Bevivino et al., 1998).

1.2.2 Endophytic bacteria

The word endophyte means "in the plant." The usage of this term is very broad and includes bacteria, fungi, and insects in plants, and also algae within algae (Schulz & Boyle, 2006). Any organ of the host can be colonized. Although, there are diverse uses for the word endophyte, "endophytes" are most commonly defined as those organisms whose "infections are inconspicuous, the infected host tissues are at least transiently

symptomless and the microbial colonization can be demonstrated to be internal" (Stone et al., 2000). Endophytes that live inside of the plant root and stem are free-living and do not include pathogens that cause disease. The effects of endophytes on plants can be neutral, deleterious, or beneficial. The use of endophytes as inoculants is focused on those endophytes that promote plant growth. These have potential for use in agribusiness and have already been used singly or as mixtures for inoculating plants to promote growth and yield (Bhattacharjee et al., 2008).

Endophytic bacteria (endophytes) inhabit a more favorable environment than rhizospheric bacteria as they are less vulnerable to competition from other soil bacteria and are shielded from various biotic and abiotic stresses (Reinhold-Hurek & Hurek, 1998). Additionally, endophytes enjoy direct provision of nutritional elements within the host and a low O_2 factor that assists optimal nitrogenase activity for endophytic strains that fix N. In return, endophytes benefit the host plant's growth and development through biological N fixation and growth-promoting substances (Sevilla et al., 2000). Apparently, this intimate association led researchers to anticipate the use of these bacteria in developing sustainable agricultural practices.

The microbial community of endophytes colonizes plant tissues (root, stem, seed, tuber, and fruit) and is capable of establishing interactions among themselves, pathogens, and plants. They can promote plant growth and interact with pathogens to induce systemic resistance (Kloepper & Ryu, 2006). In addition, a variety of diazotrophic bacteria has been found and quantified in different plants, which may contribute substantially to the N nutrition of the plant (Döbereiner et al., 1995).

The main micro-organisms used as biofertilizers and their functions

1.2.3 Nitrogen fixing bacteria

Nitrogen-fixing bacteria include symbiotic (*Rhizobium*) and free-living genera (*Pseudomonas* spp., *Bacillus* spp., *Azospirillum* spp, and *Burkholderia* spp.). These have frequently been reported as plant growth promoters. The symbiotic rhizobia that fix atmospheric N_2 gas in plant root nodules have a mutually beneficial relationship with their host plants. Because of the N-fixing activity, less inorganic N fertilizers are applied to legumes than to non-legume crops. The free-living, N-fixing bacteria, such as *Azospirullum*, *Azotobacter*, and *Pseudomonas*, that fix atmospheric nitrogen do not need a specific host plant compared to the legume *Rhizobia*. These free-living, N-fixing bacteria thrive within the plant, successfully colonizing roots, stems, and leaves. During the association, the invading bacteria benefit the acquired host with a marked increase in plant growth, vigor, and yield (Bhattacharjee et al., 2008).

1.2.3.1 Symbiotic nitrogen fixing bacteria

Two groups of symbiotic, N-fixing bacteria that have been extensively studied include *Rhizobia* and *Frankia*. *Rhizobia* are known for their ability to establish symbiotic interactions with leguminous plants by the formation and colonization of root nodules, where bacteria fix N to ammonia and make it available for the plant. The bacteria are mostly rhizospheric microorganisms, despite their ability to live in the soil for long periods of time (Shridhar, 2012). *Frankia* form root nodules on more than 280 species of woody plants from 8 different families. However, their symbiotic relationship is not as well understood. *Frankia* are known to form effective symbiosis with the species of

Alnus and *Casuarina* (Dash & Gupta, 2011). Bradyrhizobia are common soil-dwelling microorganisms that can form symbiotic relationships with leguminous plant species where they fix N in exchange for carbohydrates from the plant. Like other rhizobia, they have the ability to fix atmospheric N into forms readily available for other organisms to use. They are slow growing in contrast to *Rhizobium* species, which are considered fast growing rhizobia. In a liquid media broth, it takes *Bradyrhizobium* species 3-5 days to create a moderate turbidity and 6-8 hours to double in population size. They tend to grow best with pentoses as a carbon source. Some studies indicate that coinoculation of *Bradyrhizobium* and certain PGPR can positively affect symbiotic N fixation by enhancing both root nodule number or mass and increasing dry weight of nodules, yield components, grain yield, soil nutrient availability, and nitrogenase activity (Saharan & Nehra, 2011).

1.2.3.2 Free-living nitrogen fixing bacteria

Free-living (non-symbiotic) N fixation has a potentially important agronomic significance, but this potential could be limited the availability of carbon and energy sources for the energy-intensive N fixation process. However, this limitation can be compensated by moving closer to or inside the plants, as is seen with diazotrophs that are present in the rhizosphere and the rhizoplane or grow endophytically. Some important, free-living, N-fixing bacteria include *Azoarcus* sp., *Gluconacetobacter diazotrophicus, Herbaspirillium* sp., *Azotobacter* sp., *Acetobacter*, *Azospirillum*, *Azomonas*, *Bacillus*, *Beijerinckia*, *Clostridium*, *Enterobacter*, *Klebsiella*, *Pseudomonas*, and *Xanthobacter* (Vessey, 2003, Saharan & Nehra, 2011).

Azosprillum: *Azospirillum* species fix N_2 under microaerophilic conditions and belong to the facultative endophytic diazotrophs groups, which have been frequently isolated from the rhizosphere and from inside the roots and shoots of many plants such as sugarcane, maize, wheat, rice, and grasses (Döbereiner et al., 1995, Shridhar, 2012). There are about 16 *Azospirillum* species that have been isolated and identified so far; however, *Azospirillum brasilense* and *Azospirillum lipoferum* have been studied in more detail than the others. Studies showed that *Azospirillum* directly benefits plants by improving shoot and root development and increasing the rate of water and mineral uptake by roots, but nitrogen fixation does not play a major role in plant growth promotion. The key signals and components of plant growth promotion effects are the production and secretion of plant growth regulators such as auxins, cytokinins, and gibberellins and nitric oxide (Fibach-Paldi et al., 2012). In the study by Bashan 2010 (Bashan & De-Bashan, 2010) , the mechanism of action of *Azospirillum* and plant interaction also include enhancement of membrane activity, water and mineral uptake, and root system development and mobilization of minerals, mitigation of environmental stressors of plant and biological control of numerous phytopathogens.

Herbaspirillum: *Herbaspirillum* spp. is a broad-host-range endophytic diazotroph found in roots, stems and leaves of several plants, including economically important gramineous species, such as rice, wheat, maize and sugarcane, which can contain high numbers of the bacterium (Rothballer et al., 2008, Olivares et al., 1996, Kirchhof et al., 2001). These organisms are also found in forage grasses and tropical species, such as pineapple and banana (Weber et al., 1999). *Herbaspirillum seropedicae* has been isolated from sugarcane stems, leaves, and roots (Baldani et al., 1986, Olivares et al., 1996).

Herbaspirillum frisingense has been isolated from C_4 fiber plants, such as *Miscanthus spp.* and *Pennisetum purpureum* (Helman et al., 2011, Kirchhof et al., 2001, Rothballer et al., 2009, Rothballer et al., 2008). *Herbaspirillum rubrisubalbicans*, which colonizes the xylem vessels of sorghum leaves and sugarcane roots, is able to fix N (Döbereiner et al., 1995). *Herbaspirillum lusitanum* is another nitrogen-fixing bacterium that was isolated from root nodules of *Phaseolus vulgaris* (Baldani et al., 1996, Baldani et al., 2000). These *Herbaspirillum* species contribute to plant growth promotion and nutrient uptake via N-fixation, auxin production, and ACC deaminase (Helman et al., 2011).

Burkholderia spp., which are also endophytes, have been repeatedly isolated from roots, stems, and rhizosphere of maize, sugarcane, and rice. They were reported as plant-associated nitrogen-fixing bacteria (Baldani et al., 2000, Helman et al., 2011, Bhattacharjee et al., 2008, Riggs et al., 2001, Estrada et al., 2002). There are two major clusters in *Burkholderia*. The first cluster includes human pathogens, such as *Burkholderia cepacia*, which also have been proven as a biocontrol agent; the second cluster includes 25 species reported as non-pathogenic, plant-associated species. Plant-associated *Burkholderia spp.*, such as *B. unamea*, *B. tropica,* and *B. silvatlantica,* can increase plant nutrient availability via auxin production, ACC deaminase, biological nitrogen fixation, phosphate solubilization, and siderophore production (Helman et al., 2011, Estrada et al., 2002, Caballero-Mellado et al., 2007). The second cluster of *Burkholderia* has potential as plant growth promoters, but the close relationship to *B. cepacia* has made these strains controversial for use as commercial products.

Pseudomonas: *Pseudomonas* is very common bacteria that can be found in any agricultural soils. Many *Pseudomonas spp.* possess many PGP traits that have

biofertilizer potential. For example, *P. putida* can produce ACC deaminase that promotes root initiation and elongation by reducing the level of ethylene that restricts root development (Glick et al. 2007). *Pseudomonas putida* strains have been used as seed inoculants on crop plants to promote growth and increase yields (Glick et al., 2007). *Pseudomonas stutzeri* A1501, which has been used as a crop inoculant, was originally isolated from rice paddy soils. It can colonize the rhizosphere and fix nitrogen under microaerophilic conditions (Yan et al., 2008).

Azotobacter: Azotobacter, which is known as a cyst-forming genus is regarded as free-living nitrogen fixer. These cysts are dormant cells resistant to deleterious conditions, which consists of a contracted oval cell, called central body, covered with a two-layer capsule. This cyst-forming characteristic is different from endospore formation by *Bacillus* spp. in their mode of synthesis, their chemical content and their resistance properties. There are six well-known species: *A. chroococcum, A. vinelandii, A. beijerinckii, A. nigricans, A. armeniacus,* and *A. paspali. Azotobacter spp.* are usually isolated from soil. *Azotobacter spp.* can form cysts. Cyst formation is similar to the endospore formation in *Bacillus* strains, which allows it to exist under unfavorable environmental conditions for a long time. Reports have shown that application of *Azotobacter*, such as *A. chroococcum* and *A. vinelandii*, singly or mixed with other PGP strains can affect the seed germination and seedling elongation and improve the yield of wheat, tomato, potato, sorghum, and maize. *Azotobacter* can also secrete plant hormones such as auxins, gibberlllins, and cytokinins, produce siderophores, and solubilize phosphate. Some research has also shown that *Azotobacter* produces antifungal

compounds that can be used as a biocontrol agent (Hussain et al., 1987, Bagyaraj & Menge, 1978, Zahir et al., 2005, Zahir et al., 1997, Shende et al., 1984, Chen, 2006).

Bacillus is the most abundant genus in the rhizosphere, and the use of this group of bacteria as a plant growth promoter and a biocontrol agent has been known for many years. The substances released by this group of strains can increase nutrient availability in the plant rhizosphere (Barriuso et al., 2008). *Bacillus subtilis* is able to maintain stable contact with higher plants and promote their growth. *Bacillus licheniformis* shows considerable colonization of pepper and tomato and can be used as a biofertilizer/biocontrol agent under conventional management (Garc á et al., 2004). Some strains, such as *Bacillus megatorium, Bacillus mucilaginosus* and *Bacillus pumilus*, not only showed nitrogen-fixing activity but also phosphate- and potassium-solubilizing activity.

1.2.4 Phosphate-solubilizing bacteria

The use of phosphate-solubilizing bacteria (PSB) as a biofertilizer dates back to the 1950s. *Pseudomonas*, *Bacillus*, *Rhizobium*, *Burkholderia*, and *Erwinia* are reported to have phosphate-solubilizing activity that can convert the insoluble inorganic phosphate compounds, such as tricalcium phosphate, dicalcium phosphate, hydroxyapatite, and rock phosphate, to plant available forms (Rodr ǵuez & Fraga, 1999, Yao et al., 2006). Inoculants containing PSB can increase crop yield up to 200-500 kg/ha and save an estimated 30-50 kg of phosphate fertilizers per hectare (Chen, 2006).

1.2.5 Potassium-solubilizing bacteria

Potassium-solubilizing bacteria (KSB) are able to solubilize plant unavailable potassium to plant available forms by secretion of organic acid. Among this group of bacteria, *Bacillus mucilaginosus* are the most studied KSB that are used as a biofertilizer (Han & Lee, 2006). Other genera such as *Burkholderia* sp., *Paenibactillus* sp., and *Acidothiobacillus* sp. were also have been reported to be KSB (Parmar & Sindhu, 2013, Diep & Hieu, 2013). KSB are heterotrophic bacteria that can obtain energy and cellular carbon from preexisting organic material. KSB also play a very important role in maintaining soil structure and helping maintain the water in soil (Han & Lee, 2005).

1.3 MECHANISM AND ASSESSMENT

1.3.1 Nitrogen fixation

1.3.1.1 Nitrogen fixation mechanism

Nitrogen fixation is a biological process that reduces molecular N_2 into ammonia (NH_3), which can be easily absorbed by plants. During this conversion, an oxygen-sensitive enzyme, nitrogenase, plays a very important role in catalysis. Synthesis of nitrogenase is controlled by several *nif* geneswhich code for proteins related and associated with the fixation of atmospheric N into a form of N available to plants. These genes are found in both N-fixing bacteria and cyanobacteria. For example, Mo-nitrogenase (FeMoCo) is the most common form of nitrogenase, and there are three structural genes coding for the enzyme, including *nifD*, *nifK*, and *nifH*. In these three nif genes, *nifD* and *nifK* are responsible for Mo protein, and *nifH* is responsible for Fe protein. Other *nif* genes are responsible for the full assembly of FeMoCo. They are *nifB*,

nifQ, *nifE*, *nifN*, *nifX*, *nifU*, *nifS*, *nifV*, *nifY* and *nifH*. *nifS* and *nifU* help in the assembly of iron sulfur clusters, and *nifW* and *nifZ* aid with the maturation of nitrogenase. In addition, some diazotrophs, such as *Klebsiella* spp., contain genes like *nifF* and *nifJ* for electron transport to nitrogenase and regulatory *nifLA* genes for controlling the *nif* gene cluster (Franche et al., 2009, Dixon & Kahn, 2004). To elucidate and further understand the molecular N-fixing mechanism, the *nif* genes cluster that codes nitrogenase and nitrogenase activity should be studied.

1.3.1.2 Isolation of nitrogen fixing bacteria

Isolations of N-fixing bacteria are typically performed in N-free semisolid media with different carbon sources and pH values as described by Dőbereiner et al. (1995) and Bashan et al. (1993). Semisolid N-free media offer the possibility for N-fixing bacteria to find the right niche, within an oxygen gradient, for optimal conditions for N fixation. In this niche, the bacteria form a growth pellicle. After surface sterilization or direct inoculation to N- free medium, usually five to seven days after incubation at approximately 28 ℃, those vials showing a veil-like pellicle near the surface of the media are considered positive. The cultures from the positive vials are subjected to further purification steps by streaking them onto plates with specific agar media. Distinct colonies grown on these media are randomly selected and transferred to a fresh semi-solid, N-free medium for final purification (Dőbereiner et al., 1995, Bashan et al., 1993).

1.3.1.3 Evaluation of nitrogen fixing activity in vitro

The nitrogenase enzyme is responsible for N_2-fixation and can also reduce acetylene to ethylene. Hence, the acetylene reduction assay provides a highly sensitive and inexpensive method to quantify the relative nitrogenase enzyme activity in N-fixing samples.

The basic procedure for the acetylene reduction analysis is to prepare the potential N-fixing strains, incubate for 2-7 days, inject 10% of acetylene, replace the cap with a rubber septum, and continue to incubate for another 24 hours. The gas samples are then collected and analyzed by a gas chromatography. The the amount of ethylene produced is calculated as the main parameter toassess the bacterial N-fixing activity.

Other studies have suggested that another method, the ^{15}N isotope dilution (Boddey et al., 1995, Vlassak et al., 1973), can be used to estimate the N-fixing activity. The ^{15}N isotope dilution was first described by MacAuliffe et al. (1958) and is the most widely used for estimating field N fixation by legumes. The advantage of the ^{15}N isotope dilution method is that it makes, possible the separation of N taken up by the plant from fertilizer and soil from that fixed in the plant. Many workers have described the ^{15}N isotope dilution method as the most reliable measure of N_2-fixation (Bal & Chanway, 2012, Bal et al., 2012, Toro et al., 1998).

1.3.2 Phosphate solubilization

1.3.2.1 Mechanisms of phosphorus solubilization

There are many proposed theories that explain the mechanism of phosphorus solubilization. Among them, acid production theory and proton/enzyme theory are the most accepted theories.

The phosphate solubilizing microorganism (PSM) can secrete organic acids, such as gluconic acid and 2-ketogluconic acid, which are accompanied by the acidification of the medium. These organic acid can dissolve/chelate plant unavailable P (PO_4^{3-}) to plant available forms such as HPO_4^{2-} and $H_2PO_4^-$ (Khan et al., 2009). During this acidification process, the pH will decrease from 7.0 to as low as 2.0 (Gaur and Sachar 1980; Gaind and Gaur 1990, 1991; Illmer and Schinner, 1992). Gluconic acid is the most common frequent agent of mineral phosphate solubilization, which is produced by *Pseudomonas* sp., *Erwinia herbicola*, *Pseudomonas cepacia*, and *Burkholderia cepacia*. 2-ketogluconic acid, which is detected from the production of *Rhizobium leguminosarum*, *Rhizobium meliloti*, and *Bacillus firmus*, is another important organic acid during the mineral phosphate solubilization process. The mixture of lactic, isovaleric, isobutyric, and acetic acids was found from the production of *Bacillus spp.*. Other organic acids, such as glycolic, oxalic, malonic, and succinic acid, have also been identified among phosphate solubilizers. (Khan et al., 2009, Rodr ǵuez & Fraga, 1999). The enzymes phytate and phosphatase are known to be involved in helping releasing phosphorus from organic phosphatic compounds (phytate) and solubilizing plant available P in aquatic environments (phosphatase).

1.3.2.2 Isolation of phosphate-solubilizing bacteria

Detection and estimation of the phosphate solubilization ability of microorganisms have been possible through the use of plate screening methods. Phosphate solubilizers produce clearing zones around the microbial colonies in media. Defined selective media used insoluble mineral phosphates such as tricalcium phosphate (TCP; $Ca_3(PO_4)_2$) or rock phosphate (hydroxyapatite), which are contained in the media as the insoluble P source. Later, some researchers demonstrated that TCP in CO_2-saturated water is several times soluble than pure water. A large number of PSB strains were isolated but were found to be insufficient to contribute to the P plant nutrition. Bashan (2013) proposed that no metal-P compound can serve as the universal selection factor for PSB. The usage of a combination of 2 or 3 metal-P compounds together would be a good choice that would reduce the isolation number of potential PSB (Lopez et al., 2011). The selection of the metal-P candidates for potential PSB will depend on the type of soil (alkaline, acidic, or organic-rich) where the PSB will be used (Bashan et al., 2013).

1.3.2.3 Evaluation of phosphate solubilizing activity

Bacterial strains were evaluated for their ability to solubilize inorganic phosphate. There are two main methods for evaluating the solubilization activity, one is qualitative method and the other is quantitative. In the study by Lopez in 2011(Lopez et al., 2011), Modified Pikovskaya's medium (MPIK) containing several metal-P compound was used. A specific amount of the purified potential PSB strain was placed on this medium and incubated at 28℃ for 2 to 7 days. Appearance of halo zone around the colonies after 5

days incubation indicated phosphate solubilization. Halo size produced by the respective strain was measured and categorized as low, medium, or high on the basis of zone diameter.

The production of a halo on a solid agar medium should not be considered the ultimate test for P solubilization. When colonies grow without a halo after several replacements of the medium, an additional test in liquid media to assay P dissolution should be performed (Bashan et al., 2013). Quantitative determination of phosphate solubilizing activity is carried out by using the molybdenum-antimony colorimetric method (Jackson & Barak, 2005) .

The overall hypothesis of this dissertation was N_2-fixing and P-solubilizing isolated from different sources will promote plant growth and nutrient uptake on selected agricultural crop. We had two overall aims, the first aim was to conduct comprehensive studies of N_2-fixing and P-solubilizing bacteria in specific crop situations in Alabama, and the second aim was to characterize and select effective strains for use as biofertilizers. The specific objectives of this study were 1) to isolate putative N_2-fixing and P-solubilizing bacteria from target crops; 2) to characterize the isolated bacteria *in vitro*; and 3) to characterize the selected bacteria *in planta*. We had two strategies for isolating the effective N_2-fixing and P-solubilizing strains, one strategy was to isolate effective strains from energy crops which need little or no fertilizer; and the other strategy was to isolate effective strains from long-term rotations where nutrient application has been controlled.

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CHAPTER 2 ISOLATION AND CHARACTERIZATION OF NITROGEN-FIXING AND PHOSPHATE-SOLUBILIZING BACTERIA FROM ENERGY CROPS

2.1 INTRODUCTION

Nitrogen and phosphorus are essential macronutrients for plant growth. The major sources of N and P in agriculture are chemical fertilizers. Nitrogen in the soil can be lost due to denitrification, leaching, and volatilization. About 50% of nitrogen (Miransari, 2010, Vessey, 2003) in the soil can be absorbed by the crops. World phosphate is being depleted because the only phosphate source, rock phosphate, is in limited supply. When it is applied to soil, phosphate is quickly immobilized and cannot be absorbed by the plants. Chemical fertilization can cause toxic waste to accumulate in the soil, which will decrease soil pH and the enzyme activities of soil microbes, thereby affecting soil structure (Böhme & Böhme, 2006, Vessey, 2003). With regard to the cost, chemical fertilizers are unaffordable for many of the world's farmers who rely on organic matter as a nutrient source, as is found in subsistence agriculture. These problems associated with chemical fertilizers increase the need to develop economically viable and environmentally sustainable fertilizers to replace or partially replace chemical fertilizers. Thus, there is a growing opportunity for efficient biofertilizers.

Biofertilizers have been used as an alternative or complementary fertilizer for plants. As defined by Vessey in 2003, biofertilizers are living microorganisms that colonize the rhizosphere or the interior of the plant and promote growth by increasing the supply or availability of nutrients to the host plant. Nitrogen-fixing and phosphate-solubilizing bacteria represent the two major groups of biofertilizers. Free-living N-fixers,

such as *Azospirillum*, *Herbaspirillum*, *Burkholderia*, *Azotobacter*, *Bacillus* and *Acetobacter*, have frequently been evaluated as biofertilizers (Pesakovic et al., 2013, Anand & Chanway, 2013, Jetiyanon & Plianbangchang, 2012, Adesemoye & Kloepper, 2009, Hayat et al., 2010). Among P-solubilizing bacteria, some *Pseudomonas* and *Bacillus* strains have also been evaluated. To date, the majority of research and development of biofertilizers has been done outside Europe and North America.

The overall strategy of the work reported in this chapter was to find effective strains from selected perennial gramineae that exhibit massive growth each year and with no or limited input of chemical fertilizers.

In this study, giant reed (*Arundo donax* L.) and switchgrass (*Panicum virgatum* L.) were chosen as the target crops. Both of these plants are perennial grasses that have been extensively evaluated as sources for biofuel (Huang, 2012). In temperate climate zones, giant reed overwinter as rhizomes underground and each year it grows very quickly, reaching 4-6 m without fertilization. Switchgrass was included in this study as a model energy crop because it grows rapidly in temperate climate zones, can reach heights of 2-3 m on marginal lands, and requires low levels of fertilization. The rapid growth of energy crops with little or no fertilizer suggests a possible involvement of microorganisms that supply nutrients to the plants. Thus, it is meaningful to assess the plant-associated bacteria of these energy crops.

The objectives of this study were 1) to isolate, characterize and identify the N-fixing and P-solubilizing bacteria from rhizosphere soil and from inside roots and stems of giant reed and switchgrass; and 2) to evaluate the selected nitrogen-fixing and

phosphate-solubilizing strains for plant growth promotion activity, effects on root morphology, and increased nutrient uptake by plants under greenhouse conditions.

2.2 MATERIALS AND METHODS

2.2.1 Experiments *in vitro*

2.2.1.1 Isolation

Different nitrogen-free media were used for isolating different N-fixing bacterial species. Selective media with different carbon sources and pH values, including NFb (Bashan et al., 1993), JNFb (Olivares et al., 1996), LGI (Cavalcante & Döbereiner, 1988), and Ashby's (Lü & Huang, 2010) semi-solid/solid, were used in this study. These semi-solid nitrogen free media offer the opportunity for putative nitrogen fixers to form growth pellicles where they can absorb atmospheric nitrogen. Isolations of putative P-solubilizing bacteria were performed in SRSM (Vazquez et al., 2000) and modified Pikovaskaya's media (MPIK) (Lopez et al., 2011). The compositions of each medium are in Appendix A.

Rhizosphere soil, root, and stem samples from giant reed and switchgrass were collected in three different locations (greenhouse, Plant Breeding Unit, and Field Crop Unit) in central Alabama, USA. Samples were taken in July of 2010, and April of 2011 and 2012. Serially diluted rhizosphere soils were placed on NFb, JNFb, LGI, Ashby's, SRSM, and MPIK solid media and incubated at 28 ℃ for up to 5 days for bacteria selection. The roots and stems were washed with sterile distilled water and were surface disinfected by immersion in 70% ethanol for 1 minute and 50% commercial bleach for 3

minutes. They were then rinsed 3 times in sterilized distilled water. Stem and root samples (1.0g) were macerated separately using a KLECO grinder in sterilized distilled water and serially diluted to 10^{-4} to 10^{-5}. One hundred microliters of these dilutions were inoculated into JNFb, NFb, and LGI semi-solid media and plated onto SRSM and MPIK solid media. To confirm the elimination of all surface bacteria on roots and stems, the sterilized samples were placed on trypticase soy agar (TSA) and incubated at 28℃ for 24 hours before being ground. The putative N-fixing bacteria isolated from different N-free media were selected and inoculated into the corresponding semi-solid media for further purification. The formation of a pellicle (Plate 2-1) on top of the semi-solid media indicated that the bacteria had putative nitrogen-fixing activity. This procedure was repeated 3 to 4 times, and the putative nitrogen fixers were saved in a -80℃ freezer. Colonies that grew on SRSM and MPIK with clear zones (Plate 2-2) were selected and streaked onto the corresponding media for further purification. Purified bacteria on SRSM and MPIK with clear zones were selected and saved in a -80℃ freezer. These bacteria were regarded as putative phosphate solubilizers.

The enumeration of culturable N-fixing and P-solubilizing bacteria was carried out on NFb and MPIK media, respectively. Samples were taken from GH, PBU and FCU on April 2013. Colony forming units (CFU) were estimated by the Miles and Misra drop-plate method (Miles et al., 1938).

2.2.1.2 Characterization of plant growth-promoting traits of the isolates

Auxin production

The capacity of the isolated putative N-fixing and P-solubilizing bacteria to produce auxin (Frankenberger Jr, 1988, Gordon & Weber, 1951, Husen, 2003) was determined *in vitro*. Bacteria were grown on M-26 for 24 hours on a shaker at 150 rpm at room temperature as seed culture. Next, 15 µl of culture were inoculated to 1.5 ml minimal salt medium (MS) amended with 5 mM L-Tryptophan and grown again on the shaker for 48 hours. Tubes containing 1.5 ml bacteria broth were centrifuged at 12,000 rpm for 5 minutes and 0.5ml of the bacteria supernatant were added into 1ml $FeCl_3$-$HClO_4$ reagent. They were then set for 25 minutes under dim light after the color density reached its maximum. The pink color reaction indicated positive auxin production (Plate 2-3). Quantitative measurements of the color intensity were made using a UV-spectrophotometer (Thermo mode: Genesys 10S UV-Vis) at 530 nm absorbance. Auxin concentration was estimated by comparison with the standard curve prepared with indole-3-acetic acid (Sigma Lot#092K1235) and expressed in µg ml^{-1}. The composition and preparation of M-26 medium, MS medium, L-trytophan stock solution and $FeCl_3$-$HClO_4$ reagent are shown in Appendix A.

Determination of nitrogenase activity

The nitrogenase activity of the isolated putative N-fixing bacteria was determined using the acetylene reduction assay (Lopez et al., 2011, Holguin et al., 1992). Purified putative N-fixing bacteria were removed from the -80 ℃ freezer and grown on NFb for 48 hours. Half a loopful of bacteria was removed and placed into 9 ml sterilized water

tubes, and 100 µl of the cultures were then added to 4 ml of NFb semisolid medium (14 ml tubes) which were incubated at 28 ℃ for 7 days. The tube caps were then replaced by rubber caps, 10% (v/v) of air (1 ml) in the culture tubes was removed, and 10% (v/v) acetylene was injected into the culture tubes. The tubes were then incubated for 24 h and analyzed by a gas chromatography using a Hewlett-Packard 5890 Series II gas chromatograph (Agilent Technologies, Santa Clara, CA, USA) equipped with a hydrogen flame ionization detector (FID) to quantity the amount of ethylene converted from acetylene. Instrument operating conditions were set as follows: column (J&W GC column GS-GASPRO 60 m x 0.320 mm Serial No: USB467916H) with the oven temperature at 50 ℃; injector temperature at 250 ℃; detector temperature at 250 ℃; helium (He) as carrier gas with a flow rate at 2 ml/min; nitrogen (N_2) as makeup gas with a flow rate at 24 ml/min; hydrogen (H2) flow rate at 30 ml/min, and air flow rate at 300 ml/min. The nitrogen-fixing bacterium, *Azospirillum brasilense* 99B-817 (ATCC# 29710), served as the positive control. Results were expressed in nmoles ethylene 24 h^{-1} ml^{-1}. Each sample was analyzed 3 times.

Inorganic phosphate solubilization activity

All the isolated putative P-solubilizing bacteria were tested for their P-solubilizing activity. Three different formulations of MPIK, each with a different P source (0.38% $Ca_3(PO_4)_2$, 0.01% Fe PO_4, and 0.01% Al PO_4) was used for quantitative determination of the inorganic phosphate solubilizing capacity. A loopful of bacteria was inoculated into 25ml of the liquid MPIK media (without yeast exact and agar), which was stirred thoroughly and shaken at 150 rpm under 28 ℃. Bacterial broth samples were taken at 3,

8, and 14 days, and bacteria cells were precipitated by centrifugation at 12,000 rpm for 15 minutes. The amount of P in the supernatant was determined by the Molybdate-blue method (Murphy & Riley, 1962). A blue color reaction indicated a positive reaction for phosphate solubilization (Plate 2-4). Phosphorus concentration was estimated by comparing with the standard curve prepared with KH_2PO_4 (sigma Lot# 069K0342) and expressed in mg P L^{-1}. The composition was attached in Appendix A.

Siderophore production

Chrome azurol S (CAS) agar (Husen, 2003, Schwyn & Neilands, 1987) was used to qualitatively describe the siderophore production. Bacteria were grown on TSA for 24-48 hours and then a half loop of bacteria was transferred to 9 ml distilled water tube. The bacterial suspension was vortexed thoroughly; CAS medium was divided into 4 quadrants; each quadrant was pipetted with 15 µl bacteria suspension. Orange halos around the growing bacterial colonies confirmed the siderophore production after 48-72 hours incubation (plates 2-5). The CAS agar was a mixture of four solutions that were prepared and sterilized separately before mixing. Fe-CAS indicator solution, buffer solution and glucose/mannitol solution were sterilized by an autoclave; casamino acid was sterilized by 0.2 µM filter. The composition of CAS medium is shown in Appendix A.

2.2.1.3 Identification of isolates--molecular characterization and phylogenetic analysis

Taxonomic classification of each strain was based on the partial sequence of 16S rDNA. DNA was extracted and amplified with universal bacterial primers: 8F (5'-AGAGTTTGATCCTGGCTCAG -3') and 1492R (5'-ACGGCTACCTTGTTACGACTT - 3'). PCR was performed using Lucigen EconoTaq Plus Green 2X master mix (Lucigen Corp.) with cycling parameters: initial denaturation at 95 ℃ for 5 min; 31 cycles of 94 ℃ for 1 min, 57 ℃ for 45 sec, 70 ℃ for 2 min; and a final extension at 70 ℃ for 10 min. All sequences were blasted against the type strains in the ribosomal database project to identify bacterial taxa of each strain.

2.2.2 Experiments *in planta*

2.2.2.1 Treatments and experimental design

All pot experiments were conducted in a greenhouse. Temperature within the greenhouse was maintained at 27 ± 2 ℃ throughout the experiment. Round pots, 15 cm in diameter and 12 cm deep, were filled with 1.7 kg of thoroughly mixed field soil, with adjustment of soil pH to 7.0 by adding lime, taken from a field at the E.V. Smith Research Center, Plant Breeding Unit of Alabama Agricultural Experiment Station near Tallassee, Alabama. The soil was loamy sand, with a texture of 76.4% sand, 2.0% clay, and 21.6 % silt. It contained 0.32% total N, 59 lbs P/acre, 161 lbs K/acre, 97 lbs Mg/acre, 886 lbs Ca/acre, 3 lbs S/acre, 0.42 lbs B/acre, 6.9 lbs Zn/acre, 47 lbs Mn/arce, 34 lbs Fe/acre, and 0.8 lbs Cu/acre.

Prior to planting, soil water content in pots was adjusted to 60% field capacity, and two fertilizers containing P, K and S were applied to all pots at the recommended rates adjusted according to the soil test. Specifically, $CaSO_4 \cdot 2H_2O$ was added into pots at the rate of 0.034 g pot^{-1} to provide adequate S nutrient for growth of corn and wheat. Because the field soil was already high in P and K, additional P and K fertilizers were not added. Two corn or eight wheat seeds were sowed in each pot at a depth of 1.5 inches (corn) or 0.5 inches (wheat). Treatments of selected N-fixing and P-solubilizing bacterial strains with the concentration of 1.0×10^7 CFU ml^{-1} were then applied to corn and wheat seeds at a rate of 1 ml $seed^{-1}$.The seeds were then covered with soil and soil water content in pots was adjusted to 80% field capacity. After germination, only one corn or six wheat seedlings were kept in each pot. All pots were watered the same amount each morning to provide enough water for germination and growth.

Plant growth promotion and nutrient uptake assay for N-fixing bacteria

The experimental design was a complete randomized design with six replicates. Six putative nitrogen-fixing bacteria (ARA: above 100 nmol ethylene 24 h^{-1} ml^{-1}) that were selected for growth promotion and nutrient uptake test in corn (*Zea mays*) and wheat (*Triticum aestivum*) included NNA-14, NNA-17, NNA-19, NNA-20, NP-3, and NXU-38. Besides the six N-fixing bacterial strains treatments, one positive control and one water blank were also included. Each crop test was repeated twice. Corn was chosen because of its fast growth, and wheat was chosen because of its sensitive response to nitrogen.

The selected bacterial strains were grown on TSA to obtain a single colony. After growing for 48-72 hours at 28 ℃, a portion of resulting growth was placed in distilled H_2O. Serial dilutions were made and the bacterial suspensions were adjusted to a concentration of 10^7 cfu ml^{-1}.

Plant growth promotion and nutrient uptake assay for Phosphate-solubilizing bacteria

The experimental design was a complete randomized design with eight replicates. The top 19 strains based on the highest average value of 3d, 8d, and 14d inorganic phosphate-solubilizing data were chosen for greenhouse test.

This phosphate-solubilizing test was designed as a quick screen test. Corn was selected as the only host crop. The test was repeated twice.

2.2.2.2 Data collection

Plant growth promotion and nutrient uptake assay for Nitrogen-fixing bacteria

Corn plants were harvested at V5 growth stage (28 DAP). Plant growth-promoting parameters, including plant height, fresh and dry weight of shoots and roots, and chlorophyll content (SPAD 502 meter), were measured. Root morphology parameters were determined by using the WinRhizo Arabidopsis software v2009c 32 bit (Regent Instruments, Quebec, Canada) connected to Epson XL1000 professional scanner. The parameters obtained from the root scanner included total root length (cm), root surface area (cm^2), root volume (cm^3), number of tips and 0-0.5 mm fine roots. After root

morphology determination, plant samples were dried in an oven at 70 ℃ for 72 hours to obtain the dry weight. All above-ground tissues were sent to Waters Agricultural Laboratories, Inc (Camilla, Georgia) for nutrient analysis.

Wheat plants were harvested at 4 weeks after planting (28 DAP). Plant growth-promoting parameters including plant height, fresh and dry weight of shoots and roots, chlorophyll content (SPAD 502 meter), number of leaves, and number of tillers were measured. Root morphology determination in wheat was conducted in the same manner as corn. Six replicates of shoot tissues from each of the repeated tests were sent to Waters Agricultural Laboratories, Inc (Camilla, Georgia) for nutrient analysis.

Plant growth promotion and nutrient uptake assay for Phosphate-solubilizing bacteria

Plant growth-promoting parameters, including plant height, fresh shoot and root weight, dry shoot and root weight (70 ℃), and chlorophyll content (SPAD 502 meter), were measured. Six replicates of shoot tissues were sent to Waters Agricultural Laboratories, Inc (Camilla, Georgia) for nutrient analysis.

2.2.2.2 Statistical analysis

Growth promotion data collected from replicated tests in greenhouse were pooled and subjected to analysis of variance using the PROC GLIMMIX procedure in SAS. The critical *P*-value of 0.05 was used as cutoff for testing fixed effects, and determination of differences in least-squares means was based on adjusted *P*-value obtained by using the

option ADJUST=DUNNETT (Differences with the control) or ADJUST=SIM (Differences between times) in the LSMEANS statement.

Given that heterogeneity in growth characteristics of wheat is too complex to be explained only under univariate analysis because all parameters are correlated, multivariate approaches were used to evaluate growth characteristics of wheat. Canonical discriminant analysis was implemented by using SAS PROC CANDISC procedure. Total-sample standardized canonical coefficients and total variation explained by each canonical variable were obtained. A graph was built for the first two canonical variables, showing the 95% confidence ellipses of the means vectors for each bacterium treatment, using SAS GPLOT procedure after computing the 95% confidence ellipse in order to visualize the multivariate trends of all treatments jointly.

2.3 RESULTS

2.3.1 Isolation and enumeration of nitrogen-fixing and phosphate-solubilizing bacteria

Based on cultural and morphological characteristics, a total of 190 putative N-fixing bacteria and 61 putative P-solubilizing bacteria were obtained from giant reed and switchgrass from rhizosphere and inside root and stem. One hundred twenty-seven putative N-fixing strains and 25 putative P-solubilizing strains were obtained from giant reed, and 63 putative N-fixers and 31 putative P-solubilizers were obtained from switchgrass.

When isolating the putative N-fixing and P-solubilizing bacteria, the rhizosphere soil pH vaules varied between 5.8 to 6.9. An average pH of 6.32 was recorded for giant reed rhizosphere soil and 6.08 for switchgrass rhizosphere soil.

The population evaluations were repeated 3 times, and averaged data are presented in table 2-4. The highest population of putative N-fixing bacteria was found in the giant reed rhizosphere at 3.6 X 10^5 cfu ml^{-1}, and the lowest was found in switchgrass stem at 6.9x10^1 cfu ml^{-1}. For putative P-solubilizing bacteria, the highest population was found in the rhizosphere of giant reed rhizosphere at 9.6x10^4 cfu ml^{-1}, and the lowest population was found inside switchgrass stems at 1.4 X 10^1 cfu ml^{-1} (Table 2-4).

2.3.2 Confirmation of N-fixing activity and evaluation of other PGP traits

All putative N-fixing bacteria that were isolated from giant reed and switchgrass were transferred to the N-free medium 3 to 4 times and afterwards, all formed pellicles in semi-solid N-free medium. Acetylene reduction assays (ARA) were conducted to confirm and assess the N-fixation. The results showed that all the strains with pellicle formation were ARA positive. In the ARA tests, the levels of bacterial conversion of acetylene (C_2H_2) to ethylene ranged (C_2H_4) from 40 to 350 nmol C_2H_4 24 hrs^{-1} ml^{-1} with different strains.

Analysis of other traits reported to be associated with plant growh promotion revealed that 96.9% of the isolated N-fixing bacteria produced auxin and ranged from 1.48 to 119.11 μg ml^{-1}, and that 19.4% of the N-fixing bacteria produced remarkably

high amounts of auxin that were more than 50 μg ml^{-1}. In addition, 85% of the isolated N-fixing bacteria produced siderophores.

2.3.3 Confirmation of P-solubilizing activity and evaluation of other PGP traits

P-solubilizing activity of the isolated putative P-solubilizing bacteria from giant reed and switchgrass was comfirmed by molybedate-blue method. Results showed that all the putative P-solubilizing bacteria had P-solubilizing activity on the 3 d test and that 16.4% of the P-solubilizers lost the P-solubilizing activity at 8 d test. The 14 d test showed that the P-solubilizing activity could either increase or decrease. Roughly half the P-solubilizers had remarkably high P-solubilizing activity: the average of 3 d, 8 d and 14 d tests data were up to 2.0 mg P ml^{-1}.

Other PGP traits showed that 88.5% of the isolated P-solubilizing bacteria produced auxin and ranged from 1.55 to 110.58 μg ml^{-1}, and that 9.8% P-solubilizing bacteria produced remarkably high amounts of auxin which were over 50 μg ml^{-1}. In additon, 91.8% of the isolated P-solubilizing bacteria produced siderophores.

2.3.3 Molecular indentification

One hundred twenty-two of the total 190 isolated N-fixing strains were identified by using 16S rDNA. Results represented a large diverse with 24 genera including *Arthrobacter* (1), *Bacillus* (3), *Beijerinckia* (1), *Burkhloderia* (3), *Chryseobacterium* (2) *Citrobacter* (1) *Curtobacterium* (3), *Ensifer* (1), *Enterobacter* (4), *Flavobacterium* (4),

Leclercia (1), *Lysobacter* (1), *Microbacterium* (5), *Pantoea* (2), *Phyllobacterium* (1), *Pseudomonas* (16), *Psychrobacillus* (1), *Rahnella* (1), *Rhizobium* (2), *Shinella* (1), *Sphinogbacterium* (2), *Sphingomonas* (3), *Sporosarcina* (1), *Stenotrophomonas* (1), and *Xanthomonas* (3). N-fixing strains in *Beijerinckia*, *Citrobacter*, *Curtobacterium*, *Ensifer*, and *Sporosarcina* genera were only isolated from giant reed. The number inside of the parentheses indicated the different species in each genus. N-fixing strains in *Lysobacter* and *Phyllobacterium* genera were only found in switchgrass rhizosphere.

Fourty-five of the total 61 of the isolated P-solubilizing strains were identified using 16S rDNA. The results represented a diverse of 9 genera including *Burkholderia* (2), *Citrobacter* (1), *Enterobacter* (1), *Flavobacterium* (2), J*anthinobacterium* (1), *Microbacterium* (1), *Panteoea* (2), *Pseudomonas* (13) and *Sphingobium* (1). The number inside the parentheses indicates the number of different species in each genus. P-solubilizing strains in *Microbacterium* genus were only isolated from giant reed rhizosphere. P-solubilizing strains in *Citrobacter*, *Flavobacterium*, and *Janthinobacterium* genera were only isolated from switchgrass.

2.3.4 Plant growth promotion and nutrient uptake tests on selected N-fixing bacteria

Plant growth promotion and nutrient uptake on corn

Application of selected N-fixing bacteria to corn seeds resulted in stimulated root growth and development of root morphology rather than plant height and biomass accumulation, except for the strain NNA-19, which significantly increased fresh weight of root by 13.2% compared to the control (Table 2-7). Applying NNA-14 to corn seeds

significantly increased root length, surface area, and fine roots by 14.4%, 11.8%, and 17.3%, respectively (P<0.05). In addition, enhancement in root surface area, root volume, and number of root tips in corn was observed after applying NNA-19 to seeds. All selected N-fixers tended to increase number of root tips for corn (Table 2-7).

For nutrient uptake, of the six selected N-fixers only NNA-14 stimulated N uptake of corn significantly (P<0.05) (Table 2-8). Nitrogen content in shoot of corn was even greater when NNA-14 was applied than when the positive control, 99B-817, was applied.

Plant growth promotion and nutrient uptake on wheat

Application of selected N-fixing bacteria to wheat seeds resulted in stimulated root growth and development in root biomass accumulation and root morphology when compared to the control (Table 2-9). Specifically, applying NNA-14 to wheat seeds resulted in significantly increased fresh and dry weights of root, surface area of roots, and number of root tips (P<0.05); NNA-19 increased fresh and dry weights of root and number of root tips; NXU-38 increased root length, surface area, number of root tips, and fine roots; NNA-17 increased number of root tips; and NP-3 increased fresh weight of root. For NNA-19, maximum increases among the parameters over the control were 31.5% (fresh weight of root), 25.0% (dry weight of root), and 95.9% (number of root tips). Increases by NXU-38 were 20.7% (root length), 12.5% (surface area), and 25.8% (fine roots), greater than the positive control, *Azospirillum brasilense* 99B-817. However, the

six selected N-fixers as well as the positive control failed to promote nutrient uptake of N, P, and K in wheat (Table-2-10).

The results presented above suggest that plant growth promotion by bacteria might not always be associated with nutrient uptake of corn and wheat in early growth stage (4 weeks after planting).

Given that the univariate ANOVA provided no information on the multivariate, all growth-promotion data of wheat were pooled together and subjected to discriminant function analysis. The first two canonical variables (Can 1 and Can 2) explained 67.8% of total variation (Table 2-11). In addition, tests of dimensionality for the discriminant analysis indicated that only the first two dimensions were statistically significant at α=0.05 level. Therefore, Can 1 and Can 2 were selected for further analysis and interpretation.

Canonical discriminant analysis facilitated the evaluation of all measured parameters through weighing each original parameter according to its contribution on each canonical variable. Can 1 was positively weighted by number of root tips (0.74) and fine roots (0.32); Can 2 was positively weighted by number of leaves (0.39), number of tillers (0.40), root fresh weight (0.50), root dry weight (0.40), root surface area (0.38), and root volume (0.63) (Table 2-11). This demonstrated that all of the parameters listed above were important both to discriminate and to classify strain treatments.

Based on the mean classes of Can 1 and Can 2, (Figure 2-1) 67.8% of the total variation could be explained and 95% confidence ellipses of the multivariate mean vectors for each strain treatment were determined. The confidence ellipses allowed us to

discriminate the six treatments from the blank control and the positive control. Overall, strains NNA-14, NNA-19, and NXU-38 significantly promoted wheat growth when compared to the blank control.

2.3.5 Plant growth-promotion and nutrient uptake tests on selected P-solubilizing bacteria

Analysis of variance indicated that both plant growth promotion and nutrient uptake in corn were significantly increased by selected P strains. Specifically, 7 of the 19 P-strains, including P-2, P-7, P-10, PM-10, PSRS-7, PXU-65, and PXU-67, promoted root biomass accumulation significantly ($P<0.05$) (Table 2-11); P-2 and P-10 also increased dry weight of corn shoot. For nutrient uptake, only PXU-56 promoted P uptake in corn, whereas N uptake was increased by 11 of the 19 P-strains, three of which, including PXU-56, PXU-69, and P-8, also promoted K uptake (Table 2-12). Maximum increases among the parameters over the blank control were 59.4% in fresh weight of root and 18.3% in dry weight of shoot by P-10, and 60.0% in dry weight of root and 6.2% in SPAD by PXU-67.

2.4 CONCLUSIONS AND DISCUSSIONS

This study evaluated the nitrogen-fixing bacteria and phosphate-solubilizing bacteria from the rhizosphere soil and internal root and stem of two energy crops, giant reed and switchgrass, by using selective media. The populations were evaluated based on the Miles and Misra method, and the diversity was evaluated based on 16S rDNA. The N-fixers were confirmed by acetylene reduction assay (ARA) and P-solubilizers were

confirmed through both qualitative and quantitative determination. Other PGP traits, such as auxin and siderophore production, were determined *in vitro* to evaluate the plant growth-promotion potential. This is the first report of culture-based isolation and evaluation of nitrogen-fixing bacteria and phosphate-solubilizing bacteria on giant reed and a relative comprehensive evaluation of cultural N-fixing and P-solubilizing bacteria on switchgrass. There are a few reports on evaluating the bacterial community on switchgrass. For example, Bahulikar et al. (2014) studied the diversity of N-fixing bacteria (culture-independent approach) on switchgrass. Gagne-Bourgue et al. (2013) evaluated endophytic bacteria from different cultivars of switchgrass and their antimicrobial and growth promotion potential.

The 25 N-fixing genera encountered in this study include *Arthrobacter*, *Bacillus*, *Beijerinckia*, *Burkholderia*, *Chryseobacterium*, *Citrobacter*, *Curtobacterium*, *Ensifer*, *Enterobacter*, *Flavobacterium*, *Leclercia*, *Lysobacter*, *Microbacterium*, *Pantoea*, *Phyllobacterium*, *Pseudomonas*, *Psychrobacillus*, *Rahnella*, *Rhizobium*, *Shinella*, *Sphinogbacterium*, *Sphingomonas*, *Sporosarcina*, *Stenotrophomonas*, and *Xanthomonas*. Some of these have been reported as N-fixers, such as *Burkholderia* (Jha et al, 2009), *Pantoea* (Feng et al, 2005), *Bacillus* (Madhiyan et al, 2011), *Pseudomonas* (Muthukumarasamy et al, 2007), *Enterobacter* (Peng et al, 2009), *Rhizobium* (Zahran, 1999), and *Sphingomonas* (Xie and Yokota, 2006).

We observed that among the putative N-fixing strains, bacterial pellicle formation was consistent with nitrogenase activity data. To observe the consistency between pellicle formation and N_2-fixing activity, several preconditions need to be met and the suggestions are: 1) the N-free selective medium should be kept pure, a slight

contamination may lead to false conclusions; 2) when isolating N-fixing bacteria, bacteria should be transferred to N-free, semi-solid medium more than 3 times in order to increase the probability of real N-fixers; and 3) the bacteria should be allowed to grow as long as possible because some N-fixing bacteria express nitrogenase activity only when they are accumulated in sufficient numbers (Wani, 1981).

Besides the N-fixing activity and P-solubilizing activity, other PGP traits such as auxin and siderophore production, were also evaluated. Both auxin production and siderophore production are very important plant growth promotion and nutrient uptake characteristics.The production of auxin could improve plant growth and nutrient uptake by increasing the number of root hairs and lateral roots (de Jesus Santos et al., 2014, Lambrecht et al., 2000). Siderophore production by bacteria may enhance plant growth and nutrient uptake by increasing the availability of Fe near the root or by inhibiting the colonization of plant pathogen or other harmful bacteria on roots. All strains from giant reed and switchgrass produced siderophores or had P-solubilizing activity.

Selected N-fixers and P-solubilizers were tested on corn and/or wheat for growth promotion and nutrient uptake in greenhouse. Consistently, two N-fixers, NNA-14 and NNA-19, promoted growth of corn in root morphology and growth of wheat in both biomass accumulation and root morphology. These findings suggest that wheat might be a better crop for testing N-fixing bacteria than corn under the same growth conditions. Seven of the 19 P-solubilizing strains promoted growth of corn, wheareas, interestingly, 11 of the P-solubilizing strains increased nutrient uptake of nitrogen rather than phosphorus.

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Table 2-1 Information for selected nitrogen-fixing bacteria.

Strain name	Colonization	Isolation medium	ARA (nmol ethylene 24 h^{-1} ml^{-1})	auxin (μg ml^{-1})	P solubilization at 8 d (mg PL^{-1})	Siderophore production (+/-)	ID
NNA-14	Giant reed root	NFb	350	50.07	N/A	-	*Sphingomonas trueperi*
NXU-38	Giant reed rhizosphere	JNFb	264	118.25	3.44	+	*Enterobacter oryzae*
NNA-19	Giant reed root	NFb	243	43.31	3.19	+	*Sphingomonas trueperi*
NNA-17	Giant reed root	NFb	183	40.38	2.74	+	*Sphingomonas trueperi*
NP-3	Giant reed rhizosphere	Ashby's	146	2.34	2.97	-	*Psychrobacillus psychrodurans*
NNA-20	Giant reed root	NFb	130	49.55	2.54	-	*Sphingomonas trueperi*
99B-817	Positive control	N/A	1265	N/A	N/A	N/A	*Azospirillum brasilense*

Table 2-2 Information for selected phosphate-solubilizing bacteria.

Strain Name	Colonization	Auxin ($\mu g\ ml^{-1}$)	P solubilization at 3/8/14 d ($mg\ P\ L^{-1}$)	Siderophore production (+/-)	ID
PXU-56	SG rhizosphere PBU	46.62	2.5/3.3/2.4	-	*N/A*
PXU-69	SG rhizosphere FCU	6.78	2.7/2.7/2.3	+	*Janthinobacterium lividum*
PXU-66	A rhizosphere GH	5.24	2.2/2.8/2.6	+	*Pseudomonas mandelii*
P-8	SG rhizosphere GH	N/A	2.8/2.8/2.7	+	*Pseudomonas mandelii*
P-6	A rhizosphere GH	31.17	2.6/2.6/2.6	-	*Microbacterium maritypicum*
PASGS-7	SG rhizosphere GH	24.48	2.9/2.3/2.5	-	*Citrobacter freundi*
PXU-54	SG rhizosphere PBU	110.58	2.9/3.0/2.2	-	*Sphingobium chungbukense*
PXU-53	SG rhizosphere PBU	11.89	2.8/2.9/2.5	+	*Pseudomonas jessenii*
P-2	SG rhizosphere GH	10.46	2.5/2.5/2.6	+	*Pseudomonas jessenii*
P-10	A rhizosphere GH	12.56	2.7/2.8/2.6	+	*Pseudomonas jessenii*
PXU-72	SG rhizosphere GH	6.63	2.5/2.1/1.8	+	*N/A*
PXU-71	SG rhizosphere FCU	13.5	3.7/1.9/1.7	+	*Pseudomonas reinekei*
PXU-67	A rhizosphere PBU	11.41	2.7/2.5/2.1	+	*Pseudomonas vancouverensis*
PXU-65	A rhizosphere PBU	9.78	3.2/2.3/2.8	+	*Pseudomonas vancouverensis/jessenii*
PM-8	SG root GH	10.84	2.0/2.9/2.7	-	*Pseudomonas frederiksbergensis*
PM-10	A rhizosphere GH	17.3	2.2/1.9/1.8	+	*Pseudomonas taiwanensis*
PSRS-7	SG rhizosphere GH	6.63	2.6/1.9/1.4	+	*Pseudomonas reinekei*
P-7	SG rhizosphere GH	20.08	2.8/2.2/2.2	+	*Pseudomonas reinekei*

Note: A:*Arundo*; SG: switchgrass; FCU: Field Crop Unit of E.V. Smith Research Center; PBU: Plant Breeding Unit of E.V. Smith Research Center; GH: field near greenhouse; N/A: not available

Table 2-3 Description of the source and the numbers of putative nitrogen-fxing bacteria (NFB) and phosphate solubilizing bacteria (PSB)

Host plant	Origin	Putative NFB	Putative PSB
Giant reed	Rhizosphere	57	22
	Root	23	3
	Stem	40	0
Switchgrass	Rhizosphere	32	27
	Root	15	5
	Stem	16	4
Giant reed or switchgrass	N/A	7	-

N/A---not available

Table 2-4 Enumeration of putativeN-fixing and P-solubilizing bacteria isolated from giant reed and switchgrass

Samples	Soil pH	NFb (mean cfu/g)	MPIK(mean cfu/g)
ARhs	6.32	$3.6x10^5$	$9.6x10^4$
AR	---	$9.0x10^3$	$3.0x10^2$
ASt	---	$3.3x10^2$	$2.1x10^1$
SGRhs	6.08	$2.3x10^5$	$6.4x10^4$
SGR	---	$7.5x10^4$	$1.1x10^3$
SGSt	---	$6.9x10^1$	$1.4x10^1$

ARhs (Giant reed rhizosphere soil); AR (Giant reed root); ASt (Giant reed stem); SGRhs (Switchgrass rhizosphere soil); SGR (Swithgrass root); and SGSt (Swtichgrass stem).

Table 2-5 Characterization of putative N-fixing bacteria isolated from different locations and different plant parts

Strain	Pellicle (+/-)	ARA (nmol c2h4/24h ml)	Auxin (ug/ml)	Siderophore (+/-)	Genus	Species	Source	Medium
N1	+	47	2.30	-	*Sphingobacterium*	*multivorum*	A-rhs	JNFb
N2	+	46	1.97	-	*Phyllobacterium*	*brassicacearum*	SG-rhs	JNFb
N3	+	56	2.08	-	*Stenotrophomonas*	*maltophilia*	SG-rhs	JNFb
N4b	+	45	1.51	-	*Flavobacterium*	*aquidurense*	A-rhs	JNFb
N5	+	43	1.59	-	*Lysobacter*	*enzymogenes*	SG-rhs	JNFb
N6	+	47	6.44	+	*Pseudomonas*	*moraviensis*	SG-rhs	JNFb
N7	+	57	19.48	-	*Rahnella*	*aquatica*	A-rhs	JNFb
N8	+	45	7.23	-	*Microbacterium*	*maritypicum*	A-rhs	JNFb
N9	+	51	5.99	-	*Microbacterium*	*maritypicum*	A-rhs	JNFb
N10	+	50	6.40	-	*Sporosarcina*	*psychrophila*	A-rhs	JNFb
N11a	+	46	40.08	+	*Microbacterium*	*resistens*	A-rhs	JNFb
N11b	+	47	1.85	-	*Chryseobacterium*	*indologenes*	A-rhs	JNFb
JX-AE1	+	48	7.45	+	*Pseudomonas*	*tremae*	A-stem	NFb
JX-AE2	+	48	6.10	-	*Curtobacterium*	*pusillum*	A-stem	NFb
JX-AE3	+	43	5.72	-	*Curtobacterium*	*flaccumfaciens*	A-stem	NFb
JX-AE4	+	50	4.67	+	*Xanthomonas*	*campestris*	A-stem	NFb

JX-AE5	+	47	10.20	+	*Bacillus*	*megaterium*	A-stem	NFb
JX-AE6	+	48	2.53	+	*Bacillus*	*pumilus*	A-stem	NFb
JX-AE7	+	41	13.62	+	*Bacillus*	*megaterium*	A-stem	NFb
JX-AE8	+	41	6.10	+	*Bacillus*	*subtilis*	A-stem	NFb
JX-AE9	+	40	15.42	+	*Curtobacterium*	*luteum*	A-stem	NFb
JX-SE2	+	47	6.06	+	*Xanthomonas*	*translucens*	SG-stem	NFb
JX-SE3	+	42	9.59	-	*Microbacterium*	*testaceum*	SG-stem	NFb
JX-SE4	+	46	11.40	+	*Xanthomonas*	*campestris*	SG-stem	NFb
JX-SE5	+	43	14.63	+	*Microbacterium*	*flavum*	SG-stem	NFb
JX-SE6	+	41	10.68	+	*Chryseobacterium*	*indologenes*	SG-stem	NFb
JX-SE7	+	46	5.69	+	*Xanthomonas*	*campestris*	SG-stem	NFb
JX-SE8	+	44	12.71	-	*Sphingomonas*	*panni*	SG-stem	NFb
JX-SE9	+	49	20.01	-	*Microbacterium*	*arborescens*	SG-stem	NFb
Arundo Stem	+	66	6.14	+	N/A		A-stem	NFb
Arundo A1	+	49	2.98	+	N/A		A-stem	NFb
Arundo A2	+	47	8.05	+	N/A		A-stem	NFb
Arundo A3	+	48	28.95	+	N/A		A-stem	NFb
Arundo A4	+	47	44.92	-	*Enterobacter*	*asburiae*	A-stem	NFb

Arundo A5	+	48	48.01	+	*Enterobacter*	*nimipressuralis*	A-stem	NFb
SG S1	+	47	50.90	+	N/A		SG-rhs	NFb
SG S2	+	46	48.83	+	N/A		SG-rhs	NFb
NJA-1	+	48	3.43	+	*Burkholderia*	*gladioli*	A-root	JNFb
NJA-2	+	48	5.69	+	*Pseudomonas*	*koreensis*	A-root	JNFb
NJA-3	+	49	4.37	+	*Burkholderia*	*plantarii*	A-root	JNFb
NJA-4	+	55	4.52	+	*Burkholderia*	*plantarii*	A-root	JNFb
NNA-1	+	47	9.07	+	*Burkholderia*	*plantarii*	A-root	NFb
NNA-2	+	47	2.19	+	N/A		A-rhs	NFb
NNA-3	+	50	10.31	+	N/A		A-rhs	NFb
NNA-4	+	48	5.35	+	*Pseudomonas*	*simiae*	A-stem	NFb
NNA-5	+	47	114.94	+	*Rahnella*	*aquatilis*	A-stem	NFb
NAAS-2	+	47	23.05	+	*Ensifer*	*adhaerens*	A-rhs	NFb
NAAS-11	+	47	113.18	+	N/A		A-rhs	NFb
NAAS-8B	+	53	113.21	+	N/A		A-rhs	NFb
NAAS-4	+	46	0.00	-	N/A		A-rhs	NFb
NAAS-8B	+	50	3.96	-	N/A		A-rhs	NFb
NAAS-8C	+	47	60.15	+	N/A		A-rhs	NFb

NAAS-11	+	46	115.32	+	N/A		A-rhs	NFb
NNA-6	+	49	2.19	+	N/A		A-rhs	NFb
NJA-5	+	48	62.78	+	*Pantoea*	*ananatis*	A-stem	JNFb
NJA-6	+	47	63.19	+	N/A		A-stem	JNFb
NJA-7	+	42	2.00	+	N/A		A-stem	JNFb
NJA-8	+	48	1.82	+	N/A		A-rhs	JNFb
NA-Soil	+	47	56.20	+	N/A		A-rhs	NFb
NAAS-6	+	47	2.08	+	*Flavobacterium*	*johnsoniae*	A-rhs	NFb
NAAS-10A	+	48	1.51	+	N/A		A-rhs	NFb
NJA-9	+	47	5.46	+	*Burkholderia*	*gladioli*	A-root	JNFb
NNA-7	+	47	45.04	+	*Pantoea*	*ananatis*	A-stem	NFb
NNA-8	+	59	47.25	-	*Sphingomonas*	*pseudosanguinis*	A-stem	NFb
NNA-9	+	49	47.89	+	N/A		A-stem	NFb
NNA-10	+	47	115.32	+	*Rahnella*	*aquatilis*	A-stem	NFb
NNA-11	+	47	3.39	+	N/A		A-rhs	NFb
NAAS-9	+	47	8.32	+	N/A		A-rhs	NFb
NAAS-9B	+	47	30.75	+	N/A		A-rhs	NFb
NJA-10	+	48	31.69	+	N/A		A-rhs	JNFb

NJA-11	+	46	29.55	+	N/A		A-rhs	JNFb
NNA-12	+	47	42.59	+	N/A		A-rhs	NFb
NJA-16	+	47	0.00	+	N/A		A-root	JNFb
NNA-21	+	45	48.80	-	*Pseudomonas*	*fragi*	A-stem	NFb
NNA-20	+	130	49.55	-	*Sphingomonas*	*trueperi*	A-root	NFb
NNA-19	+	243	43.31	+	*Sphingomonas*	*trueperi*	A-root	NFb
NNA-18	+	196	48.19	+	*Sphingomonas*	*trueperi*	A-root	NFb
NNA-17	+	183	40.38	+	*Sphingomonas*	*trueperi*	A-root	NFb
NNA-16	+	46	4.22	+	*Burkholderia*	*gladioli*	A-stem	CR-NFb
NNA-15	+	46	57.40	+	*Pseudomonas*	*fragi*	A-stem	CR-NFb NFb
NNA-14	+	350	50.07	-	N/A		A-root	NFb
NNA-13	+	47	49.47	+	*Bacillus*	*megaterium*	A-rhs	NFb
NJA-16	+	50	0.00	+	N/A		A-root	JNFb
NNA-12	+	41	3.58	+	N/A		A-root	NFb
NJA-11	+	49	51.20	+	N/A		A-rhs	JNFb
NJA-10	+	48	2.64	+	N/A		A-rhs	JNFb
NAAS-9B	+	45	0.00	+	N/A		A-rhs	NFb
NAAS-9	+	42	0.00	+	*Pseudomonas*	*umsongensis*	A-rhs	NFb

NNA-11	+	52	3.39	+	N/A		A-rhs	NFb
NNA-10	+	47	115.32	+	N/A		A-stem	NFb
NNA-9	+	45	45.86	+	N/A		A-stem	NFb
NNA-8	+	45	46.05	+	N/A		A-stem	NFb
NNA-7	+	47	34.89	+	N/A		A-stem	NFb
NJA-9	+	46	42.03	+	N/A		A-root	JNFb
NJA-10b	+	47	44.66	+	N/A		A-rhs	JNFb
NAAS-6	+	44	1.59	+	N/A		A-rhs	NFb
NA-soil	+	45	43.68	+	*Pantoea*	*ananatis*	A-rhs	NFb
NJA-8	+	45	2.68	+	N/A		A-rhs	JNFb
NJA-7	+	43	42.89	+	N/A		A-stem	JNFb
NJA-6	+	46	43.95	+	N/A		A-stem	JNFb
NJA-5	+	50	45.64	+	*Pantoea*	*ananatis*	A-stem	JNFb
NNA-6	+	47	2.12	+	*Sphingomonas*	*pseudosanguinis*	A-rhs	NFb
NAAS-1	+	50	107.16	+	*Rahnella*	*aquatilis*	A-rhs	NFb
NAAS-8C	+	45	8.88	+	*Burkholderia*	*plantarii*	A-rhs	NFb
NAAS-8	+	51	23.99	+	*Burkholderia*	*plantarii*	A-rhs	NFb
NAAS-4	+	48	21.10	+	*Rhizobium*	*nepotum*	A-rhs	NFb

NAAS-8B	+	45	14.78	+	*Burkholderia*	*plantarii*	A-rhs	NFb
NAAS-11	+	47	106.37	+	*Rahnella*	*aquatilis*	A-rhs	NFb
NAAS-2	+	48	0.00	+	*Ensifer*	*adhaerens*	A-rhs	NFb
NNA-5	+	48	115.32	+	N/A		A-stem	NFb
NNA-4	+	45	38.38	+	*Pseudomonas*	*simiae*	A-stem	NFb
NNA-3	+	47	35.15	+	*Pseudomonas*	*oryzihabitans*	A-rhs	NFb
NNA-2	+	42	40.75	-	N/A		A-rhs	NFb
NNA-1	+	45	48.23	+	N/A		A-rhs	NFb
NJA-4	+	54	47.25	+	N/A		A-rhs	JNFb
NJA-3	+	47	18.92	+	N/A		A-rhs	JNFb
NJA-2	+	51	25.68	+	*Pseudomonas*	*koreensis*	A-rhs	JNFb
NJA-1	+	46	48.68	+	*Burkholderia*	*gladioli*	A-rhs	JNFb
NASGS-2	+	48	9.48	-	N/A		SG-rhs	NFb
NAAS-11	+	52	20.79	+	*Rahnella*	*aquatilis*	A-rhs	NFb
NASGS-6	+	46	25.79	+	*Rahnella*	*aquatilis*	SG-rhs	NFb
NASGS-1	+	47	17.26	+	*Rhizobium*	*tibeticum*	SG-rhs	NFb
NASGS-3	+	50	93.07	+	*Pantoea*	*agglomerans*	SG-rhs	NFb
NASGS-9	+	51	1.89	+	N/A		SG-rhs	NFb

NNS-18	+	48	3.06	+	N/A		SG-rhs	NFb
NNS-19/NNS-9	+	44	5.54	+	*Pseudomonas*	*moraviensis*	SG-root	NFb
NASGS-5	+	55	2.00	+	N/A		SG-rhs	NFb
NNS-20	+	47	3.58	+	*Bacillus*	*megaterium*	SG-rhs	NFb
NNS-21	+	50	4.26	+	*Baciullus*	*megaterium*	SG-stem	NFb
NNS-22	+	44	2.72	+	*Pseudomonas*	*aeruginosa*	SG-stem	NFb
NNS-23	+	48	2.30	+	N/A		SG-root	NFb
NNS-24	+	52	3.47	+	*Bacillus*	*megaterium*	SG-rhs	NFb
NNS-17	+	45	2.72	+	N/A		SG-rhs	NFb
NNS-16	+	52	2.72	+	N/A		SG-rhs	NFb
NNS-15	+	49	60.60	+	*Pseudomonas*	*psychrophila*	SG-stem	NFb
NNS-14	+	61	12.45	+	*Pseudomonas*	*umsongensis*	SG-root	NFb
NNS-13	+	43	7.34	+	*Pseudomonas*	*arsenicoxydans*	SG-root	NFb
NNS-12	+	49	3.17	+	N/A		SG-root	NFb
NNS-11	+	45	8.09	+	*Pseudomonas*	*umsongensis*	SG-root	NFb
NNS-10	+	46	89.24	+	N/A		SG-rhs	NFb
NNS-1	+	45	11.70	+	*Pseudomonas*	*fulva*	SG-rhs	NFb
NNS-2	+	46	3.06	+	N/A		SG-root	NFb

NNS-3	+	57	6.36	+	*Pseudomonas*	*moraviensis*	SG-root	NFb
NNS-4	+	42	3.06	+	N/A		SG-root	NFb
NNS-5	+	50	63.08	+	*Bacillus*	*megaterium*	SG-stem	NFb
NASGS-6	+	48	1.93	+	*Rahnella*	*aquatilis*	SG-rhs	NFb
NASGS-7	+	48	1.48	-	*Flavobacterium*	*chungangense*	SG-rhs	NFb
NNS-6	+	47	81.23	+	*Pseudomonas*	*fragi*	SG-stem	NFb
NNS-7	+	49	81.79	+	*Pseudomonas*	*fragi*	SG-root	NFb
NJS-3	+	45	8.32	+	*Pseudomonas*	*jessenii*	SG-root	JNFb
NJS-4	+	50	7.60	+	*Pseudomonas*	*moraviensis*	SG-root	JNFb
NJS-5	+	49	3.28	+	N/A		SG-stem	JNFb
NJS-6	+	46	11.14	+	*Pseudomonas*	*fragi*	SG-stem	JNFb
NJS-7	+	45	6.78	+	*Pseudomonas*	*tremae*	SG-root	JNFb
NJS-8	+	48	8.62	+	*Pseudomonas*	*jessenii*	SG-root	JNFb
NJS-9	+	47	56.27	+	*Pseudomonas*	*moraviensis*	SG-root	JNFb
NJS-10	+	46	8.09	+	N/A		SG-stem	JNFb
NJA-12	+	70	6.70	+	*Pseudomonas*	*reinekei*	Arundo root	JNFb
NJA-13	+	48	30.98	+	*Beijerinckia*	*fluminensis*	A-stem	JNFb
NJA-14	+	47	69.09	+	*Shinella*	*zoogloeoides*	Arundo root	JNFb

NJA-15	+	48	7.23	+	*Pseudomonas*	*moraviensis*	Arundo root	JNFb
AstII-1	+	48	73.34	+	*Pantoea*	*ananatis*	A-stem	JNFb
AstII-2	+	43	79.16	+	*Pantoea*	*ananatis*	A-stem	JNFb
AstII-5	+	48	77.32	+	*Pantoea*	*agglomerans*	A-stem	JNFb
NXU-27	+	43	55.07	+	*Citrobacter*	*youngae*	Arundo green house	NFb
NXU-28	+	49	2.19	-	N/A		SG-rhs	NFb
NXU-29	+	48	7.15	+	*Chryseobacterium*	*rhizosphaerae*	A-rhs	NFb
NXU-30	+	44	119.11	+	*Enterobacter*	*oryzae*	rhs	NFb
NXU-31	+	45	88.71	+	*Rahnella*	*aquatilis*	rhs	NFb
NXU-32	+	45	5.99	+	N/A		rhs	NFb
NXU-33	+	46	1.89	+	*Sphingobacterium*	*detergens*	SG-rhs	NFb
NXU-34	+	48	2.04	+	*Sphingobacterium*	*detergens*	SG-rhs	NFb
NXU-35	+	45	52.25	+	*Enterobacter*	*asburiae*	SG-rhs	NFb
NXU-36	+	58	53.87	+	*Citrobacter*	*youngae*	A-rhs	NFb
NXU-37	+	50	7.90	+	*Chryseobacterium*	*rhizosphaerae*	A-rhs	NFb
NXU-38	+	264	118.25	+	*Enterobacter*	*oryzae*	rhs	NFb
NXU-39	+	50	2.38	-	N/A		rhs	NFb
NXU-40	+	47	8.24	+	*Pseudomonas*	*umsongensis*	A-rhs	NFb

NXU-41	+	45	53.79	+	*Enterobacter*	*ludwigii*	SG GH	NFb
NXU-42	+	51	52.29	+	*Enterobacter*	*nimipressuralis*	SG FCU	NFb
NXU-43	+	56	54.62	+	*Leclercia adecarboxylata*	*Enterobacter asburiae*	Soil	NFb
NXU-44	+	48	49.25	+	*Flavobacterium*	*anhuiense*	SG GH	NFb
NXU-45	+	45	30.79	+	N/A		A-rhs	NFb
NXU-46	+	42	3.47	+	*Burkholderia*	*metallica*	SG Fcu	NFb
NXU-47	+	48	21.02	-	N/A		SG PBU	NFb
NXU-48	+	51	2.53	+	*Psychrobacillus*	*psychrodurans*	A-rhs	NFb
NXU-49	+	67	9.52	+	*Chryseobacterium*	*indologenes*	SG-rhs	NFb
NXU-50	+	51	2.30	+	N/A		rhs	NFb
NXU-51	+	49	1.59	+	*Arthrobacter*	*nicotinovorans*	A-rhs	NFb
NXU-52	+	46	2.75	+	*Arthrobacter*	*nicotinovorans*	SG-rhs	NFb

n.d not determined, + positive reaction, - negative reaction, *ARA* acetylene reduction assay,

Table 2-6 Characterization of putative P-solubilizing bacteria isolated from different locations and different plant parts.

Strain	Auxin (ug/ml)	Siderophore	P-test			Genus	Species	Source	Medium
			3d	8d	14d				
P1	10.46	+	2.6	2.4	2.5	*Pseudomonas*	*jessenii*	A-rhs	SRSM
P2	6.81	+	2.5	2.5	2.6	*Pseudomonas*	*jessenii*	SG-rhs	SRSM
P3	8.62	+	1.6	2.7	0.0	*Pseudomonas*	*rhodesiae*	A-rhs	SRSM
P4	8.66	+	1.9	0.6	0.2	N/A		A-rhs	SRSM
P5	95.62	+	2.1	0.2	0.2	*Pseudomonas*	*extremorientalis*	A-rhs	SRSM
P6	31.17	-	2.6	2.6	2.6	*Microbacterium*	*maritypicum*	A-rhs	SRSM
P7	20.08	+	2.8	2.2	2.2	*Pseudomonas*	*reinekei*	SG-rhs	SRSM
P8	0	+	2.8	2.8	2.7	*Pseudomonas*	*mandelii*	SG-rhs	SRSM
P9	0	+	2.7	2.6	2.3	*Pseudomonas*	*mohnii*	SG-rhs	SRSM
P10	12.56	+	2.7	2.8	2.6	*Pseudomonas*	*jessenii*	A-rhs	SRSM
PM-2	9.82	+	2.1	0.3	1.1	N/A		SG-root	SRSM
PM-5	6.17	+	1.1	0.0	0.0	*Burkholderia*	*gladioli*	A-root	SRSM
PM-6	5.76	+	0.8	0.0	0.0	*Burkholderia*	*gladioli*	A-root	SRSM
PM-7	10.95	+	2.7	2.6	2.4	N/A		SG-root	SRSM
PM-8	10.84	-	2.0	2.9	2.7	*Pseudomonas*	*frederiksbergensis*	SG-root	SRSM

PM-10	17.30	+	2.2	1.9	1.8	*Pseudomonas*	*taiwanensis*	A-rhs	SRSM
PM-11	31.77	+	1.7	0.0	0.0	N/A		SG-stem	SRSM
PM-12	9.41	+	2.2	0.0	0.0	N/A		SG-root	SRSM
PM-14	5.61	+	0.7	0.0	0.0	*Burkholderia*	*gladioli*	A-root	SRSM
PAS-8	0	+	2.6	1.9	2.0	N/A		A-rhs	SRSM
PAS-7	5.57	+	2.5	2.2	2.2	*Pseudomonas*	*jessenii*	A-rhs	SRSM
PAS-6	1.44	+	2.8	2.1	2.4	*Pseudomonas*	*jessenii*	A-rhs	SRSM
PARS-10	6.25	+	1.9	0.7	1.8	N/A		A-rhs	SRSM
PARS-8	6.66	+	2.7	0.9	0.1	*Pseudomonas*	*extremorientalis*	A-rhs	SRSM
PARS-6	0	+	2.80	1.20	1.30	N/A		A-rhs	SRSM
PARS-4	6.55	+	2.8	0.7	0.4	*Pseudomonas*	*extremorientalis*	A-rhs	SRSM
PARS-2	6.21	+	2.0	0.7	0.1	N/A		A-rhs	SRSM
PARS-1	5.72	+	2.70	2.20	0.50	N/A		A-rhs	SRSM
PSRS-9	3.99	+	1.8	1.4	1.3	*Pseudomonas*	*moraviensis*	SG-rhs	SRSM
PSRS-8	4.93	+	2.1	1.7	0.8	*Pseudomonas*	*reinekei/moraviensis*	SG-rhs	SRSM
PSRS-7	6.63	+	2.6	1.9	1.4	*Pseudomonas*	*reinekei*	SG-rhs	SRSM
PSRS-6	4.30	+	2.5	1.9	1.5	*Pseudomonas*	*moraviensis*	SG-rhs	SRSM
PSRS-1	0	+	2.0	1.5	1.4	N/A		SG-rhs	SRSM

PASGST-2	52.63	+	1.4	0.0	0.0	*Pseudomonas*	*fragi*	SG-stem	SRSM
PASGR-2	11.77	+	2.3	1.7	1.7	*Pseudomonas*	*migulae*	SG-root	SRSM
PAAS-2	9.41	+	1.5	0.4	0.4	*Pseudomonas*	*costantinii*	A-rhs	SRSM
PASGST-4	58.04	+	1.4	0.0	0.0	*Pseudomonas*	*fragi*	SG-stem	SRSM
PASGS-7	4.33	-	2.9	2.3	2.5	*Citrobacter*	*freundii*	SG-rhs	SRSM
PASGS-1	24.48	+	0.4	1.5	0.0	*Pantoea*	*dispersa*	SG-rhs	SRSM
PASGS-1	0	+	1.80	1.90	1.80	*Enterobacter*	*ludwigii*	SG-rhs	SRSM
PASGST-a	53.31	+	1.5	0.0	0.0	*Pseudomonas*	*fragi*	SG-stem	SRSM
PXU-53	11.89	+	2.8	2.5	2.9	*Pseudomonas*	*jessenii*	SG-rhs	SRSM
PXU-54	110.58	-	2.9	2.2	3.0	*Sphingobium*	*chungbukense*	SG-rhs	SRSM
PXU-55	22.00	+	0.0	2.5	3.0	*Flavobacterium*	*chungangense*	SG-rhs	SRSM
PXU-56	46.62	-	2.5	3.3	2.4	N/A		SG-rhs	SRSM
PXU-57	29.06	+	1.8	2.2	2.3	N/A		SG-rhs	SRSM
PXU-58	9.93	+	1.5	1.2	0.9	*Burkholderia*	*metallica*	SG-rhs	SRSM
PXU-59	1.55	+	2.3	2.0	1.8	*Flavobacterium*	*aquidurense*	SG-rhs	SRSM
PXU-60	14.63	+	2.7	2.2	2.0	N/A		SG-rhs	SG-rhs
PXU-61	11.06	+	2.6	1.9	2.3	*Pseudomonas*	*moraviensis*	SG-rhs	SRSM
PXU-62	44.06	+	2.7	2.2	0.0	N/A		A-rhs	SRSM

PXU-63	60.41	+	1.8	0.0	0.0	*Panteoea*	*eucalypti*	A-rhs	SRSM
PXU-64	41.69	+	1.2	0.0	0.0	N/A		SG-rhs	SRSM
PXU-65	9.78	+	3.2	2.3	2.8	*Pseudomonas*	*jessenii*	A-rhs	SRSM
PXU-66	5.24	+	2.8	2.2	2.6	*Pseudomonas*	*mandelii*	A-rhs	SRSM
PXU-67	11.47	+	2.7	2.1	2.5	*Pseudomonas*	*vancouverensis*	A-rhs	SRSM
PXU-68	4.67	+	1.0	0.0	0.0	*Burkholderia*	*metallica*	SG-rhs	SRSM
PXU-69	6.78	+	2.7	2.3	2.7	*Janthinobacterium*	*lividum*	SG-rhs	SRSM
PXU-70	5.05	+	2.7	1.4	1.6	*Pseudomonas*	*rhodesiae*	SG-rhs	SRSM
PXU-71	13.50	+	3.7	1.9	1.7	*Pseudomonas*	*reinekei*	SG-rhs	SRSM
PXU-72	6.63	+	2.5	1.8	2.1	N/A		SG-rhs	SRSM

N/A not available + positive reaction, - negative reaction, P-test quantitative phosphate-solubilizing test

Table 2-7 Growth promotion tests of selected N strains in corn in greenhouse.

Time	Treatment	SPAD	Plant height (cm)	Fresh weight (g)		Dry weight (g)		Root length (mm)	Surface area	Volume	No. of tips	Fine roots
				Shoot	Root	Shoot	Root					
A			68.9 a	18.70 a	13.20 a	1.79 a	0.83 a	443 b	307 a	17.6 a		170 b
B			70.3 a	17.17 b	8.15 b	1.56 b	0.82 a	756 a	324 a	11.3 b		334 a
	Control		70.0	18.63	10.17	1.68	0.74	549	296	14.0		225
	NNA-14		70.5	18.05	10.60	1.71	0.82	628 *	331 *	14.8		264 *
	NXU-38		71.4	19.24	10.70	1.75	0.87	575	298	13.7		242
	NNA-17		69.6	18.38	10.60	1.70	0.85	601	320	15.2		256
	NP-3		68.7	17.49	9.96	1.62	0.79	531	286	13.9		219
	NNA-20		70.0	16.56	10.45	1.58	0.79	613	307	13.4		265 *
	99B-817		66.8	17.22	12.25	1.69	0.91	700 ***	367 ***	16.1		294 ***
A	Control	40.3	69.2	20.17	13.61	1.91	0.83	417	306	18.5	561	159
A	NNA-14	41.2									633	
A	NXU-38	40.6									622	
A	NNA-19	40.1	70.3	19.32	15.40 *	1.89	0.95	414	335 *	21.9 *	847 ***	153
A	NNA-17	40.6									663	
A	NP-3	40.4									749 *	
A	NNA-20	40.7									742 *	
A	99B-817	41.6									995 ***	
B	Control	39.6									797	
B	NNA-14	39.5									1823 ***	
B	NXU-38	39.9									977	
B	NNA-17	40.6									1282 ***	
B	NP-3	38.3									936	
B	NNA-20	38.2									1095 **	
B	99B-817	36.2 ***									1398 ***	

* $P<0.05$, ** $P<0.01$, and *** $P<0.001$, compared with control.
Means within each column and time factor with different letters differ ($P<0.05$)

Table 2-8 Effect of selected N strains on nutrient uptake of corn in greenhouse

Time	Strain treatment	Nitrogen (%)	P (%)	K (%)
A	Control	3.37	0.22	4.08
A	NNA-14	3.81 *	0.23	3.73
A	NXU-38	3.39	0.20	3.97
A	NNA-19	3.03 *	0.18 *	3.99
A	NNA-17	3.12	0.20	4.21
A	NP-3	2.86 **	0.19	4.19
A	NNA-20	2.94 *	0.16 ***	3.58 *
A	99B-817	3.53	0.25 **	4.66 *

* $P<0.05$, ** $P<0.01$, and *** $P<0.001$, compared with control.

Table 2-9 Growth-promotion tests of selected N strains in wheat in greenhouse.

Time	Treatment	Height (cm)	Number of leaves	Number of tillers	Fresh weight (g)		Dry weight (g)		Root length (mm)	Surface area	Volume	Number of tips	Fine roots
					Shoot	Root	Shoot	Root					
C		34.7 a	15.6 a	3.9 a	2.68 a	1.51	0.32 a	0.14 a	234 b	92 b	3.0 a	325 a	100 b
D		32.0 b	14.0 b	4.1 a	1.94 b	1.84	0.27 a	0.12 a	369 a	115 a	2.9 a	323 a	182 a
	Control	34.3	14.1	3.7	2.32	1.43	0.29	0.12	285	96	2.7	243	132
	NNA-14	33.2	15.9	4.3	2.30	1.81 *	0.30	0.14 *	305	107 *	3.0	369 *	144
	NXU-38	32.7	15.2	4.1	2.26	1.68	0.29	0.12	344 **	107*	2.7	401 ***	166 **
	NNA-19	32.9	15.3	4.3	2.39	1.88 **	0.31	0.15 **	318	108	3.0	476 ***	156
	NNA-17	33.7	13.5	3.6	2.15	1.34	0.29	0.12	283	90	2.4	333 *	138
	NP-3	33.1	14.3	4.1	2.32	1.78 *	0.30	0.13	305	108	3.1	274	138
	NNA-20	33.2	15.4	4.1	2.33	1.68	0.28	0.13	296	107	3.1	256	133
	99B-817	33.7	15.1	4.2	2.41	1.76 *	0.31	0.14 *	274	105	3.4 *	239	122

* $P<0.05$, ** $P<0.01$, and *** $P<0.001$, compared with control.

Means within each column and time factor with different letters differ ($P<0.05$).

Table 2-10 Effect of selected N strains on nutrient uptake of wheat in greenhouse

Time	Strain treatment	Nitrogen (%)	P (%)	K (%)
C	Control	4.83	0.41	5.18
C	NNA-14	4.16 *	0.31 **	4.67 ***
C	NXU-38	4.58	0.36 **	4.98
C	NNA-19	4.38	0.38 *	4.93
C	NNA-17	4.54	0.39	4.82 *
C	NP-3	4.55	0.39	4.89 *
C	NNA-20	4.62	0.40	4.98
C	99B-817	4.46 *	0.32 ***	4.80 ***

* $P<0.05$, ** $P<0.01$, and *** $P<0.001$, compared with control.

Table 2-11 Total-sample standardized canonical coefficients and total variation explained by each canonical variable (Can)

Variable	Can1	Can2	Can3	Can4
Height	-0.29	-0.17	-0.01	0.33
Number of leaves	0.20	0.39	-0.15	0.04
Number of tillers	0.24	0.40	0.14	-0.09
Shoot fresh weight	-0.02	0.14	0.09	0.02
Root fresh weight	0.26	0.50	0.27	-0.18
Shoot dry weight	0.11	0.07	0.24	0.06
Root dry weight	0.24	0.40	0.34	0.11
Root length	0.25	0.03	-0.10	-0.38
Root surface area	0.12	0.38	0.07	-0.27
Root volume	-0.13	0.63	0.33	0.05
Number of root tips	0.74	-0.11	-0.05	-0.19
Fine roots	0.32	-0.06	-0.10	-0.31
Variation (%)	38.7	29.1	14.9	11.35

Table 2-12 Growth promotion tests of selected P strains in corn in greenhouse in 2013

Treatment	Height (cm)	Fresh weight (g)		Dry weight (g)		
		Shoot	Root	Shoot	Root	SPAD
Control	64.00	11.64	3.64	1.11	0.45	39.34
P-6	70.75 **	12.24	2.00 ***	1.16	0.47	39.88
P-8	70.50 *	13.9	3.45	1.27	0.51	37.77
PASGS-7	66.33	11.68	2.80	1.09	0.38	38.90
PXU-53	62.75	10.58	2.46 *	1.01	0.52	39.68
PXU-54	66.33	11.12	1.36 ***	1.04	0.34	41.60 *
PXU-56	68.00	12.14	3.29	1.16	0.43	35.05 ***
PXU-60	66.33	11.88	1.80 ***	1.02	0.38	38.92
PXU-66	70.33	12.74	2.09 ***	1.28	0.46	41.98 *
PXU-69	60.93	9.79	3.36	0.89	0.42	38.57
Control	70.75	12.18	3.15	0.82	0.20	40.68
P-10	73.42	13.21	5.02 ***	0.97 *	0.31 ***	39.67
P-2	71.00	12.52	4.71 ***	0.90 *	0.29 ***	41.18
P-7	68.67	11.84	3.78 *	0.77	0.20	40.28
PM-10	69.75	12.63	4.93 ***	0.91	0.29 ***	41.82
PM-8	68.01	11.44	3.62	0.78	0.20	42.65 *
PSRS-7	73.25	13.00	4.69 ***	0.91	0.27 *	39.97
PXU-65	71.73	13.17	4.74 ***	0.95	0.27 *	41.82
PXU-67	69.43	11.92	4.66 ***	0.87	0.32 ***	43.20 ***
PXU-71	67.50	11.32	3.65	0.80	0.24	42.47 *
PXU-72	68.92	11.62	3.58	0.80	0.21	42.58 *

* $P<0.05$, ** $P<0.01$, and *** $P<0.001$, compared with control.

Table 2-13 Effect of selected P strains on nutrient uptake of corn in greenhouse

Strain	Nitrogen (%)	P (%)	K (%)
Control	3.26	0.30	3.98
PXU-56	3.94 ***	0.35 *	4.45 ***
PXU-69	3.98 ***	0.30	4.23 *
PXU-66	4.29 ***	0.30	4.12
PXU-60	4.47 ***	0.26 **	4.10
P-8	4.54 ***	0.28 *	4.20 *
P-6	4.29 ***	0.27	3.96
PASGS-7	3.76 ***	0.29	4.03
PXU-54	3.67 **	0.29	3.83
PXU-53	3.58 *	0.27 **	3.92
Control	4.69	0.31	5.59
P-2	3.63 ***	0.30	4.83 **
P-10	3.86 ***	0.30	5.03
PXU-72	5.13 **	0.32	5.49
PXU-71	5.28 ***	0.32	5.38
PXU-67	4.94	0.27 *	4.97 *
PXU-65	4.52	0.28	5.06 *
PM-8	3.72 ***	0.27	5.12
PM-10	3.72 **	0.28	5.19
PSRS-7	4.18 *	0.27 *	4.78 **
P-7	4.83	0.34	5.62

* $P<0.05$, ** $P<0.01$, and *** $P<0.001$, compared with control.

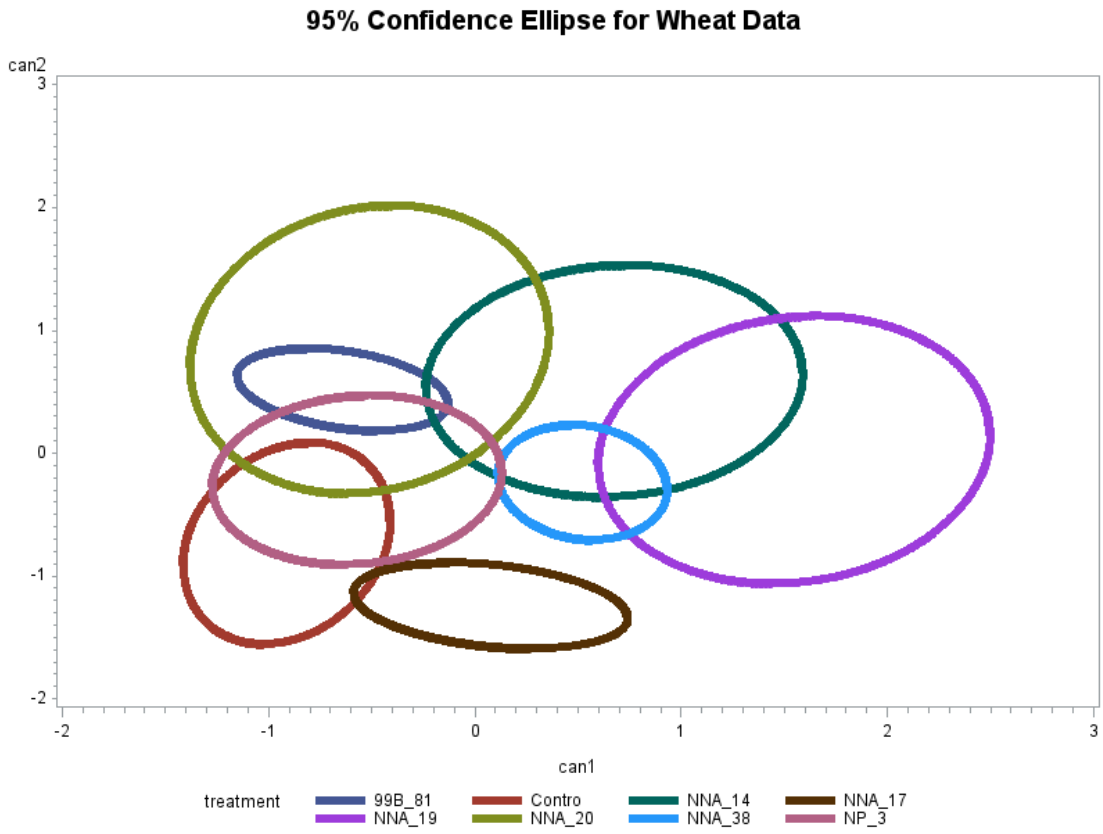

Figure 2-1 95% confidence ellipse for pooled wheat data collected from time C and D.

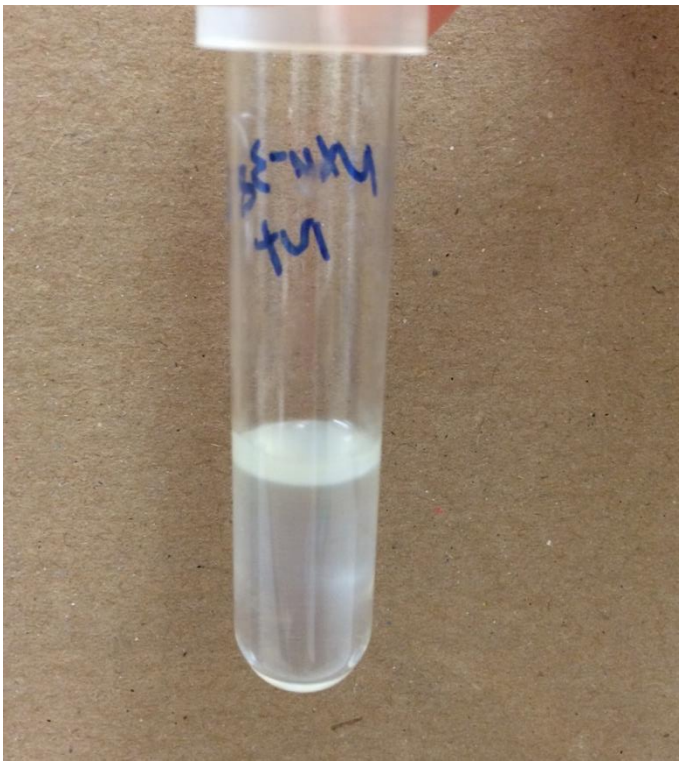

Plate 2-1 Pellicle formation on NFb medium

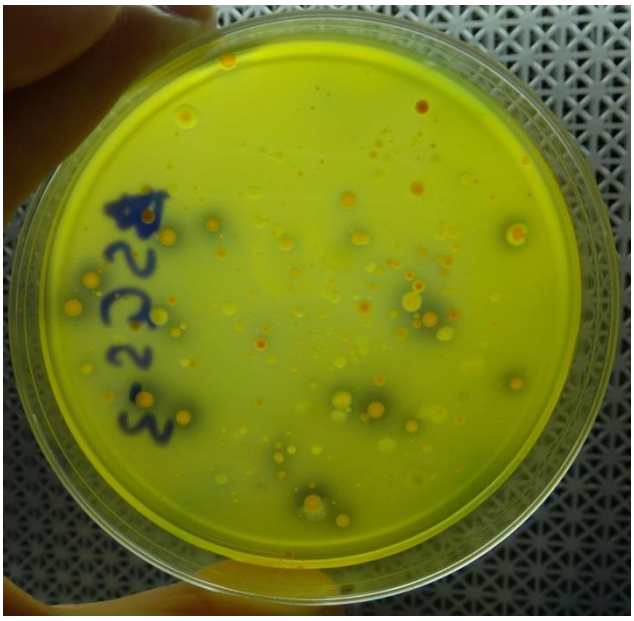

Plate 2-2 Isolation of putative P-solubilizing bacteria

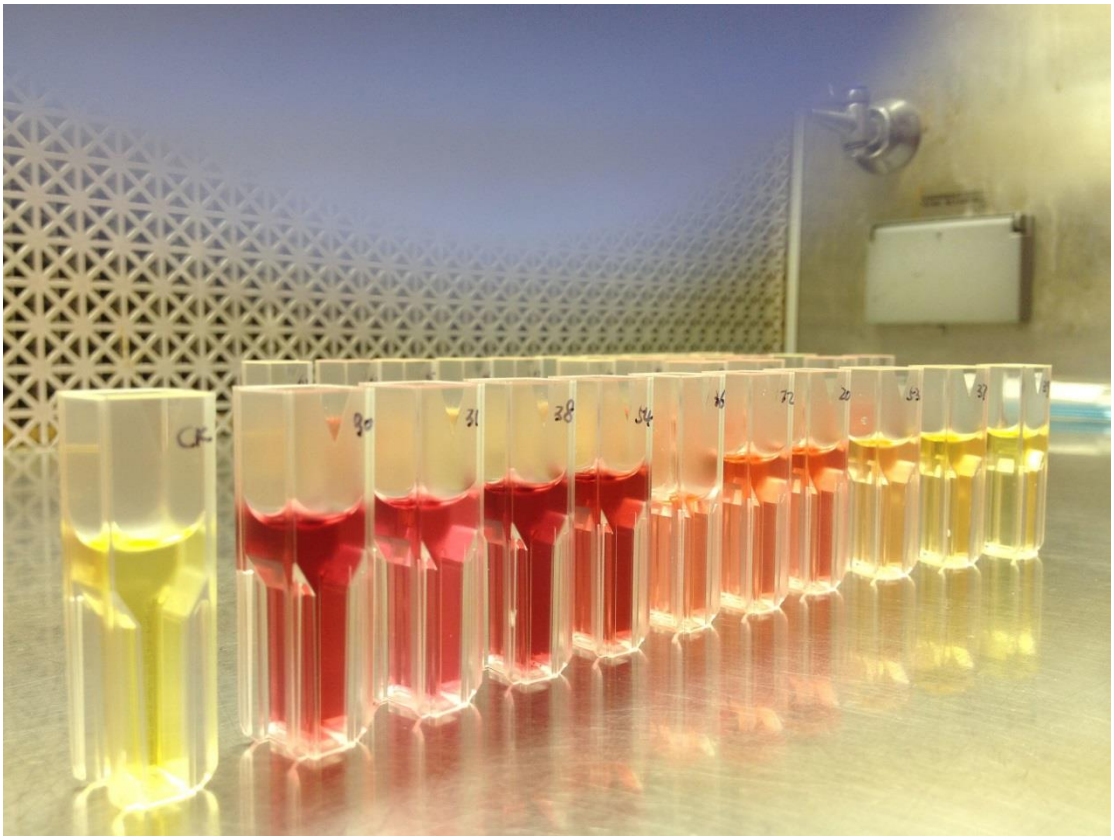

Plate 2-3 Pink color reaction of auxin production

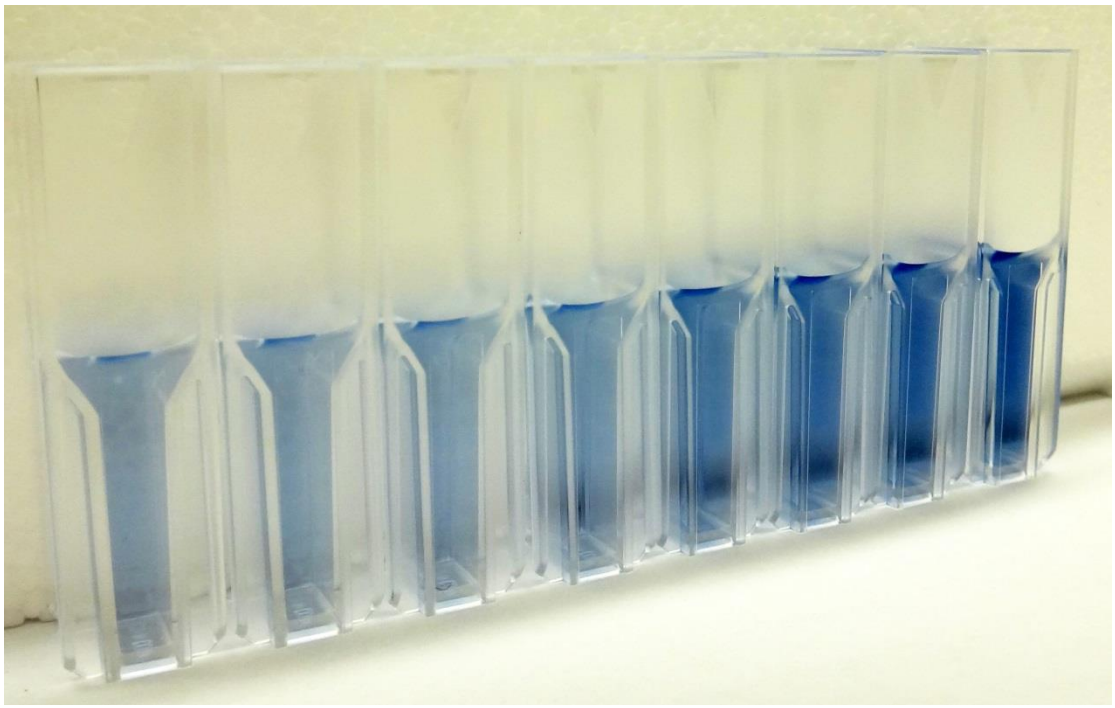

Plate 2-4 Blue color reaction of quantitative evaluation of bacterial P-solubilization

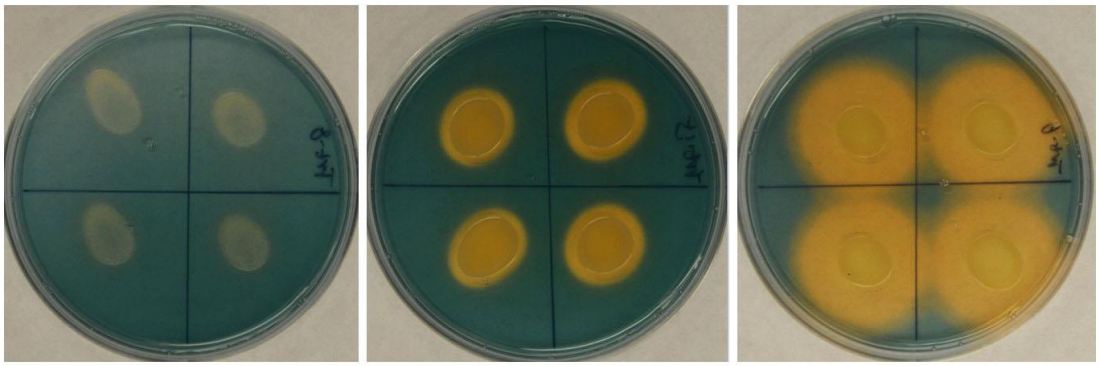

Plate 2-5 Orange halo of evaluating siderophore production

CHAPTER 3 ISOLATION, CHARACTERIZATION AND IDENTIFICATION OF FREE-LIVING NITROGEN-FIXING BACTERIA FROM A NITROGEN-LIMITED COTTON FIELD IN THE OLD ROTATION IN ALABAMA

3.1 INTRODUCTION

Nitrogen is the most important macronutrient to plant growth and productivity. Large amounts of chemical nitrogen fertilizers are applied to agricultural crop soils, but only about 50% of the nitrogen in the soil can be absorbed by crops (Miransari, 2010). Nitrogen in the soil can be lost due to denitrification, leaching and volatilization. Biological nitrogen fixation has been considered as an alternative or complementary way to meet the plant nitrogen requirement, which can help plant fix atmospheric nitrogen and reduce the input of chemical nitrogen fertilizer. Symbiotic and free-living nitrogen-fixing bacteria are two main groups of diazotrophic bacteria. Rhizobia, the symbiotic nitrogen-fixing bacteria, are well studied; however, a limitation is that they only fixe atmospheric nitrogen in the roots of the appropriate legume. Free-living nitrogen- fixing bacteria, such as *Azospirillum*, *Azotobacter*, *Burkholderia*, *Bacillus*, *Pseudomonas* and *Herbaspirillum*, are studied as promising strains that can significantly promote plant growth and replace or partially replace chemical nitrogen fertilizers (Jetiyanon & Plianbangchang, 2012, Nguyen et al., 2003, Berg, 2009, Adesemoye & Kloepper, 2009, Hayat et al., 2010). In this study, for isolation of free-living N-fixing bacteria, we selected cotton (*Gossypium hirsutum* L.) plants growing in the "Old Rotation" experiment on the campus of Auburn University.

This experiment was established in 1896 and is the oldest continuous cotton experiment in the world. The main focus of the study is cotton rotation with other crops and the controlled application of nitrogen.

We hypothesized that i. there would be diverse N-fixing bacteria in fields with a long history of little or no chemical nitrogen use, and ii. free-living N-fixing bacteria would be isolated in soil and inside roots and stems of cotton growing in the rotation. The overall objective of this study was to find strong N-fixing bacteria that have the potential for use as biofertilizers. The two specific objectives of this experiment were to 1) enumerate and compare the culturable putative N-fixing bacterial populations in different no- nitrogen-input cropping systems of cotton; and 2) isolate, characterize and identify the N-fixing bacteria from rhizosphere soil and from inside roots and stems of selected cotton crops.

3.2 MATERIAL AND METHODS

3.2.1 Field sampling

In 2012, five different cropping systems of cotton under natural condition without any irrigation growing in the Old Rotation were chosen for testing. The systems, also referred to as treatments, included treatment 1 (cotton every year, no input of N-fertilizer), treatment 2 (cotton every year, winter legume, no input of N-fertilizer), treatment 4 (2-yr cotton-corn rotation, winter legume, no input of N-fertilizer), treatment 6 (the same as treatment 1) and treatment 8 (cotton every year,

winter legume, no input of N fertilizer) (Table 3-1). Six cotton plants and rhizosphere soil samples were randomly taken from each plot or cropping system treatment at 15 feet apart at 30, 60, and 90 days after planting (DAP) for bacterial isolation and enumeration.

3.2.2 Methods for isolation

NFb Nitrogen-free medium (Bashan et al., 1993) was used for the bacteria isolation. The enumeration of putative N-fixing bacteria was carried out in NFb solid medium, and NFb semi-solid medium was used for further purification.

Rhizosphere soil and root and stem samples from cotton were collected, sealed in plastic bags, and placed in a 4 ℃ refrigerator after sampling. One hundred microliters of serially diluted (from 10^0 to 10^{-5}) rhizosphere soil solution were spread on NFb solid medium for enumeration and isolation. The stem and root samples were washed with sterilized distilled water to remove surface dust. Surface sterilization was carried out by immersing samples in 70% ethanol for 1 minute and in 50% commercial bleach for 3 minutes. They were then rinsed 3 times in sterilized distilled water. Stem and root samples (1.0g) were macerated separately using a KLECO grinder in sterilized distilled water and serially diluted to10^{-3}. To confirm the elimination of all surface bacteria on roots and stems, the sterilized samples were placed on trypticase soy agar (TSA) and incubated at 28 ℃ for 24 hours before being ground.

For isolating putative N-fixing bacteria, 100 µl of the serial dilutions were inoculated onto solid NFb medium for enumeration and isolation. Single colonies were removed and inoculated into semi-solid NFb medium for further purification. Putative nitrogen-fixing bacteria were transferred to semi-solid NFb. This procedure was repeated for 3-4 times to confirm the consistency of nitrogen-fixing activity. Colony forming units (CFU) were estimated by the Miles and Misra drop-plate method (Miles et al., 1938). Purified putative N-fixing bacteria were saved in 1.5 ml vials filled with 70% TSB + 30% glycerol liquid nutrient medium in -80 ℃ freezer for future use.

3.2.3 Methods for characterization

3.2.3.1 Determination of nitrogenase activity

The nitrogenase activity of the isolated putative N-fixing bacteria was determined using the acetylene reduction assay (Lopez et al., 2011, Holguin et al., 1992). Purified putative N-fixing bacteria were removed from the -80 ℃ freezer and grown on NFb for 48 hours. Half a loopful of bacteria was removed and placed into 9 ml sterilized water tubes, and 100 µl of the cultures were then added to 4 ml of NFb semisolid medium (14 ml tubes), which were incubated at 28 ℃ for 7 days. The tube caps were then replaced by rubber caps, 10% (v/v) of air (1 ml) in the culture tubes was removed, and 10% (v/v) acetylene was injected into the culture tubes. The tubes were then incubated for 24 h and analyzed by gas chromatography using a Hewlett-Packard 5890 Series II gas chromatograph (Agilent Technologies, Santa Clara, CA,

USA) equipped with a hydrogen flame ionization detector (FID) to quantity the amount of ethylene converted from acetylene. Instrument operating conditions were set as follows: column (J&W GC column GS-GASPRO 60 m x 0.320 mm Serial No: USB467916H) with the oven temperature at 50 ℃; injector temperature at 250 ℃; detector temperature at 250 ℃; helium (He) as carrier gas with a flow rate at 2 ml/min; nitrogen (N_2) as makeup gas with a flow rate at 24 ml/min; hydrogen (H2) flow rate at 30 ml/min; and air flow rate at 300 ml/min. The nitrogen-fixing bacterium, *Azospirillum brasilense* 99B-817 (ATCC# 29710), served as the positive control. Results were expressed in nmoles ethylene 24 h^{-1} ml^{-1}. Each sample was analyzed 3 times.

3.2.3.2 Auxin production:

The capacity of the isolated putative N-fixing bacteria to produce auxin (Frankenberger Jr, 1988, Gordon & Weber, 1951, Husen, 2003) was determined *in vitro*. Bacteria were grown on M-26 for 24 hours on a shaker at 150 rpm at room temperature as seed culture. Next, 15 µl of culture were inoculated to 1.5 ml minimal salt medium (MS) amended with 5 mM L-Tryptophan and grown again on the shaker for 48 hours. Tubes containing 1.5 ml bacteria broth were centrifuged at 12,000 rpm for 5 minutes, and 0.5 ml of the bacteria supernatant were added into 1ml $FeCl_3$-$HClO_4$ reagent. They were then set for 25 minutes under dim light after the color density reached its maximum. The pink color reaction indicated positive auxin production (Plate 2-3). Quantitative measurements of the color intensity were made

using a UV-spectrophotometer (Thermo mode: Genesys 10S UV-Vis) at 530 nm absorbance. Auxin concentration was estimated by comparison with the standard curve prepared with indole-3-acetic acid (Sigma Lot#092K1235) and expressed in µg ml^{-1}. The composition and preparation of M-26 medium, MS medium, L-trytophan stock solution and $FeCl_3$-$HClO_4$ reagent are shown in Appendix A.

3.2.3.3 Siderophore production:

Chrome azurol S (CAS) agar (Husen, 2003, Schwyn & Neilands, 1987) was used to describe the siderophore production qualitatively. Bacteria were grown on TSA for 24-48 hours and then a half loop of bacteria was transferred to 9 ml distilled water tube. The bacterial suspension was vortexed thoroughly, and CAS medium was divided into 4 quadrants. Each quadrant was pipetted with 15 µl bacteria suspension. Orange halos around the growing bacterial colonies confirmed the siderophore production after 48-72 hours incubation (plates 2-5). The CAS agar was a mixture of four solutions that were prepared and sterilized separately before mixing. Fe-CAS indicator solution, buffer solution and glucose/mannitol solution were sterilized by an autoclave; casamino acid was sterilized by 0.2 µM filter. The composition of CAS medium is shown in Appendix A.

3.2.4 Molecular Identification

Taxonomic classification of each strain was based on the partial sequence of 16S rDNA. DNA was extracted and amplified with universal bacterial primers: 8F (5'- AGAGTTTGATCCTGGCTCAG -3') and 1492R (5'- ACGGCTACCTTGTTACGACTT - 3'). PCR was performed using Lucigen EconoTaq Plus Green 2X master mix (Lucigen Corp.) with cycling parameters: initial denaturation at 95 ℃ for 5 min; 31 cycles of 94 ℃ for 1 min, 57 ℃ for 45 sec, 70 ℃ for 2 min; and a final extension at 70 ℃ for 10 min. All sequences were blasted against the type strains in the ribosomal database project to identify bacterial taxa of each strain.

3.2.5 Data analysis

Given that the variation of putative N-fixing bacterial population among sub-samples was the same order of magnitude as the variation among experimental units for two of the cropping system treatments (1 and 2), subsamples were treated as replicates for data analysis. Putative N-fixing bacterial population data were transformed into log scale and then subjected to analysis of variance using the PROC GLIMMIX procedure in SAS. The critical *P*-value of 0.05 was used as cutoff for testing fixed effects, and determination of differences in least-squares means was based on adjusted *P*-value obtained by using the option ADJUST=SIMULATE in the LSMEANS statement.

3.3 RESULTS

3.3.1 Enumeration of free-living nitrogen-fixing bacteria under selected cropping system treatments

Culturable N-fixing bacterial populations in rhizosphere soil and in root and stem of cotton plants were evaluated for selected cropping system treatments at 30, 60, and 90 DAP. Analysis of putative N-fixing bacterial population data indicated a sampling position × sampling time × cropping system treatment interaction ($P<0.05$).

The effect of cropping system treatment on culturable N-fixing bacterial population was consistent with each sampling time and sampling position except at 60 DAP in root and stem of cotton and at 90 DAP in stem (Figure 3-1). Generally, culturable N-fixing bacterial populations tended to be greater under cropping system treatments 2, 4, and 8 than under cropping system treatments 1 and 6, probably because the former cropping systems added winter legume, resulting in increases in culturable N-fixing bacterial populations in rhizosphere soil and root and stem of cotton. There was no difference in culturable N-fixing bacterial populations under cropping system treatments1 and 6 or cropping system treatments 2, 4, and 8.

Changes in populations of culturable N-fixing bacteria over time in rhizosphere soil were different from those inside cotton roots and stems (Figure 3-2). In rhizosphere soil, culturable N-fixing bacterial populations were higher at 90 DAP than at 30 and 60 DAP, regardless of cropping system treatment. In root and stem of

cotton, culturable N-fixing bacterial populations under all selected cropping system treatments were highest at 30 DAP, followed by 90 DAP and 60 DAP. Regardless of sampling time and cropping system treatment, culturable N-fixing bacterial populations were consistently highest in rhizosphere soil, followed by root and stem of cotton (Figure 3-3)

At 30 DAP, highest culturable N-fixing populations in rhizosphere soil and root and stem of cotton were approximately 4.46 X 10^6 per gram soil (under cropping system treatment 2), 5.08 X 10^5 per gram root (under cropping system treatment 4), and 9.55 X 10^4 per gram stem (under cropping system treatment 2). Lowest culturable N-fixing bacterial populations in rhizosphere soil and root and stem of cotton were approximately 1.44 X 10^6 per gram soil, 2.23 X 10^4 per gram root, and 2.2 X 10^3 per gram stem under cropping system treatments 2.

3.3.2 Confirmation of N-fixing activity and evaluation of other PGP traits

A total of 65 putative N-fixing bacterial strains were isolated from rhizosphere soil and inside root and stem of selected treatments. The N-fixing activity and PGP traits of these strains are presented in Table 3-4.

The acetylene reduction assay (ARA) was used to determine the nitrogenase activity. The nitrogenase activity of all the isolated strains were confirmed by ARA. From the ARA results, there was little difference among the N-fixing bacteria, which ranged from 40 to 59 nmol C_2H_4 24 hrs^{-1} ml^{-1}.

All the isolated strains had auxin production activity, which ranged from 1.8 to 47.4 μg ml^{-1}. Of the isolated strains, 72.3% had siderophore production activity, and 12.3% had very strong siderophore production activity. The orange halo and colony ratio was up to 2 (Plate 3-1).

3.3.3 Molecular identification

Fifty one out of 65 of theN-fixing strains were identified. A total of 13 bacterial genera were obtained from the old cotton rotation, including *Bacillus* (9 strains), *Beijerinckia* (1strain), *Burkholderia* (3 strains), *Caulobacter* (2strains), *Curtobacterium* (1 strain), *Gordonia* (1 strain), *Massilia* (1 strain), *Methylobacterium* (1 strain), *Microbacterium* (1 strain) *Paenibacillus* (1 strain), *Pseudomonas* (9 strains), *Sphingomonas* (1 strain), and *Variovorax* (1 strain). The identification results are presented in Table 3-4.

3.4 CONCLUSIONS AND DISCUSSION

This is the first report evaluating culturable N-fixing bacteria in a selected cotton rotation without any input of chemical N fertilizer. Overall, the results support the starting hypotheses that a diverse of culturable free-living N-fixing bacteria were isolated from rhizosphere, and endophytically in roots and stem in fields with a long history of little or no chemical nitrogen use. The strain characterization confirmed the isolated N_2-fixing bacteria have nitrogenase activity. Other auxin and siderophore

production test showed these isolates have plant growth promotion potentials. Based on the molecular identification, we could see the distribution of each genus isolated, which suggests that the N-fixing bacterial genera may colonize specific preferred niches. For example, some endophytic N-fixing bacteria, such as *Methylobacterium* and *Microbacterium,* were only isolated from stem, while *Variovorax* was only isolated from roots, and *Spingomonas* was isolated from inside of root and stem. In contrast, other N-fixing bacteria, such as *Bacillus, Pseudomonas, Caulobacter,* and *Beijerinckia*, have very wide niches and can be isolated both from the rhizosphere and from inside roots or stems. For example, Xu (2014) found that *Burkholderia* could be isolated from both the rhizophere and inside roots and stems of giant reed (*Arundo donax* L.). In contrast, in the current study, *Burkholderia* was only found in rhizosphere soil and not from inside plants grown in the N-limited cotton field.

From the enumeration data, the cropping system treatments with winter legumes showed a larger culturable free-living N-fixing bacterial population in rhizosphere soil, inside of stems and roots than the cropping system treatments without winter legumes. This result is consistent with other studies that legumes (Biederbeck et al., 2005, Cai et al., 2009) increase the bacterial population in rhizosphere.

Although this study was aimed at finding strong culturable free-living N-fixing bacteria for use as biofertilizers, the ARA data showed that all the isolated N-fixing strains have little difference in nitrogenase activity. The PGP traits showed that most of the strains have strong auxin and siderophore production, indicating that these

isolates have plant growth-promotion potential. The future work on these isolates should focus on evaluating plant growth promotion and nutrient uptake in cotton and other agricultural crops such as winter wheat, pepper, tomato and cucumber. Other PGP traits test in *vitro* such as P-solubilizing activity test, K-solubilizing activity and other plant hormone production tests like gibberellin and cytokine production should also be conducted for better understanding the strain's characteristics.

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Table 3-1 Cotton sampling on The Old Rotation

Treatment No.	Winter legume	Summer crop	N-fertilizer application	
1	No	Cotton	No	
2	Yes	Cotton	No	
4	Yes	Cotton/Corn	No	
6	No	Cotton	No	Same as #1
8	Yes	Cotton	No	Same as #2

Table 3-2 Log-scaled N-fixing bacteria population in stem and root of cotton plants over the three sampling times.

Sampling time (DAP[1])	Log-scaled putative N-fixer population per gram soil	
	Cotton stem	Cotton root
30	3.95 (0.19) a	5.00(0.28) a
60	1.15 (0.28) c	3.53(0.18) b
90	3.12 (0.12) b	4.40(0.11) a

1-DAP- Days after planting.

Values in parentheses are standard errors.

Means within each column with different letters are significantly different ($P<0.05$)

Table 3-3 Characterization of putative N-fixing bacteria isolated from different locations and different plant part

Strains	Pellicles	ARA (nmol C_2H_4/24h ml)	Auxin (ug/ml)	Siderophore (+/-)	Genus	Species	Source
NOR-1	+	47	40.1	-	*Beijerinckia*	*fluminensis*	Stem/trt.1@30DAP
NOR-2	+	46	7.8	+	*Pseudomonas*	*taiwanensis*	Stem/trt.1@30DAP
NOR-3	+	47	6.1	+	N/A		Stem/trt.2@30DAP
NOR-4	+	47	9.0	+	*Pseudomonas*	*thivervalensis*	Root/trt.2@30DAP
NOR-5	+	50	5.8	+	N/A		Root/trt.2@30DAP
NOR-6	+	46	6.0	-	*Bacillus*	*megaterium*	Rhs/trt.1@30DAP
NOR-7	+	47	14.6	-	*Bacillus*	*megaterium*	Rhs/trt.8@30DAP
NOR-8	+	47	32.1	-	*Beijerinckia*	*fluminensis*	Root/trt.4@30DAP
NOR-9	+	47	30.0	-	*Beijerinckia*	*fluminensis*	Root/trt.4@30DAP
NOR-10	+	47	7.4	+	N/A		Root/trt.4@30DAP
NOR-11	+	46	4.6	+	*Bacillus*	*anthracis*	Root/trt5@30DAP
NOR-12	+	45	12.3	-	*Beijerinckia*	*fluminensis*	Root/trt.1@30DAP
NOR-13	+	47	6.6	+	N/A		Rhs/trt.1@60DAP
NOR-14	+	45	5.0	+	N/A		Root/trt.1@60DAP
NOR-15	+	51	2.2	-	*Bacillus*	*gibsonii*	Rhs/trt.1@60DAP

NOR-16	+	48	2.5	+	*Bacillus*	*safensis*	Root/trt.1@60DAP
NOR-17	+	42	8.2	+	*Bacillus*	*aryabhattai*	Rhs/trt.4@60DAP
NOR-18	+	43	4.1	+	*Bacillus*	*pseudomycoides*	Rhs/ trt.4@60DAP
NOR-19	+	42	19.3	+	*Pseudomonas*	*psychrotolerans*	Stem/ trt.1@60DAP
NOR-20	+	46	11.2	+	*Bacillus*	*megaterium*	Rhs/trt 8,6,1@60DAP
NOR-21	+	59	10.5	+	*Pseudomonas*	*frederiksbergensis*	Rhs/trt.2@60DAP
NOR-22B	+	48	5.4	+	*Burkholderia*	*metallica*	Rhs/trt.4@60DAP
NOR-22	+	48	15.4	+++	*Bacillus*	*aryabhattai*	Rhs/trt.8@60DAP
NOR-23	+	48	5.7	++	*Bacillus*	*methylotrophicus*	Root/trt.2@60DAP
NOR-24	+	56	7.1	+	*Caulobacter*	*segnis*	Stem/trt.6@60DAP
NOR-25	+	46	9.8	+	*Burkholderia*	*diffusa*	Rhs/trt. 8@60DAP
NOR-26	+	44	9.6	++	*Pseudomonas*	*lini/thivervalensis0.948*	Rhs/trt.1@60DAP
NOR-27	+	45	3.0	+	N/A		Rhs/trt.1@60DAP
NOR-28	+	48	10.5	+	*Burkholderia*	*diffusa*	Rhs/trt.8@60DAP
NOR-29	+	47	6.8	+	N/A		Rhs/trt.2@60DAP
NOR-30	+	50	4.8	+	*Variovorax*	*paradoxus*	Root/trt.1@60DAP
NOR-31	+	47	3.4	+	N/A		Root/trt.4@60DAP

NOR-32	+	47	13.4	+	*Pseudomonas*	*lini*	Root/trt.4@60DAP
NOR-33	+	43	3.8	+	*Variovorax*	*paradoxus*	Root/trt.4@60DAP
NOR-34	+	48	13.5	+	*Massilia*	*flava*	Rhs/trt.4@60DAP
NOR-35	+	49	2.4	+	N/A		Root , stem/trt.8, 2@60DAP
NOR-36	+	47	2.6	+	*Curtobacterium*	*flaccumfaciens*	Stem/trt.2@60DAP
NOR-37	+	49	3.8	+	*Gordonia*	*terrae*	Rhs/trt.6@60DAP
NOR-38	+	45	3.2	+	*Methylobacterium*	*thiocyanatum*	Stem/trt.2@60DAP
NOR-39	+	42	1.9	+	*Gordonia*	*terrae*	Rhs/trt.2@60DAP
NOR-40	+	51	34.1	+	N/A		Stem/trt.2 @60DAP
NOR-41	+	43	2.0	+	*Bacillus*	*aerophilus*	Stem/trt. 6@60DAP
NOR-42	+	41	24.3	+	N/A		Root/trt.2@90DAP
NOR-43	+	47	47.4	-	*Beijerinckia*	*fluminensis*	Rhs/trt.2@90DAP
NOR-44	+	47	31.5	+	*Paenibacillus*	*lautus*	Rhs./trt.4@90DAP
NOR-45	+	48	4.5	+++	*Burkholderia*	*cenocepacia*	Rhs/trt.4@90DAP
NOR-46	+	47	8.4	-	*Bacillus*	*megaterium*	Rhs/trt.4@90DAP
NOR-47	+	47	4.1	-	*Bacillus*	*anthracis*	Rhs/trt.4@90DAP
NOR-48	+	47	2.8	++	*Bariovarax*	*soli*	Rhs/trt.4@90DAP

NOR-49	+	56	2.8	+++	*Bacillus*	*subtilis*	Stem/trt.6@90DAP
NOR-50	+	53	12.8	+	*Microbacterium*	*resistens*	Stem/trt.6@90DAP
NOR-51	+	47	23.5	-	*Sphingomonas*	*pseudosanguinis*	Ste,/trt.6@90DAP
NOR-52	+	47	7.3	+	*Pseudomonas*	*oleovorans*	Stem/trt.2@90DAP
NOR-53	+	41	11.2	+	*Bacillus*	*megaterium*	Rhs/trt.1@90DAP
NOR-54	+	47	19.7	-	*Caulobacter*	*vibrioides*	Rhs/trt.6@90DAP
NOR-55	+	47	8.1	+	*Pseudomonas*	*kilonensis*	Root/trt.10@90DAP
NOR-56	+	46	7.6	-	N/A		Stem/trt.1@90DAP
NOR-57	+	45	44.0	+++	*Pseudomonas*	*sp.*	Stem/trt.1 @90DAP
NOR-58	+	48	40.8	-	*Beijerinckia*	*fluminensis*	Root/trt.4 @90DAP
NOR-59	+	45	3.7	-	*Beijerinckia*	*fluminensis*	Root/trt. 8@90DAP
NOR-60	+	40	5.4	+	N/A		Root/trt.8@90DAP
NOR-61	+	45	15.0	-	*Sphingomonas*	*pseudosanguinis*	Root/trt.8 @90DAP
NOR-62	+	48	20.2	-	N/A		Stem/trt.2@90DAP
NOR-63	+	56	1.8	+++	*Pseudomonas*	*argentinensis*	Stem/trt.8@90DAP
NOR-64	+	48	18.0	-	*Sphingomonas*	*pseudosanguinis*	Stem/trt.8@90DAP

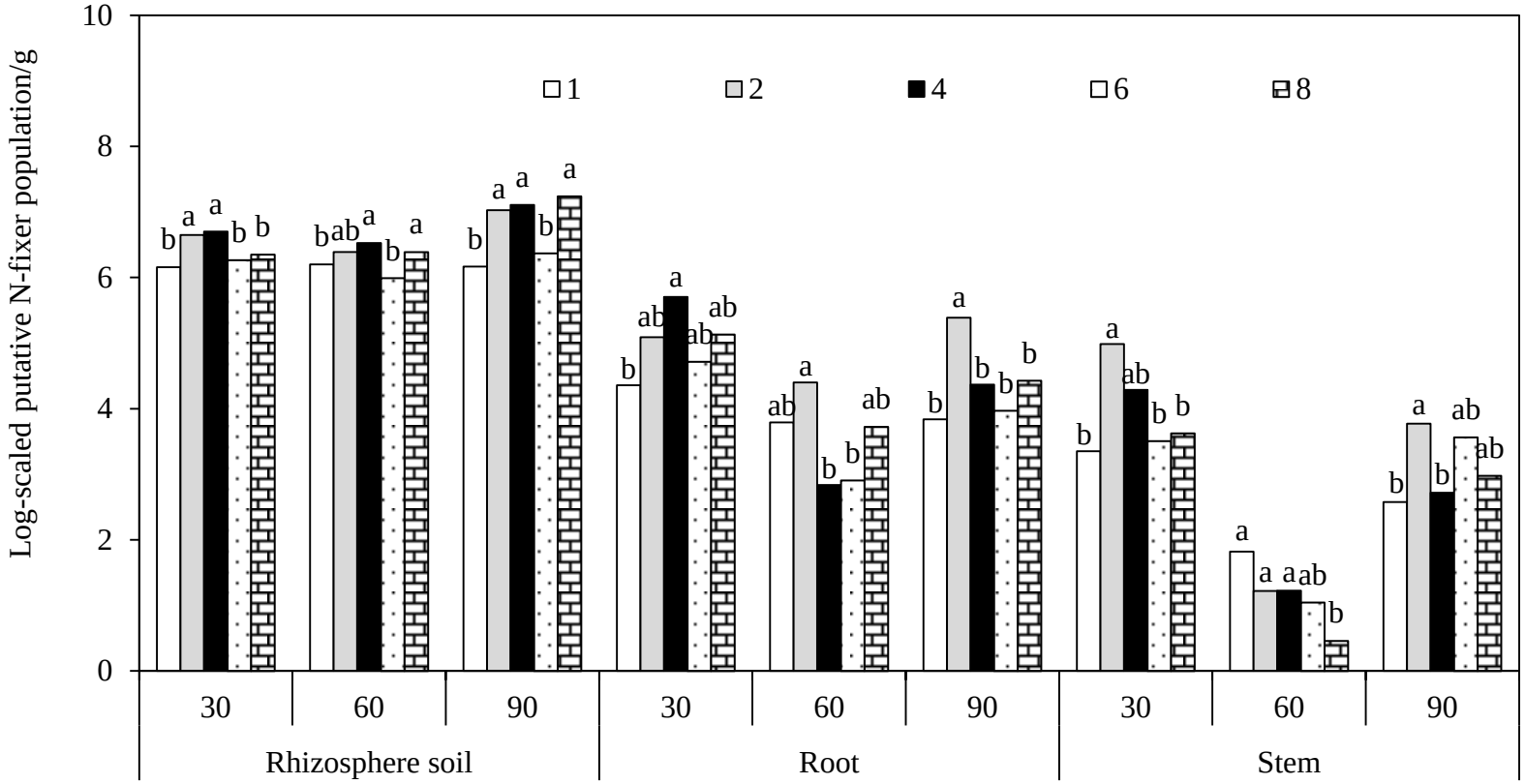

Figure 3-1 Populations of culturable N-fixing bacteria in rhizosphere soil and inside roots and stems of cotton sampled at 30, 60, and 90 days after planting across the five cropping system treatments (P<0.05).

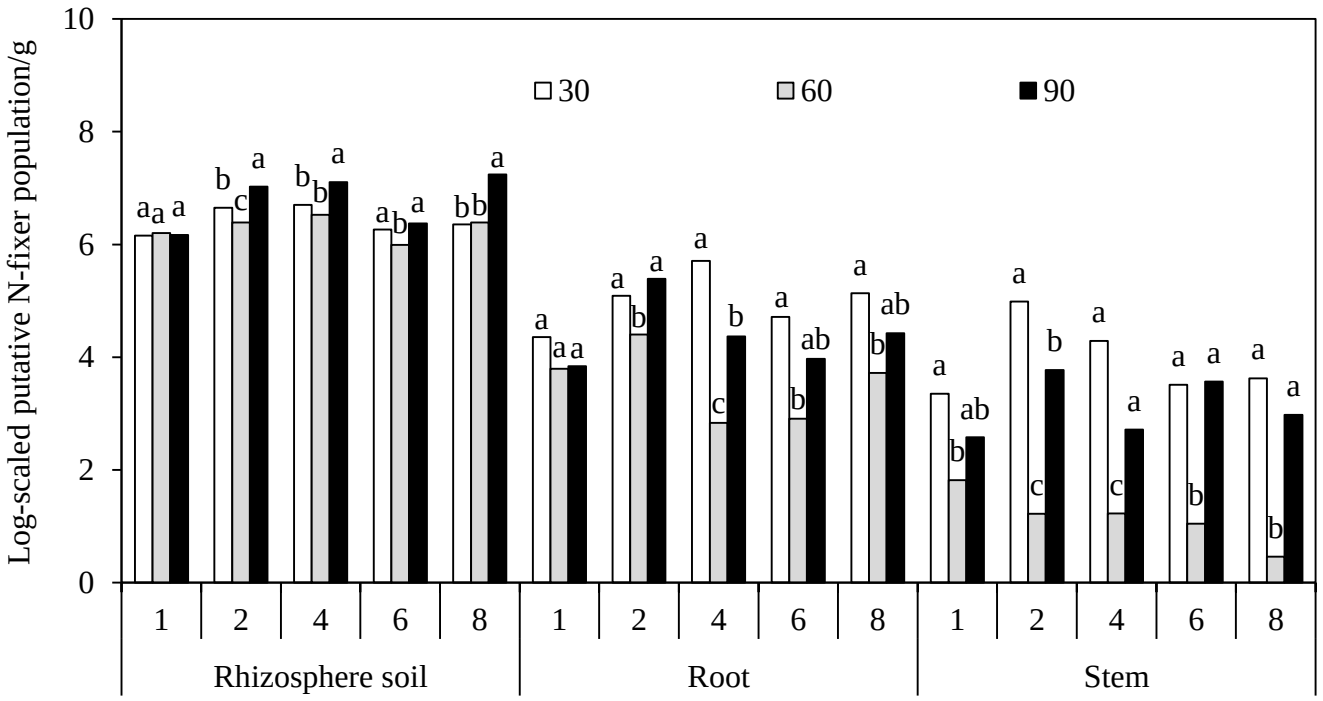

Figure 3-2 Change in populations of culturable N-fixing bacteria over sampling times (30,60, and 90 days after planting) in rhizosphere soil and inside roots and stems of cotton under selected cropping system treatments (P<0.05)

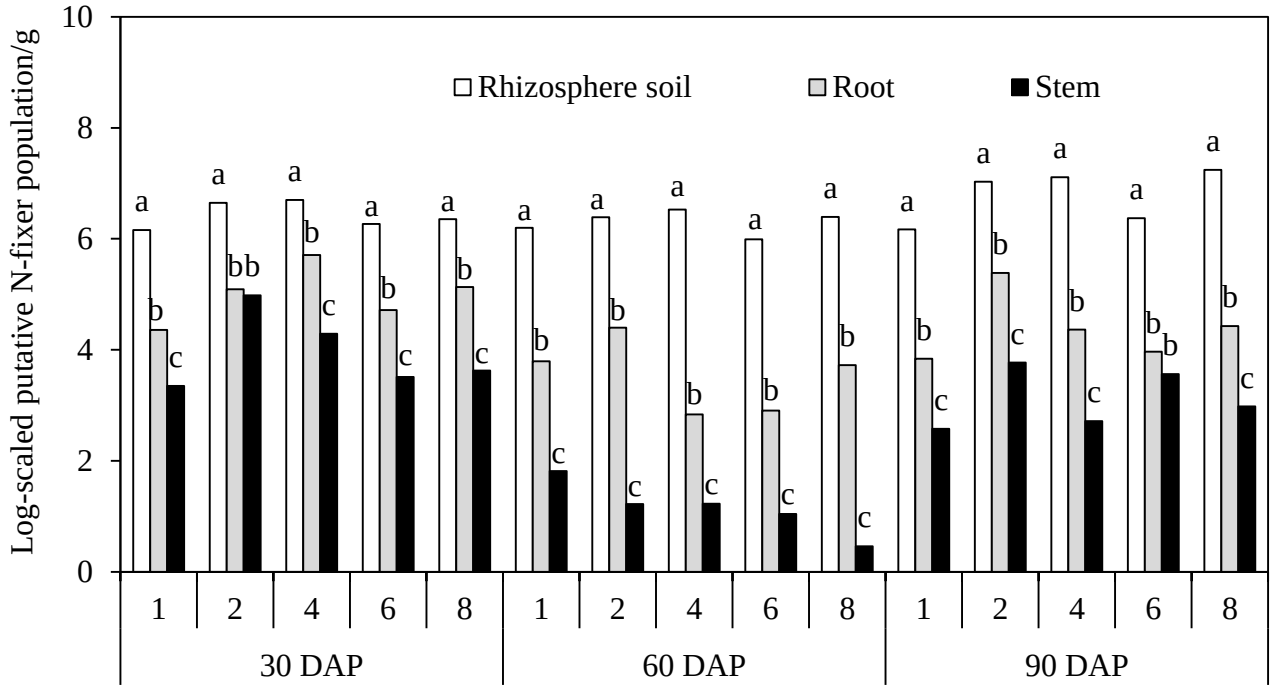

Figure 3-3 Log-scaled culturable N-fixing bacterial populations in rhizosphere soil and root and stem of cotton plant by cropping system treatment and sampling time.

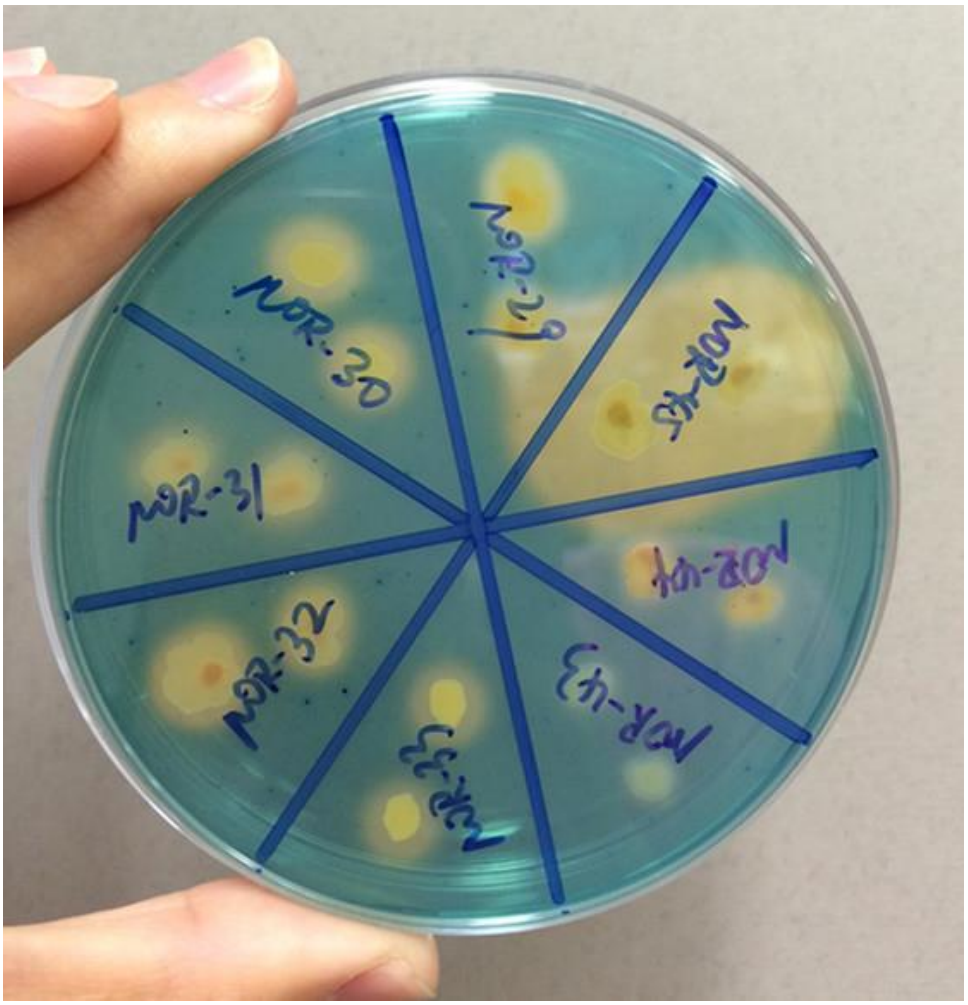

Plate 3-1 Siderophore production on CAS plate was indicated by orange halo around bacterial colonies.

Note NOR-45 siderophore production index of 2 (siderophore production index=orange halo diameter/colony diameter)

CHAPTER 4 CULTURABLE PHOSPHATE-SOLUBILIZING BACTERIA DIVERSITY AND ACTIVITY IN PHOSPHATE-LIMITED COTTON FIELD OF THE CULLARS ROTATION IN ALABAMA

4.1 INTRODUCTION

Phosphorus is one of the major macronutrients involved in plant growth and productivity. Large amounts of inorganic phosphate fertilizers are applied to agricultural soils, but the applied phosphate is quickly immobilized and becomes unavailable to plants. Many inorganic phosphates in the soil are present in insoluble forms such as calcium phosphate, which is found mostly in neutral to alkaline soil conditions, and iron phosphate and aluminum phosphate, which are found mostly under acidic conditions. Only phosphate iron forms in $H_2PO_4^-$ (dihydrogen phosphate ion) and HPO_4^{2-} (monohydrogen phosphate ion) can be taken up by plants. Therefore, the soil pH and the capacity to release insoluble phosphate as plant-available forms are important aspects affecting soil phosphorus availability (Mehta & Nautiyal, 2001, Kumar & Narula, 1999). Some soil microorganisms play important roles in converting insoluble P to plant-available forms by lowering the rhizosphere pH and by producing chelating substances (Kumar & Narula, 1999). Bacteria such as *Bacillus*, spp., *Burkholderia* spp., *Rhizobium* spp., and *Pseudomonas* spp. are reported as phosphate-solubilizers that can convert the insoluble inorganic phosphate compounds, such as tricalcium phosphate, dicalcium phosphate, hydroxyapatite, and rock

phosphate, to plant available forms (Rodr ǵuez & Fraga, 1999, Yao et al., 2006, Baig et al., 2010).

In this study, cotton in The Cullars Rotation (circa 1911), where phosphate application had been controlled, were chosen as the target crop. The Cullars Rotation was established in 1911 one century ago on the campus of Auburn University and is the oldest, continuous soil fertility study in the United States. The name refers to the framer, J. A. Cullars, who once farmed this land. Three cotton blocks were chosen for this study, including one block without P input, one block with rock phosphate as the P-source, and one block in which plants were provided with complete nutrition. We hypothesized that there would be diverse P-solubilizing bacteria in blocks without any P application and strong P-solubilizing bacteria would be isolated from blocks with a long history of rock phosphate use. The overall objective of this study was to find strong and efficient phosphate-solubilizing bacteria for use as biofertilizers. The specific objectives were 1) to enumerate and compare the putative P-solubilizing bacteria in selected blocks of cotton crops (no P input, only rock phosphate input and complete nutrient blocks) and 2) to isolate and assess the P-solubilizing bacteria from the cotton rhizosphere soil and inside of roots and stems from the selected blocks.

4.2 MATERIAL AND METHODS

4.2.1 Experiments *in vitro*

4.2.1.1 Field sampling

Three different P-treated blocks of cotton plants were chosen to study and included treatment #2 with no P input, treatment #5 with only rock phosphate as P source, and treatment #10 with complete N-P-K fertilization plus micronutrients. The detailed sampling information is listed in Table 4-1. Six cotton plants and rhizosphere soil samples were randomly taken from each of the three treatments (treatment 2, treatment 5 and treatment 10) at 30, 60, and 90 days after planting in 2012 for bacterial isolation, enumeration, and assessment.

4.2.1.2 Methods for isolation

Modified Pikovskaya's medium (MPIK) (Vazquez et al., 2000) medium was used for isolation and enumeration of bacteria. This medium contains three different insoluble phosphorus compounds $FePO_4 \cdot 2H_2O$, $AlPO_4$, and $Ca_{10}(OH)_2(PO)_6$. The calcium phosphate is not $Ca_3(PO_4)_2$, but replaced by $Ca_{10}(OH)_2(PO)_6$. The detailed medium compounds are attached in Appendix A.

Rhizosphere soil and roots and stems were collected from each cotton plant. One hundred microliters of serially diluted (from 10^0 to 10^{-5}) rhizosphere soil solution were placed on SRSM medium which contains $Ca_3(PO_4)_2$ as the only P-source for

enumeration and isolation. The composition of SRSM was attached in the Appendix A. The stem and roots were washed with sterilized distilled water to remove surface soil. They were surface sterilized through immersion in 70% ethanol for 1 minute and 50% commercial bleach for 3 minutes and then rinsed 3 times in sterilized distilled water. Stem and root samples (1.0g) were macerated separately using a KLECO grinder in sterilized distilled water and serially diluted to 10^{-3}. One hundred microliters of these dilutions were inoculated onto SRSM medium for enumeration and isolation. Bacterial single colonies that grew on SRSM with clear zones were selected and streaked on a new SRSM plate for further purification. Putative P-solubilizing bacteria were transferred on SRSM for 3 times to test the consistency of P-solubilizing activity. Purified putative P-solubilizing bacteria were maintained in 1.5 ml vials filled with 70% TSB and 30% glycerol liquid nutrient medium and stored in a -80 ℃ freezer.

The isolated putative P-solubilizing strains were tested *in vitro* for their phosphate solubilization activity and other plant growth-promoting traits such as auxin and siderophore production.

4.2.1.3 Methods for characterization

Quantitative determination of phosphate solubilization activity

Three different versions of modified Pikovskaya's media (MPIK) (Lopez et al., 2011), each with a different P source (0.38% $Ca_3(PO_4)_2$, 0.01% Fe PO_4, and 0.01% Al PO_4) were used for quantitative determination of the capacity to solubilize inorganic phosphate. One loopful of bacteria was inoculated into 25ml of liquid MPIK media (without yeast extract and agar), which was stirred thoroughly and shaken at 150 rpm at 28 ℃. Bacterial broth samples were taken at 3, 8, and 14 days, and bacteria cells were precipitated by centrifugation at 12,000 rpm for 15 minutes. The amount of P in the supernatant was determined by the Molybdenum-blue method (Murphy & Riley, 1962). A blue color indicated a positive reaction for phosphate solubilization (Plate 2-4). Phosphorus concentration was estimated by comparison with the standard curve prepared with KH_2PO_4 (sigma Lot# 069K0342) and expressed in mg P L^{-1}. The composition is attached in Appendix A.

Qualitative determination of phosphate solubilization activity

The solubilization index was used to describe qualitatively the capacity of the bacteria to solubilize insoluble inorganic phosphate. The solubilization index consists of the ratio of the total diameter (colony + halo) to the colony diameter (Kumar & Narula, 1999) (Figure 4-1).

Auxin production

Auxin is a plant hormone produced by bacterial and improves plant growth by increasing the number of root hairs and lateral roots. This auxin production is a very

important plant growth promotion and nutrient uptake characteristic of bacterial inoculants.

The capacity of the isolated putative P-solubilizing bacteria to produce auxin (Frankenberger Jr, 1988, Gordon & Weber, 1951, Husen, 2003) was determined *in vitro*. Bacteria were grown on M-26 for 24 hours on a shaker at 150 rpm at room temperature as seed culture. Next, 15 µl of culture were inoculated to 1.5 ml minimal salt medium (MS) amended with 5 mM L-Tryptophan and grown again on the shaker for 48 hours. Tubes containing 1.5 ml bacterial broth were centrifuged at 12,000 rpm for 5 minutes, and 0.5ml of the bacterial supernatant were added into 1ml $FeCl_3$-$HClO_4$ reagent. After the color density reached its maximum, the tubes were set for 25 minutes under dim light. A pink color reaction indicated positive auxin production (Plate 2-3). Quantitative measurements of the color intensity were made using a UV-spectrophotometer (Thermo mode: Genesys 10S UV-Vis) at 530 nm absorbance. Auxin concentration was estimated by comparison with the standard curve prepared with indole-3-acetic acid (Sigma Lot#092K1235) and expressed in µg ml^{-1}. The composition and preparation of M-26 medium, MS medium, L-tryptophan stock solution and $FeCl_3$-$HClO_4$ reagent are shown in Appendix A.

Siderophore production

Siderophore production is an important plant growth promotion and nutrient uptake characteristic which can be tested *in vitro*. Siderophores produced by bacteria

may enhance plant growth by increasing the availbability of Fe nearby the root or by inhibiting the colonization of plant pathogen or other harmful bacteria on roots.

Chrome azurol S (CAS) agar (Husen, 2003, Schwyn & Neilands, 1987) was used to obtain a qualitative description of siderophore production. Bacteria were grown on TSA for 24-48 hours and then half loopfuls of bacteria were transferred to 9 ml distilled water tubes. The bacterial suspension was vortexed thoroughly, and CAS medium was divided into 4 quadrants. Each quadrant was pipetted with 15 µl bacterial suspension. Orange halos around the growing bacterial colonies confirmed the siderophore production after 48-72 hours incubation (plates 2-5). The CAS agar was a mixture of four solutions that were prepared and sterilized separately before mixing. Fe-CAS indicator solution, buffer solution and glucose/mannitol solution were sterilized by an autoclave; casamino acid was sterilized by 0.2 µM filter. The composition of CAS medium is shown in Appendix A.

Molecular characterization and phylogenetic analysis

Taxonomic classification of each strain was based on the partial sequence of 16S rDNA. DNA was extracted and amplified with universal bacterial primers: 8F (5'- AGAGTTTGATCCTGGCTCAG -3') and 1492R (5'- ACGGCTACCTTGTTACGACTT- 3'). PCR was performed using Lucigen EconoTaq Plus Green 2X master mix (Lucigen Corp.) with cycling parameters: initial denaturation at 95 ℃ for 5 min; 31 cycles of 94 ℃ for 1 min, 57 ℃ for 45 sec, 70 ℃

for 2 min; and a final extension at 70 ℃ for 10 min. All sequences were blasted against the type strains in the ribosomal database project to identify bacterial taxa of each strain.

4.2.1.4 Data analysis

Culturable P-solubilizing bacterial population data were transformed into log-scaled data and then subjected to analysis of variance using the PROC GLIMMIX procedure in SAS. The critical *P*-value of 0.05 was used as cutoff for testing fixed effects, and determination of differences in least-squares means was based on adjusted *P*-value obtained by using the option ADJUST=SIMULATE in the LSMEANS statement.

4.2.2 Experiments *in planta*

4.2.2.1 Bacterial strain selection and inoculum preparation

Bacterial selection

Phosphate-solubilizing bacteria were selected for two greenhouse tests based on the 3d, 8d and 14d inorganic phosphate-solubilizing data (the average of 3d, 8d and 14d data up to 2.0 mg P L^{-1}) and halo-zone diameter index data separately (the index up to 2.0). The bacterial information is listed on Table 4-2.

Inoculum preparation

All selected bacterial strains were grown on TSA to obtain a single colony. After growing for 48-72 hours at 28 ℃, portions of the resulting growth were placed into distilled H_2O. Serial dilutions were made, and the bacterial suspensions were adjusted to a concentration of 10^7 cfu/ml. The bacterial concentrations were evaluated and recorded by the Miles and Misra method.

4.2.2.2 Greenhouse experiment setup for selected P-solubilizing strains

Two experiments were both organized in a complete randomized design (CRD) with 6 replications plus 1 water blank. Corn (*Zea mays*) was used as the host plant for testing the selected P-solubilizing strains. The two tests were each repeated twice..

In greenhouse test A, ten strains were randomly selected based on having a diameter index larger than 2. They were CUP-7, CUP-11, CUP-12, CUP-15, CUP-16, CUP-17, CUP-19, CUP-20, CUP-23, and CUP-32. Sunshine mix was used for this test. This test was conducted from September to December of 2012; corn and pepper were used as the test plants. Two corn and pepper seeds were sown in each pot, but only one germinated seed was kept. Plants were harvested after 4 weeks before V6 growth stage (28 DAP). Plant growth-promoting parameters that were measured included chlorophyll content (SPAD 502 meter), plant height, fresh shoot and root weight, and dry shoot and root weight (70 ℃). Plant samples were dried in a 70 ℃

oven for 72 hours to obtain the dry weight. No nutrient analysis was conducted for this test.

In greenhouse test B, eight bacterial strains were randomly selected. The selection is based the phosphate-solubilizing data up to 2.0 mg P L^{-1} which the average of three-day (3 d, 8 d and 14 d) data. They were CUP-4, CUP-7, CUP-9, CUP-18, CUP-19, CUP-28, CUP-38 and CUP-39. Field soil was used for this test. This test was conducted from December 2013 to April 2014; corn was used as the test crop. Two corn seeds were sown in each pot, but only one germinated seed was kept in. Plants were harvested after 4 weeks before V6 growth stage (28 DAP). Plant growth-promoting parameters that were measured included chlorophyll content (SPAD 502 meter), plant height, fresh shoot and root weight, and dry shoot and root weight (70 ℃). Plant samples were dried in 70 ℃ oven for 72 hours to obtain the dry weight. All the above-ground tissues of greenhouse test A were sent to Waters Agricultural Laboratories, Inc (Camilla, Georgia) for nutrient analysis.

4.2.2.3 Data analysis

Growth promotion data were subjected to analysis of variance using the PROC GLIMMIX procedure in SAS. The critical *P*-value of 0.05 was used as cutoff for testing fixed effects, and determination of differences in least-squares means was based on adjusted *P*-value obtained by using the option ADJUST=DUNNETT in the LSMEANS statement.

Culturable phosphorus-solubilizing bacterial populations in rhizosphere soil and in roots and stems of cotton plants were evaluated for selected fertilization treatments at 30, 60, and 90 DAP. Analysis of putative P-solubilizing bacterial population data indicated a sampling position × sampling time × fertilization treatment interaction ($P<0.05$).

4.3 RESULTS

4.3.1 Enumeration of culturable P-solubilizing bacteria from selected fertilization treatments

The effect of fertilization treatment on culturable P-solubilizing bacterial populations in rhizosphere soil was inconsistent, while the effect was more consistently found in roots and stems of cotton plants (Figure 4-2). There were no significant differences for culturable P-solubilizing bacteria in rhizosphere soil among the three fertilization treatments at 30 DAP, while differences were noted at 60 but not at 90 DAP. Based on these results, the results from rhizosphere soil at 60 DAP were examined. Fertilization treatment No. 10 with complete N-P-K fertilization plus micronutrients had significantly higher culturable P-solubilizing bacterial populations than the other two P treatments. In roots and stems of cotton plants, putative P-solubilizing bacteria were isolated only from plant samples taken at 30 DAP. Fertilization treatment No. 10 had significantly higher culturable P-solubilizing bacterial populations than the other two P treatments when sampled at 30 DAP.

Figure 4-3 shows the variation in P-solubilizing bacterial populations over time by fertilization treatment and in rhizosphere soil and roots and stems of cotton plants. The variation for rhizosphere soil was not consistent for the three P treatments. Generally, P-solubilizing bacterial populations in rhizosphere soil were higher at 30 DAP than at 60 and 90 DAP, probably because soil temperature and moisture conditions at 30 DAP were good for bacteria growth.

Regardless of sampling time and fertilization treatment, culturable P-solubilizing bacterial populations were consistently highest in rhizosphere soil, followed by populations in stems and roots of cotton (Figure 4-4). In rhizosphere soil, the highest culturable P-solubilizing bacterial populations at 30, 60 and 90 DAP were approximately 1.82 X 10^5 per gram soil (under fertilization treatment 10), 2.1 X 10^5 per gram soil (under fertilization treatment 10), and 1.67 X 10^5 per gram soil (under fertilization treatment 5). The lowest culturable P-solubilizing bacterial populations at 30, 60 and 90 DAP were approximately 5.7 X 10^4 per gram soil (under fertilization treatment 5), 3.5 X 10^4 per gram soil (under fertilization treatment 2), and 7.4 X 10^4 per gram soil (under fertilization treatment 2).

4.3.2 Confirmation of P-solubilizing activity and evaluation of other PGP traits

P-solubilizing activity of the isolated bacteria was comfirmed by quantitative determination and qualitative determination. P-solubilizing activity results from both quantitative determination and qualitative determination showed no clear relation between these two methods. For example, CUP-15 showed very strong P-solubilizing

activity by using qualitative determination, but rarely showed any P-solubilzing activity by quantitative determination. CUP-10 showed very weak P-solubilizing activity in qualitative determination but very strong P-solubilizing activity consistently from 3 d to 14 d in quantitative determination. From qualitative determination method, 61% of the isolated strains had P-solubilizing activity in which the index reached 1.5, and 31% of the isolated strains had P-solubilizing activity in which the index reached 2.0. From quantitative determination method, 50% of the isolated strains maintained strong P-solubilizing activity, which reached 2.0 mg P ml^{-1} from 3 d to 14 d, and 20.5% of the isolated strains lost P-solubilizing activity at 8 d.

Other PGP traits showed that 100% of the isolated P-solubilizing bacteria produced auxin in amounts ranging from 5.3 to 79.16 μg ml^{-1}, and that 23% P-solubilizing bacteria produced remarkably high amounts of auxin that were over 50 μg ml^{-1}. In additon, 82% of the isolated P-solubilizing bacteria produced siderophores.

4.3.3 Molecular Identification

Thirty of the total 39 isolated culitivable P-solubilizing bacterial strains were identified by using 16S r DNA. Results represented a diversity with 9 genera including *Pseudomonas* (11), *Burkholderia* (2), *Enterobacter* (1), *Sphingomonas* (1), *Pantoea* (2), *Rhizobium* (1), *Acinetobacter* (1), and *Erwinia* (1). The number inside the parentheses indicates the number of different species in each genus. Of the isolated P-solubilizing bacterial strains, 77% were from cotton rhizosphere soil, 15% from inside cotton stems and 8% from inside cotton roots.

4.3.4 Plant growth promotion and nutrient uptake tests on selected P-solubilizing bacteria

The results from greenhouse test A indicated that plant growth promotion in either corn or pepper was significantly affected by selected P-solubilizing strains. Specifically, 6 of the 10 selected P-solubilizing strains in greenhouse test A, including CUP-17 significantly promoted shoot dry weight on corn, CUP-12 promoted plant height on corn and CUP-7 promoted root dry weight on pepper ($P<0.05$) (Table 4-3).

The results from greenhouse test B indicated that plant growth promotion and nutrient uptake in corn were significantly affected by selected P-solubilizing strains. Specifically, CUP-18 and CUP-28 significantly promoted plant height of corn, CUP-9 and CUP-19 significantly promoted plant fresh weight on corn ($P<0.05$) (Table 4-3). The results of the nutrient uptake analysis were notable in that 3 (CUP-39, CUP-38, CUP-18) out of the 8 selected P-solubilizing strains promoted nitrogen uptake rather than phosphorus uptake (Table 4-4 & 4-5).

4.4 DISCUSSION AND CONCLUSIONS

This is the first report on evaluating culturable P-solubilizing bacteria diversity and population in cotton receiving controlled phosphorus fertilization and complete nutrient plus micronutrient fertilization in a long-term fertility study. Based on the culturable P-solubilizing bacterial population studies, plants with vigorous growth and high biomass yield under complete nutrition have the most abundant P-solubilizing bacteria near the rhizosphere and inside the roots and stems. This indicates that

healthy and vigorous plants have more abundant and more active microbial populations.

A total of 15 strains were isolated from block 10 (with complete nutrient plus micronutrients); 13 strains of P-solubilizing bacteria were isolated from cotton rhizosphere,1 strain from inside of stem, and 1 strain from inside of root. These strains were from 7 different genera and 10 different species. Where no P has been applied (block 2), 6 strains were isolated from the rhizosphere, 5 from stem and 0 from root. These represent 4 different genera and 6 different species. Where only rock phosphate has been applied since 1911 (block 5) 8 different strains were isolated, 6 from rhizosphere, 0 from stem and 2 from root. These represent 2 genera and 7 different species. Based on these results, it is hard to make a solid conclusion to meet the hypothesis since only culturable bacteria were evaluated in this research. After comparing with two different P-solubilizing tests; neither of those tests were promising on evaluating the strain's P-solubilizing activity. But just based on the culturable isolation and the population, treatments with complete nutrient and micronutrient has a diverse P-solubilizing bacteria species, genera and population which is opposite to our starting hypothesis that there would be diverse P-solubilizing bacteria in blocks without any P application. To meet the other hypothesis that strong P-solubilizing bacterial would be isolated from block with a long history of rock phosphate use, the methods to determine P-solubilizing activity gave inconsistent results. We were unable to determine which strain is the strongest. All the isolated

putative P-solubilizing bacteria that showed activities for solubilizing inorganic P also resulted in other plant growth promotion traits such as IAA production and siderophore production. There was no relation between the qualitative and quantitative determinations of P-solubilization results. Therefore, it is difficult to determine which method is best for evaluating bacterial P-solubilizing activity. Baig et al. (2010) found that the qualitative method did not reflect the real P-solubilizing potential of the bacteria and suggested the use of the quantitative method when studying P-solubilization by phosphate solubilizing microbes.

Based on the greenhouse tests involving P-solubilizing bacteria, we suggest that either the quantitative or qualitative method can be used as a tool to screen P-solubilizing bacteria *in vitro*. Any bacteria that have P-solubilizing activity have the potential to promote plant growth and nutrient uptake. Other mechanism of action should also be studied, including other plant hormone secretion, N-fixation, K-solubilization and stress tolerance. Since the strains promoted plant growth didn't increase nutrient uptake and the strains increase nutrient uptake didn't promote plant growth, the relation between growth promotion and nutrient uptake showed be further studied. Test crops by using these inoculants are important and need to be further studied.

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Table 4-1 Cotton sampling from the Cullars Rotation

Treatment No.	Treatment
2	No P
5	Rock phosphate as the only P source
10	Complete N-P-K fertilization plus micronutrients

Table 4-2 Characterization of putative P strains isolated from cotton at the Cullars Rotation

Strain	Auxin (µg/ml)	Siderophore (+/-)	P-test (qualitative)	P-test 3d	P-test 8d	P-test 14d	Genus	Species	Source
CUP-1	10.84	+	1.8	2.4	2.3	1.9	*Pseudomonas*	*corrugata*	cotton rhizosphere 5
CUP-2	34.33	+	1.6	2.2	1.6	1.3	*Burkholderia*	*metallica*	cotton rhizosphere 2
CUP-3	39.77	+	1.1	2.0	1.9	1.8	*Psedomonas*	*jessenii*	cotton rhizosphere 5
CUP-4	11.96	+	1.2	2.6	2.3	2.4	*Pseudomonas*	*vancouverensis*	cotton rhizosphere 5
CUP-5	12.34	+	1.8	2.1	0.5	0.4	*Pseudomonas*	*taiwanensis*	cotton rhizosphere 5
CUP-6	30.75	+	1.8	2.40	2.30	1.80	*Pseudomonas*	*chlororaphis*	cotton rhizosphere 10
CUP-7	6.78	+	2.5	0.8	2.4	2.8	*Burkholderia*	*spp.*	cotton rhizosphere 2
CUP-8	33.80	+	2.0	1.20	1.50	1.30			cotton rhizosphere 5
CUP-9	34.78	+	1.9	2.4	1.2	2.8			cotton rhizosphere 10
CUP-10	18.35	+	1.1	2.0	1.9	2.0	*Pseudomonas*	*mohnii*	cotton rhizosphere 10
CUP-11	55.03	-	2.4	0.40	0.20	0.10	*Pantoea*	*dispersa*	cotton rhizosphere 5
CUP-12	73.00	+	2.0	1.4	0.0	0.0			cotton rhizosphere 10/5
CUP-13	51.80	+	1.9	1.9	1.2	-0.1	*Acinetobacter*	*soli*	cotton rhizosphere 10
CUP-14	47.29	-	1.8	2.20	2.50	2.60	*Erwinia*	*persicina*	cotton rhizosphere 10
CUP-15	50.07	-	2.3	0.10	0.00	0.00	*Pantoea*	*eucrina*	cotton rhizosphere 2

CUP-16	56.95	-	2.4	0.22	0.12	0.00	*Pantoea*	*eucrina*	cotton rhizosphere 2
CUP-17	52.52	+	2.5	0.1	0.0	0.0	*Pantoea*	*eucrina*	cotton rhizosphere 2
CUP-18	52.29	+	2.1	2.9	2.6	2.9	*Enterobacter*	*ludwigii*	cotton rhizosphere 10
CUP-19	6.89	+	2.1	2.1	1.7	2.1	*Pseudomonas*	*psychrotolerans*	cotton rhizosphere 10
CUP-20	9.97	+	2.0	1.1	0.0	2.3	*Burkholderia*	*metallica*	cotton rhizosphere 10
CUP-21	77.58	+	1.1	1.7	0.9	1.1			cotton rhizosphere 10
CUP-22	6.51	+	1.2	1.50	1.80	1.62	*Pseudomonas*	*moorei*	cotton rhizosphere 10
CUP-23	5.46	+	2.0	1.3	-0.2	0.0	*Burkholderia*	*spp.*	cotton rhizosphere 10
CUP-24	10.68	+	1.2	1.6	0.6	0.8	*Pseudomonas*	*fulva*	cotton stem 10
CUP-25	8.43	+	1.4	2.2	1.6	1.9	*Pseudomonas*	*taiwanensis*	cotton rhizosphere 5
CUP-26	6.17	+	1.9	2.40	2.50	1.80	*Pseudomonas*	*moorei*	cotton rhizosphere 5
CUP-27	5.57	+	1.9	2.80	2.50	2.20			cotton rhizosphere 5
CUP-28	6.51	+	1.8	2.8	2.4	2.7	*Enterobacter*	*ludwigii*	cotton rhizosphere 10
CUP-29	79.16	+	1.8	1.4	0.9	1.0			cotton rhizosphere 5
CUP-30	6.33	+	1.2	1.70	1.90	1.80	*Pseudomonas*	*mohnii*	cotton rhizosphere 2
CUP-31a	14.78	+	1.4	1.6	0.0	0.0	*Rhizobium*	*multihospitium*	cotton stem 2
CUP-31b	23.61	+	1.3	1.50	1.20	0.90	*Pseudomonas*	*oleovorans*	cotton stem 2

CUP-32	5.39	+	1.2	0.8	0.0	0.0	*Burkholderia*	*metallica*	cotton rhizosphere 2
CUP-33	6.93	+	1.2	1.50	1.00	0.20			cotton rhizosphere 5
CUP-34	7.57	+	1.4	1.80	2.90	2.30	*Pseudomonas*	*mohnii*	cotton root 5
CUP-35	11.70	+	1.2	2.40	2.50	2.00			cotton stem 2
CUP-36	6.55	+	1.1	1.5	-0.1	0.0	*Pseudomonas*	*psychrotolerans*	cotton stem 2
CUP-37	47.67	-	1.1	0.80	0.50	0.40			cotton stem 2
CUP-38	2.87	-	2.0	2.4	2.5	2.9	*Sphingomonas*	*pituitosa*	cotton root 10
CUP-39	2.94	-	2.3	2.8	2.6	3.0	*Bacillus*	*cereus*	cotton root 5

Table 4-3 Effect of selected P strains on growth of corn and pepper in greenhouse in 2012

Crop	Strain treatment	Plant height (cm)	Shoot dry weight (g)	Root dry weight (g)	Total dry weight (g)
Corn	Control	91.2	2.32	0.82	3.14
	CUP-7	92.4	2.44	0.86	3.30
	CUP-19	91.4	2.55	0.82	3.37
	CUP-17	94.1	2.72 **	0.85	3.58 **
	CUP-16	95.1	2.42	0.63 *	3.05
	CUP-15	93.9	2.42	0.74	3.16
	CUP-12	94.5 *	2.33	0.70	3.03
	CUP-11	91.7	2.15	0.73	2.88 *
	CUP-32	91.6	2.25	0.62 **	2.87
	CUP-23	92.9	2.29	0.76	3.05
	CUP-20	91.0	2.34	0.71	3.05
Pepper	Control	27.6	4.99	0.27	5.26
	CUP-7	28.4	5.06	0.35 *	5.41
	CUP-19	27.5	4.98	0.34	5.32
	CUP-17	27.3	5.02	0.32	5.34
	CUP-16	27.8	5.00	0.33	5.33
	CUP-15	26.8	5.00	0.32	5.31
	CUP-12	25.4	4.85	0.32	5.17
	CUP-11	28.3	4.93	0.25	5.18
	CUP-32	27.2	4.90	0.31	5.21
	CUP-23	26.3	4.84	0.29	5.13
	CUP-20	28.1	4.97	0.31	5.28

* $P<0.05$, ** $P<0.01$, and *** $P<0.001$, compared with contro

Table 4- 4 Effect of selected P strains on growth of corn in greenhouse in 2013-2014

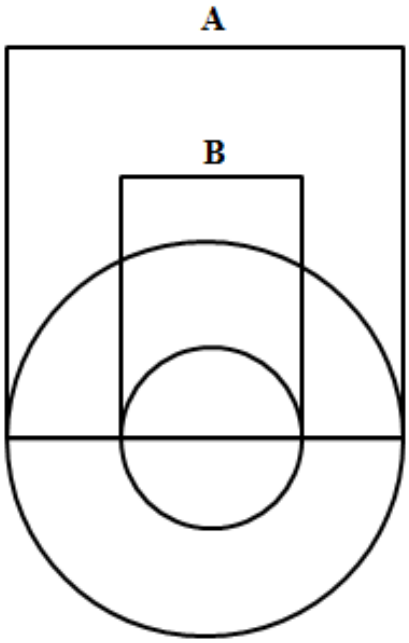
Treatment	Height (cm)	Root dry weight (g)	Root fresh weight (g)	Shoot dry weight (g)	Shoot fresh weight (g)	SPAD
Control	64	0.45	3.64	1.11	11.64	39.34
CUP-39	70.33	0.54	4.29	1.4	14.89	38.82
CUP-18	69.33 *	0.4	1.54 ***	1.18	12.47	40.5
CUP-28	71.42 **	0.41	3.91	1.26	13.49	38.62
CUP-38	68.67	0.46	3.71	1.17	12.49	37.78
Control	70.75	0.2	3.15	0.82	12.18	40.68
CUP-4	71.42	0.23	3.76	0.86	12.52	40.97
CUP-7	72.92	0.26	3.79	0.96	13.28	40.35
CUP-9	69.17	0.25	3.85 *	0.84	11.59	40.68
CUP-19	72.33	0.2	3.76 *	0.87	13.67	41.08

* $P<0.05$, ** $P<0.01$, and *** $P<0.001$, compared with control.

Table 4-5 Effect of selected P strains on nutrient uptake of corn in greenhouse in 2013-2014

Strain	Nitrogen (%)	P (%)	K (%)
Control	3.26	0.30	3.98
CUP-39	3.56 *	0.29	3.82
CUP-38	3.98 ***	0.31	4.52 ***
CUP-18	3.69 **	0.32	4.19
CUP-28	3.36	0.30	3.83
Control	4.69	0.31	5.59
CUP-7	4.09 ***	0.33	5.30
CUP-9	4.64	0.30	5.32
CUP-19	4.89	0.33	5.60
CUP-4	4.65	0.30	5.48

* $P<0.05$, ** $P<0.01$, and *** $P<0.001$, compared with control.



Solubilization index = A/B

A = diameter (colony + halozone)

B = diameter colony

Figure 4-1 Phosphate solubilization index

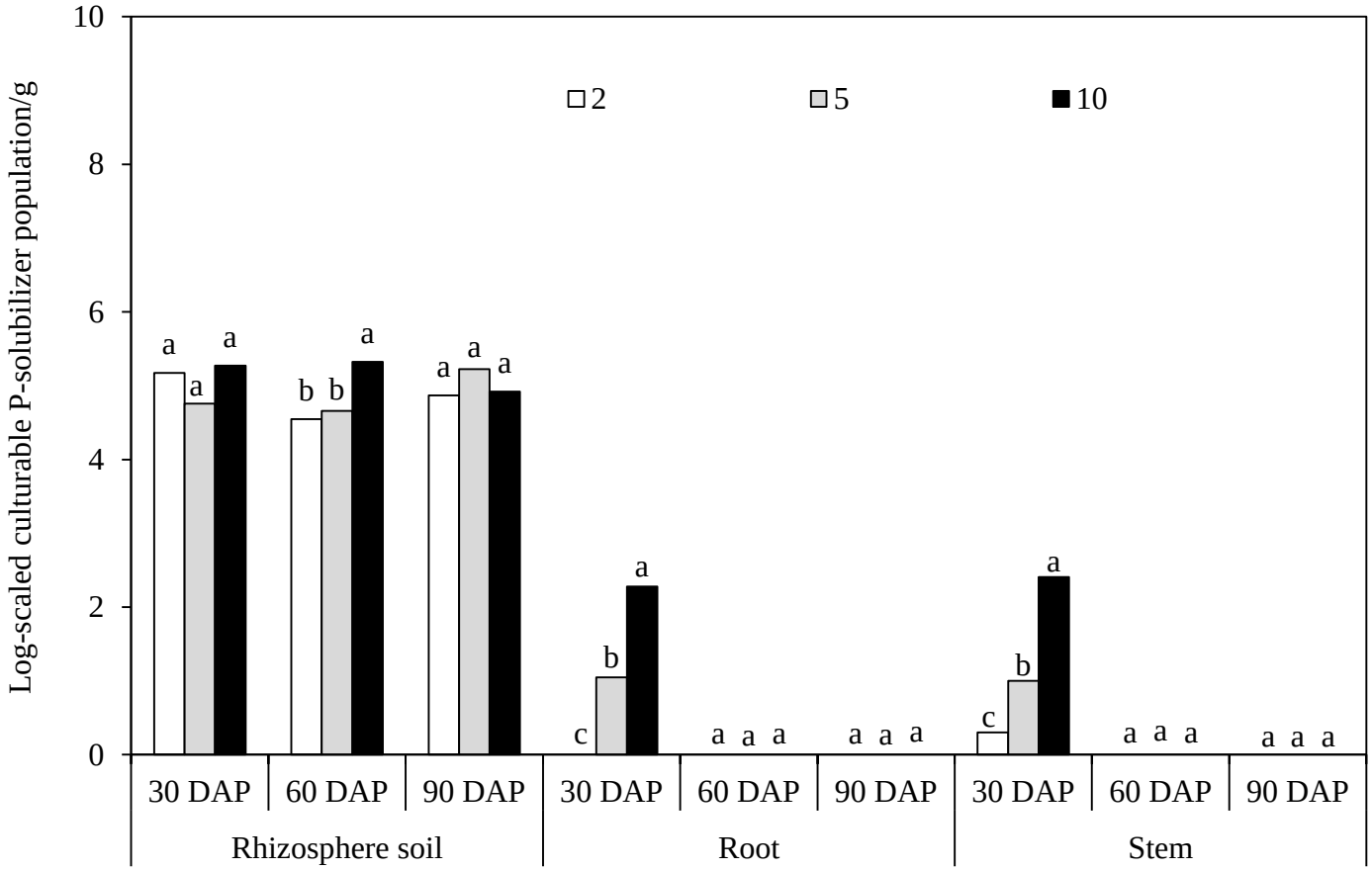


Figure 4-2 Log-scaled culturable P-solubilizing bacterial population in rhizosphere soil and in roots and stems of cotton sampled at 30, 60, and 90 days after planting across the three fertilization treatments ($P<0.05$).

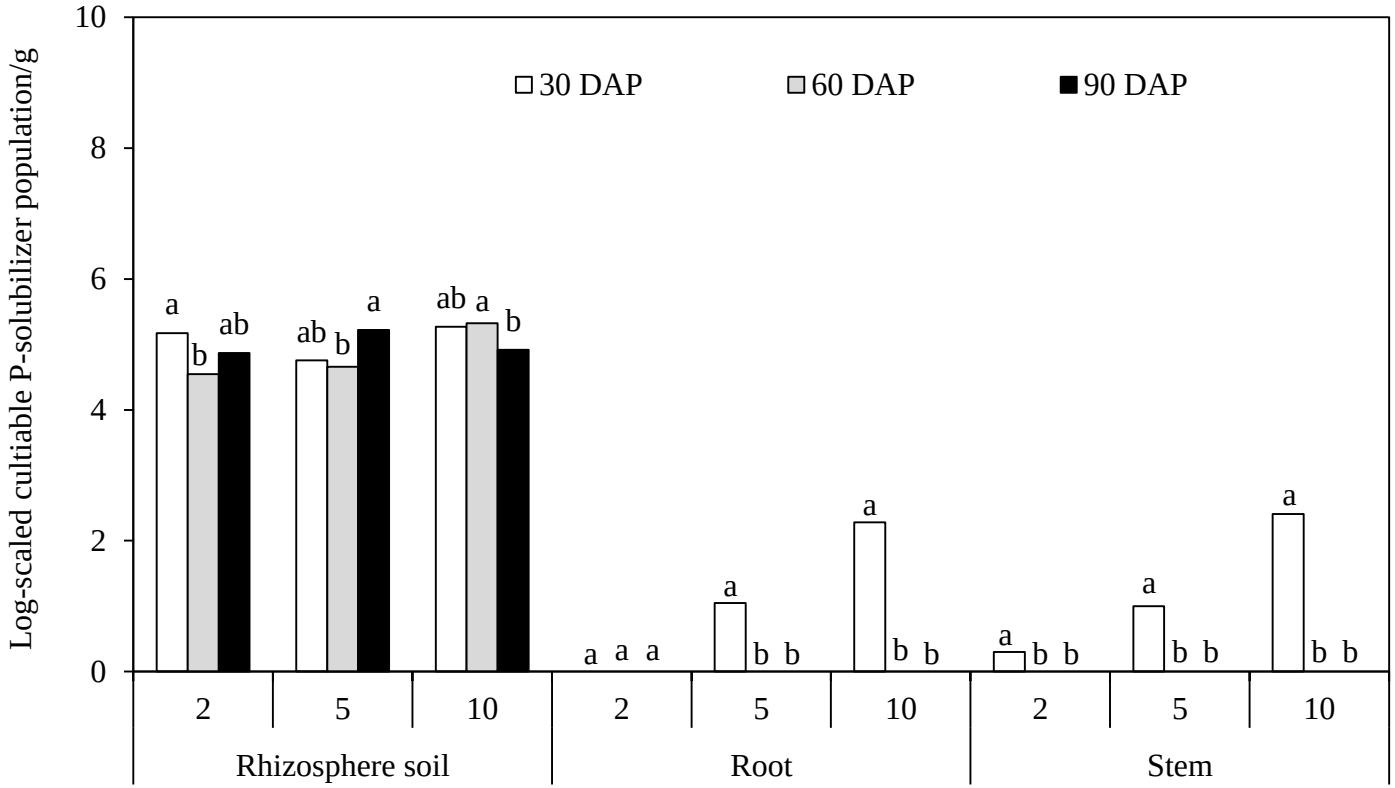


Figure 4-3 Change in log-scaled culturable P-solubilizing bacterial population over sampling times (30, 60, and 90 days after planting) in rhizosphere soil and in roots and stems of cotton under selected fertilization treatments (P<0.05).

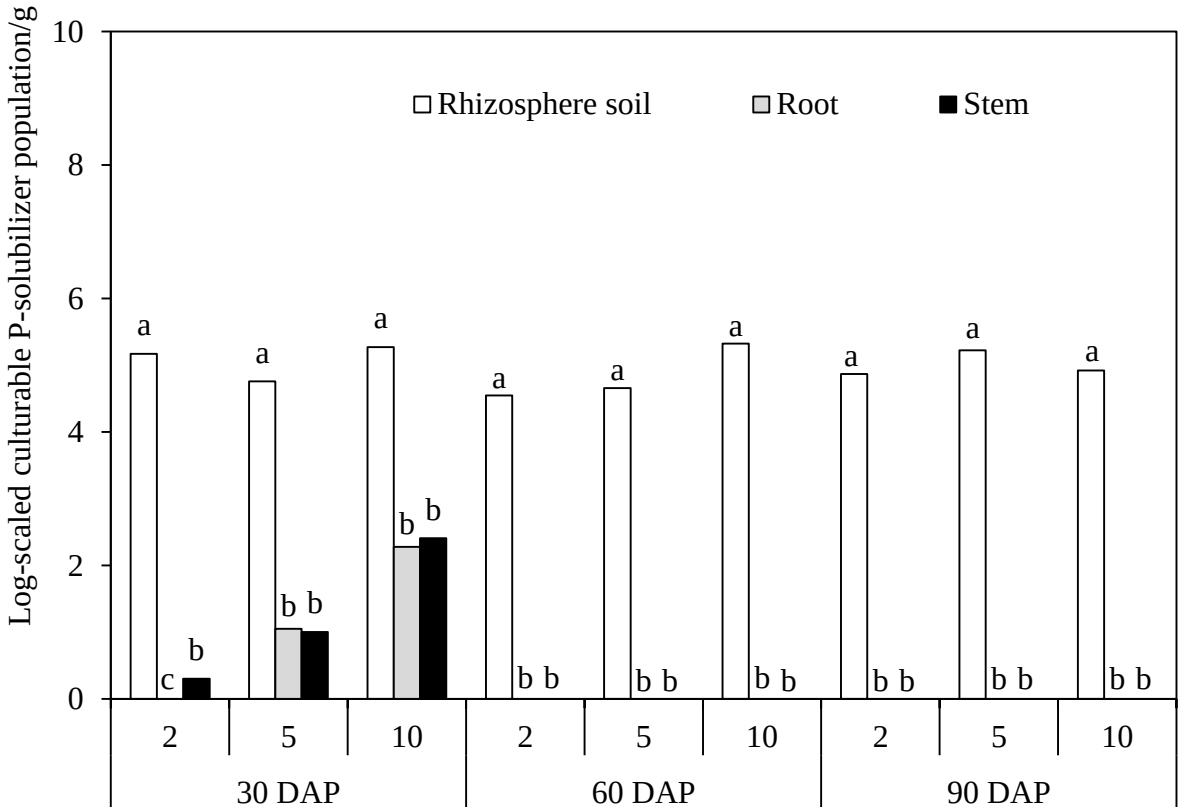


Figure 4-4 Log-scaled culturable N-fixer population in rhizosphere soil and in roots and stems of cotton plant by fertilization treatment and sampling time (P<0.05).

APPENDIX A

NFb medium: DL-malic acid, 5 g/L; KOH, 4 g/L; K2HPO4, 0.5 g/L ; MgSO4 ·7H2O, 0.2 g/L; CaCl2, 0.02 g/L; NaCl, 0.1 g/L; FeSO4 ·7H2O, 0.5 g/L; NaMoO4 ·2H2O, 2 mg/L; MnSO4 ·H2O , 10 mg/L; Bromothymol blue solution (0.5% in 0.2 N KOH), 2 ml; 0.5% alcoholic solution (or dissolved in 0.2N KOH), and Distilled water , 1000ml, pH 6.8. The semi-solid NFb medium add agar 1.9g/L, the solid NFb medium add agar 18g/L and yeast extract, 20.0 mg/L.

JNFb medium: DL-malic acid, 5 g/L; K2HPO4, 0.6 g/L; KH2PO4, 1.8 g/L; MgSO4 ·7H2O, 0.2 g/L; NaCl 0.1 g/L; CaCl2 ·2H2O, 0.02 g/L, Bromothymol blue solution (0.5% in 0.2 N KOH), 2ml; vitamin solution (Biotin 100mg/L, pyridoxol-HCl, 200 mg/L), 1.0 ml/L; micronutrient solution (CuSO4 ·5H2O , 0.4 g/L; ZnSO4 ·7H2O 0.12 g/L; H2BO3 1.4 g/L, NaMoO4 ·2H2O, 1.0 g/L, MnSO4 ·H2O , 1.5 g/L), 2.0ml; 1.63 Fe EDTA solution (1.64 in 100ml), 4.0 ml/L, KOH 4.5 g/L, pH 5.4-5.8. Semi-solid medium, agar, 1.9 g; solid medium, use 6.0 ml of bromothymol blue stock solution, 20.0 mg/L of yeast extract and 18 g/L agar.

MPIK (modified Pikovskaya's medium): Glucose, 10.0 g/L; MgCl2·6H2O, 5.0 g/L; MgSO4 ·7H2O, 0.25 g/L; KCl, 0.2 g/L; (NH4)2SO4, 0.1 g/L; FePO4 ·2H2O, 0.1 g/L; AlPO4, 0.1 g/L; Ca10(OH)2(PO)6, 3.8 g/L, pH7.0.

SRSM: Glucose, 10.0 g/L; Ca3(PO)2, 5.0 g/L; (NH4)2SO4, 0.5 g/L; KCl, 0.2 g/L; MgSO4 ·7H2O, 0.2g/L; MnSO4, 0.004 g/L; FeSO4, 0.002 g/L; NaCl, 0.2, g/L; yeast extract, 0.5 g/L; bromothymol blue, 0.1 g/L, pH7.2.

M-26: NaCl, 5 g/L; peptone, 10 g/L; beef extract, 10 g/L.

Minimal salt (MS) medium: KH2PO4, 1.36 g/L; Na2HPO4, 2.13 g/L; MgSO4 ·7H2O, 0.2 g/L; Trace elements (CaCl2 ·H2O,700 mg/L; FeSO4 ·7H2O, 200 mg/L; MnSO4 ·H2O, 20 mg/L; CuSO4 ·5H2O, 40 mg/L; H2SO4, 1 ml; Na2MoO4 ·2H2O, 4 mg/L; ZnSO4 ·7H2O, 20 mg/L; H3BO4, 3 mg/L and CoCl2 ·6H2O, 7 mg/L), 10ml; pH~7.0.

Chrome azurol (CAS) agar:

Solution 1 (Fe-CAS indicator solution): 10ml of 1mM FeCl3 ·6H2O (in 10 mM HCl); 50ml of aqueous solution of CAS (1.21 mg/ml); 40ml of aqueous solution of hexadecyl-trimetylammonium bromide (HDTMA)1.82 mg/ml.

Solution-2 (Buffer solution): dissolve 30.24 g of PIPES (peperazine-N, N'-bis [2-ethanesulfonic acid]) in 750ml of salt solution; add distilled water to bring the

volume to 800 ml after adjusting the pH to 6.8 with 50% KOH; autoclave after adding 15g agar.

Solution 3: 2g glucose, 2g mannitol, and trace elements in 70ml distilled water

Solution-4: 30ml filtered-sterilized 10% (W:V) casamino acid.

At 50 ℃ after autoclaving, solution 3 and 4 were added to the buffer solution. Solution-1 was added last with sufficient stirring to mix the ingredients. The mixture (Fe-CAS dye complex) yielded blue to dark green color.