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Natural Farming: Comparison of phosphorus-solubilizing and nitrogen-fixing bacteria among Korean Natural Farming (KNF), organic (ORG), and conventional (CON) farming methods.

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Abstract

Korean natural farming (KNF) is promoted as a self-farming system that involves culturing indigenous microorganisms (IMO) and reintroducing them into ecosystem-disturbed soil to enhance soil microbial activity and fertility. Although there has been a growing interest in this system amongst subsistence/permaculture farmers, much is still unknown about how it works. This research was designed to provide a greater understanding of the types of bacteria prevalent in the indigenous microorganisms cultured in KNF. Two experiments were conducted to compare changes in the bacterial population over time. Experiment I, conducted in Waialua, Hawai'i, consisted of three treatments: 1) KNF-SH (KNF-treated soil covered with sunn hemp (*Crotalaria juncea*) mulch, 2) ORG-SH (organically treated soil covered with sunn hemp mulch), and 3) ORG-WM (organically treated soil covered with weed mat in place of sunn hemp mulch). Grape tomato plants (*Lycopersicon esculentum*) were transplanted into the treated soil, and soil samples were collected from between the tomato plants at 14 (T_{14}) and 28 (T_{28}) days after transplanting. Experiment II, conducted in Kula, Hawai'i, consisted of three soil treatments: 1) KNF, 2) ORG (organic), and 3) CON (conventional). All three treatments were covered with bamboo mulch. Zucchini (*Cucurbita pepo* var. *cylindrica*) plants were transplanted into the treated soil, and soil samples were collected on four occasions: before transplanting the zucchini (represented as T_0), at 21 and 56 days after transplant from between the plants (T_{21} and T_{56}), and post-harvest from within the rhizosphere where plants were removed (T_{56r}).

Soil samples collected from both the Poamoho and Kula studies were plated on agar that specifically selects for nitrogen-fixing and phosphorus-solubilizing bacteria: Azospirillum (Azo; pH-adjusted to 6.8) and phosphorus-solubilizing media (Phos), respectively. The soil samples collected from Kula were also plated on De Man, Rogosa and Sharpe (MRS) agar. Each microbial colony was isolated, purified, and subjected to polymerase chain reaction (PCR) followed by DNA-sequencing analysis to identify the specific strains of bacteria isolated. The bacterial colony-forming units (CFUs) were determined. Results showed that *Bacillus megaterium* and *Bacillus aryabhattai* were prevalent in all soil samples collected. The soils treated with KNF had a greater diversity of bacteria overall in both experiments. At T_{21} (Experiment II, Kula trial), KNF-treated soil contained significantly higher numbers of CFUs compared to CON (6.03×10^6 CFU/g vs 5.3×10^5 CFU/g; $P < 0.001$). Additionally, KNF-treated soil was the only farming system that contained high amounts

of *Bacillus subtilis* and *Bacillus licheniformis* in every soil sample. The bacterial population for all farming systems increased soon after treatment but decreased over time. The bacterial population was greatest in the soil from within the rhizosphere, compared to that from between plants.

Introduction

At a population growth rate of 0.8 % per year, Hawai‘i continues to maintain its dependency on imported food to feed residents and visitors. Historically, the conventional agricultural production in the state also has relied on imported inputs such as feeds, fertilizers, composts, herbicides, and pesticides (DBED&T 2012). In order for Hawai‘i to move toward self-sufficiency, reliance on imported food and agricultural inputs must be reduced. Hawai‘i’s farmers cannot continue on their present course without serious repercussions to their sustainability, both economically and environmentally. The key to running an econimically sustainable farm is to minimize overhead costs, most of which come from imported inputs that the farms require to operate successfully. It’s imperative for Hawai‘i to implement some solutions to this problem. Farmers have tried time and time again to provide locally grown produce, but it’s difficult to keep up with the demand, turn a profit, and compete with foreign competition. One plausible solution, advocated here, is to implement a farming method that can address these issues.

Farmers have been slowly shifting towards alternative farming methods such as organic farming and natural farming. These types of farming systems incorporate little to no reliance on the use of inorganic pesticides, fertilizers, and genetically modified organisms, but rather advocate for an emphasis on ecological processes, biodiversity, and crop cycles adapted to local conditions (Woo 2010). One such natural farming method that is practiced in Hawai‘i and some parts of Asia is referred to as Korean Natural Farming (KNF) (Essoyan 2011). Farmers have found this method to be an effective and self-sufficient farming approach.

KNF is a sustainable system developed by Master Han Kyu Cho of the Janong Natural Farming Institute in South Korea. It is based on generations of sustainable farming methods practiced in Japan, China, and Korea. The KNF optimizes the production of plants or livestock through farming methods that maintain a balance in nutrient input and output, thus minimizing any detrimental effects on the environment. The balance is maintained by encouraging the growth of naturally occurring indigenous microorganisms (IMO)—fungi, bacteria, and protozoa—which in turn produce nutrients that are used in the production of crops and livestock (Essoyan 2011). Virtually all of the inputs used in the KNF, as compared to those used in conventional agricultural practices, are available locally at a fraction of the cost of imported feeds, composts, and fertilizers (Cho and Cho 2010). Numerous studies suggest that there is a correlation between soil fertility and the amount of microorganisms (including nematodes as well as bacteria, fungi, and protozoa) present in the soil (Olsson 1997, Smith 2008, Franklin and Mills 2009, Koorem et al. 2014). In a study conducted at the University of Hawai‘i that compared KNF to organic (ORG) and conventional (CON) farming methods, KNF supported better soil–food web structure, as indicated by more enriched and structured nematode communities (Wang et al. 2012). However, the microorganisms, specifically bacteria, that might be enhanced by KNF practices have yet to be determined. This research focused on determining common soil bacteria found in KNF systems across two soil types that were different from the bacteria found in soils from CON and ORG farming systems.

The overall goal of this research was to determine the types of bacteria prevalent in the KNF system compared to the CON and ORG farming systems, and to examine the dynamic of the bacterial population over time. Two experiments were done, piggy-backed onto an existing planned experiment, in attempt to achieve the overarching goal. The objectives for Experiment I were to 1) identify and quantify N-fixing and P-solubilizing bacteria present in KNF compared to ORG in a tomato agroecosystem; and 2) determine the number of colony-forming units (CFU) or bacterial population. The objectives for Experiment II were to 1) identify and quantify the predominant soil bacteria present in KNF-, CON-, and ORG-managed zucchini agroecosystems, 2) compare the identified bacteria between the three farming systems, 3) determine the bacterial population within the rhizosphere, and 4) determine the bacterial population after application over a period of time.

The soil samples used in these experiments were acquired from another research project conducted by Dr. Koon-Hui Wang, whose primary goal was to

evaluate the benefits of Korean natural farming on tropical vegetable crop-production methods. It's worth noting that natural farming methods had never been implemented in these selected sites prior to Dr. Wang's research. We are thankful that she allowed us to take soil samples from her plots.

Materials and Methods

Two field trials were conducted by Dr. Wang to compare soil bacterial population changes over time in vegetable cropping systems managed using KNF (Korean natural farming), ORG (organic), and CON (conventional) methods in two distinct climates in Hawai'i. The locations were, for Experiment I, Poamoho Experiment Station, Waialua, on O'ahu, HI (GPS coordinates: 21.5366667°N, -157.9741667°W); and Experiment II, Kula Experiment Station, Kula, Maui, HI (GPS coordinates: 20.790970°N, -156.326935°W). The soil type at Poamoho Experiment Station is Tropeptic Eutrustox: Wahiawa silty, clayey, kaolinitic, isohyperthermic soil, containing 18.6% sand, 37.7% silt, and 43.7% clay in the top 25 cm of soil. Soil organic matter was approximately 2%, and soil pH was 6.5. The soil at Kula Experiment Station is Torroxic Haplustolls: dark reddish-brown silty, clayey isohyperthermic soil with smectite, kaolinite, and aluminum and iron oxides. The topsoil has a pH of 5.2.

Experiment I

This field trial was conducted in the spring of 2013. There were three plots in total (3 replication plots; 2.44 x 9.144 m^2) each containing three subplots (KNF-SH, ORG-SH, and ORG-WM). Two months prior to the treatments being added to the soil, the KNF-SH and ORG-SH (SH = sunn hemp used as mulch/weed mat) subplots were planted with a sunn hemp cover crop, which was later terminated using a roller crimper. The dense cover of sunn hemp mulch was left in place to serve as an organic weed mat. A commercial weed mat was used in place of sunn hemp mulch for the ORG-WM (WM = weed mat) subplots. For the KNF-SH subplots, IMO4 was prepared as described by Cho (2010) and applied at 1.36 kg/30 m^2 by broadcasting onto the soil surface after the sunn hemp was cut down and before it was laid on the ground as mulch. *Lycopersicon esculentum* (grape tomato) plantlets were drenched with SES seed treatment solution (Cho and Cho 2010) at planting, followed by Type II and Type III foliar spray rotation (2 weeks of Type II, once a week; 1 week of Type III) (Reddy 2011). For the ORG-SH and ORG-WM subplots, the planting holes were fertilized with Sustane 8-2-4 (18.18 kg N/acre). The tomato plantlets were planted 609.6 mm apart within the row and spaced 1.219 m apart between rows. On day 14, the first set of soil samples (n=3, T_{14}) was randomly collected from between the plants in each farming system. The second set of samples (n=3, T_{28}) was collected on day 28. Upon collection, these soils samples were spread out on a tray and allowed to air-dry overnight. Once dried, the soil was sifted through a 2-mm mesh sieve and placed in sterile containers. The soil samples were then subjected to serial dilutions and plating on two types of selective agar, phosphorus-solubilizing and nitrogen-fixing media.

Experiment II

This trial was conducted at the Kula Research Station in Maui, Hawai'i. There were three plots in total (3.048 x 9.144 m^2), each containing three subplots (KNF, ORG, and CON) measuring 2.44 x 3.048 m^2. The experimental design was a random complete block design (RCBD). There were 10 zucchini (*Cucurbita pepo*) plants per subplot (30 plants per treatment). The zucchini plantlets in the KNF system were drenched with SES solution at planting, followed by Type II and Type III foliar spray rotation (2 weeks of Type II, once a week; 1 week of Type III) (Reddy 2011). The IMO4 was prepared and applied to the KNF subplots in the same manner as for Experiment I. For the ORG subplots, the planting holes were inoculated with mycorrhizae + Azospirillum + Sustane 8-2-4 (18.18 kg N/acre). Additionally, each zucchini plant was drenched with 236.6 ml of Mykos mycorrhizal inoculant (188 ml Mykos per 10 gallons of water) at time of planting. Lastly, for the CON plots, a 16-16-16 Nitrogen-Phosphorus-Potassium (81.81 kg N/acre) complete fertilizer was applied. Bamboo-leaf mulch was applied to all subplots. The first set of samples (n=3, T_0) were collected at random from the plot prior to soil treatment (day 0). The second set of soils was sampled on day 21 post application (n=3, T_{21}). These samples were randomly collected from between the plants in each farming system. The third and fourth sets of samples (n=3 each) were collected on day 56. The third set (T_{56}) was collected in the same manner as the second set (T_{21}), from between the plants. The fourth set (T_{56r}) was obtained from within the rhizosphere (soil in contact

with the roots) after the plants were pulled and the soil within the roots was separated out. As in experiment I, all the soil samples were air-dried overnight, sifted, and subjected to serial dilutions. In this experiment, three selective agar media were used: phosphorus-solubilizing, nitrogen-fixing, and MRS media.

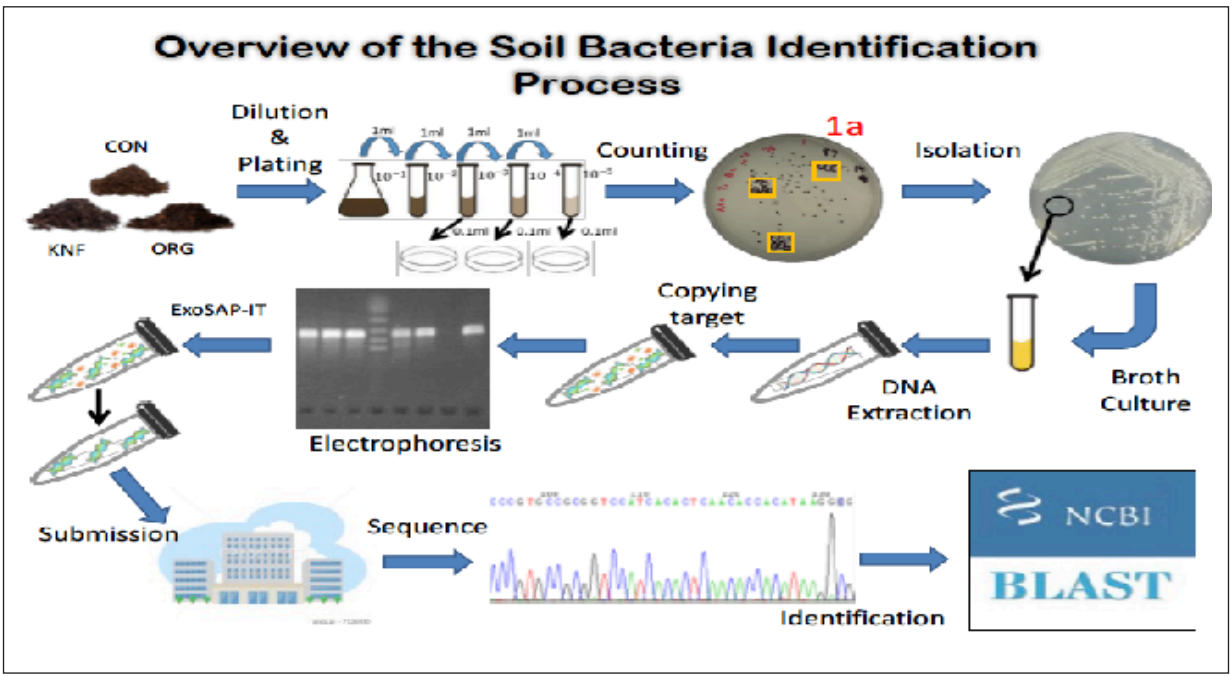
Selective Media Preparation

Many variations of selective media could have been used to target certain groups of bacteria present in the soil sample. In this experiment, the primary goal was to specifically target and identify phosphorus-solubilizing and nitrogen-fixing bacteria. Potassium (K), nitrogen (N), and phosphorus (P) are three major nutrients which are vital for plant growth and development (Scholberg et al. 2000, Singh 2009). According to Sharma et al. (2013), nitrogen is the most important mineral nutrient in terms of measurable plant requirements, followed by phosphorus. Three selective media were used to culture the soil samples: 1) MRS (De Man, Rogosa and Sharpe) media, 2) Azospirillum media, and 3) phosphorus-solubilizing media. The MRS media contained the following ingredients: Difco Lactobacilli MRS Broth, 55 g and Difco Agar, 15 g (BD™, Franklin Lakes, New Jersey). The Azospirillum media contained K_2HPO_4, 5 g; $MgSO_4{\cdot}7H_20$, 0.975 g; NaCl, 1 g; yeast extract, 0.5 g; and Difco Agar, 15 g; the pH was adjusted with 1M HCl to 6.8 prior to autoclaving (Hurst et al. 2000). The phosphorus-solubilizing medium contained Difco Plate Count agar (PCA), 23.2 g; $Ca(PO_4)_2$, 5 g; and Difco agar, 25g (Atlas 2010). A broth of each of the three selective media, devoid of agar, was also prepared for growth of purified isolates.

Serial Dilution Preparation and Plating

From the sifted samples, 8 g of soil was added to a container containing 72 ml of 0.1% peptone water (10^{-1} dilution). The sample was homogenized with a vortex mixer for approximately 5 minutes. One mL of the sample was placed into a tube containing 9 mL of 0.1% peptone water (Figure 1a). This process was repeated until the samples were serially diluted a total of five times (10^{-1} to 10^{-5}). Each serial dilution (0.1 mL) was plated onto selective agar media. The technique used to inoculate the plates was the "spread-plate" technique described by Mulder and Deinema (1981). Plates were incubated at 35°C for approximately 16 h.

Figure 1a. This is a flow chart showing an overview of the soil bacteria identification process: 1) sample collection, 2) serial dilution and plating, 3) plate count to determine CFU, 4) isolation of selected bacteria to obtain a pure isolate, 5) DNA extraction, and 6) sequencing and identification of bacteria.



The colonies appearing on the solid media were counted and recorded to determine the CFU. In Experiment I, Poamoho trial, bacterial colonies (7–10 colonies per plate) were selected at random and sub-cultured once more via streak-plate method to obtain pure cultures. In Experiment II, Kula trial, the cultured plates were placed (at a fixed point) onto a grid containing 1 cm x 1 cm blocks; three blocks located on the grid (within the area of the plate) were randomly selected. Each bacterial colony located within these boxes was sub-cultured once more via streak-plate method to obtain pure cultures. The inclusion of these blocks kept the selection of bacteria completely random (Figure 1b).

Identification of Isolates

Partial sequencing of the 16S rRNA genes of new isolates was carried out as described by Hall et al. (2003) after the 16S rRNA gene was amplified by PCR with oligonucleotide primers 16S1-F (5′-GGAGAGTTTGATCCTGGCTCAG-3′) and 16S1-R (TATTACCGCGGCTGCTGGCAC) (Integrated DNA Technologies, Coralville, Iowa.) The amplified samples were submitted to the Advanced Studies in Genomics, Proteomics and Bioinformatics (ASGPB) laboratory located at the University of Hawai‘i at Mānoa and subjected to DNA sequencing. ChromasPro was used to view the DNA sequencing. The sequences were compared with those in the GenBank databases by u sing the BLAST program (www.ncbi.nlm.nih.gov/blast).

Results

The results for Experiment I, Poamoho trial, showed that on day 14 (T_{14}), *Bacillus subtilis*, *Streptomyces collinus*, *Bacillus licheniformis*, and *Bacillus pumilis* were found only in KNF-SH samples (Figure 2). *Arthrobacter nitroguajacolicus* was present only in ORG-WM samples. All three farming systems contained *Bacillus aryabhattai*, *Bacillus megaterium*, *Burkolderia* sp., and *Paenibacillus polymyxa*. Soil samples collected on day 28 (T_{28}) showed *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus pumilis*, and *Aneurinibacillus migulanus* only in KNF-SH samples (Figure 3). *Cellulosimicrobium* sp. and *Promicromonospora* sp. were present only in the ORG-SH samples. All three farming systems contained *Arthrobacter globiformis*, *Bacillus aryabhattai*, *Bacillus megaterium*, *Burkholderia terricola*, and *Paenibacillus polymyxa.* The ORG-SH soil samples collected on day 14 and 28 (plated on phosphorus-solubilizing media) contained higher amounts of bacterial colonies (78.31 x 10^4 CFU/g and 63.52 x 10^4 CFU/g, respectively, $P<0.001$) than the ORG-WM samples (51.23 x 10^4 CFU/g on day 14 and 46.56 x 10^4 CFU/g on day 21, $P<0.001$; Figure 4). The KNF-SH soil samples collected on day 14 and 28 contained more CFUs (104.32 x 10^4 CFU/g, $P<0.001$) than the ORG-SH and ORG-WM samples ($P<0.001$). The soil samples plated on Azospirillum media showed similar results (Figure 5). There was no significant difference between the ORG-SH and ORG-WM samples

Figure 1b. In Experiment II, Kula Trial, the underside of each plate was pre-marked (a) with a red line prior to plating/culturing of soil samples. A piece of transparency film containing 1 cm x 1cm squares and a black mark (b) was constructed. Three squares located within the film were selected at random. The incubated plates were then placed onto the transparency film, making sure both lines (a,b) were superimposed onto one another (c). Any bacteria located within the three marked boxes were streaked to obtain a pure strain/isolate. These pure strains were later sequenced and identified. The transparency film was used as a template for all cultured plates.

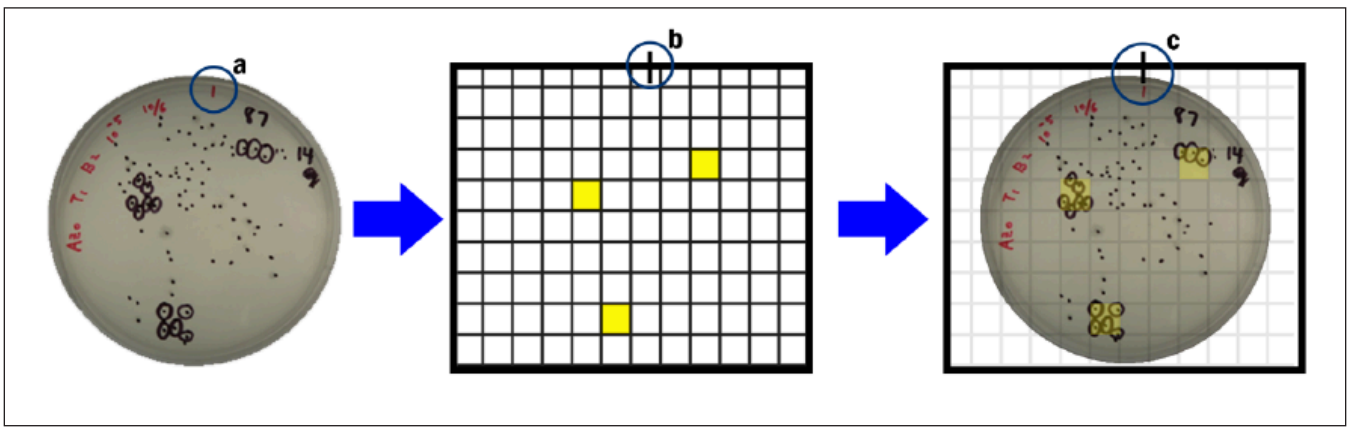


Figure 2. Experiment I, Poamoho Trial. Identification and count of isolated bacteria on day 14 post treatment, T_{14}.

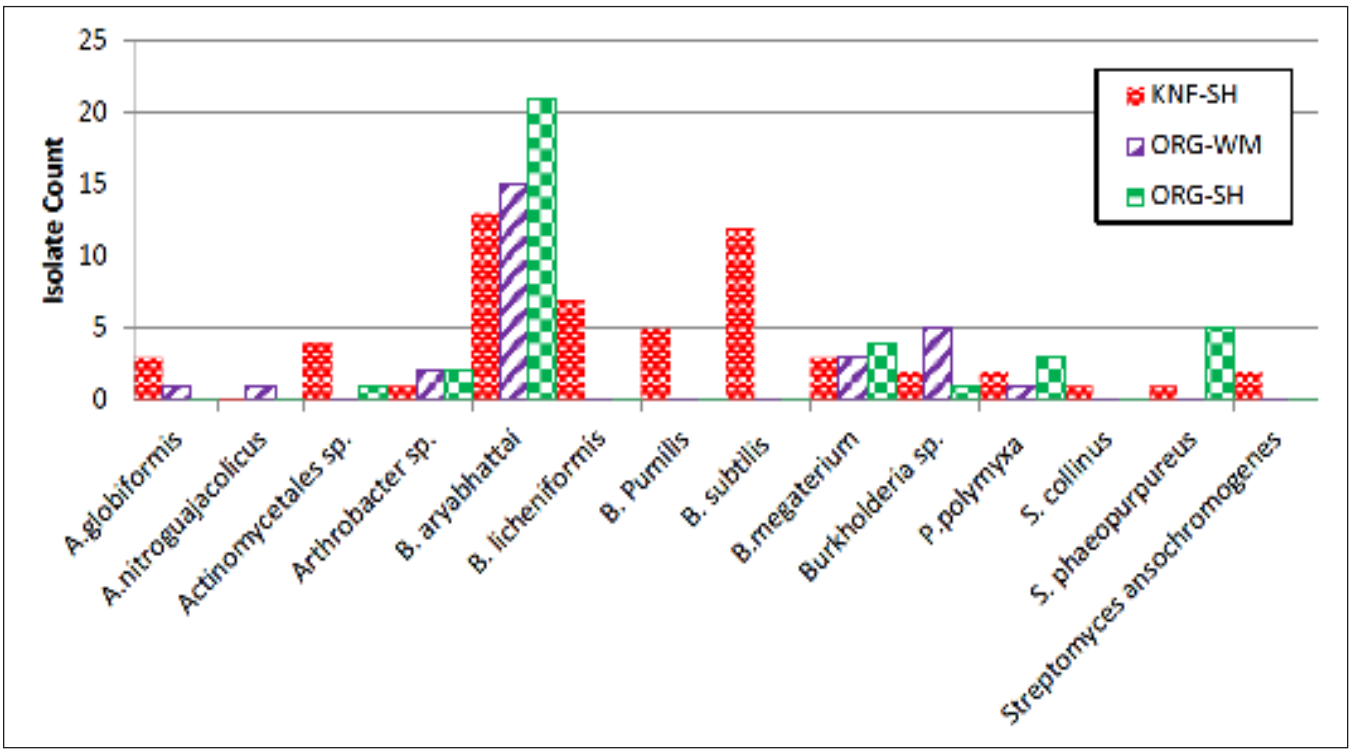
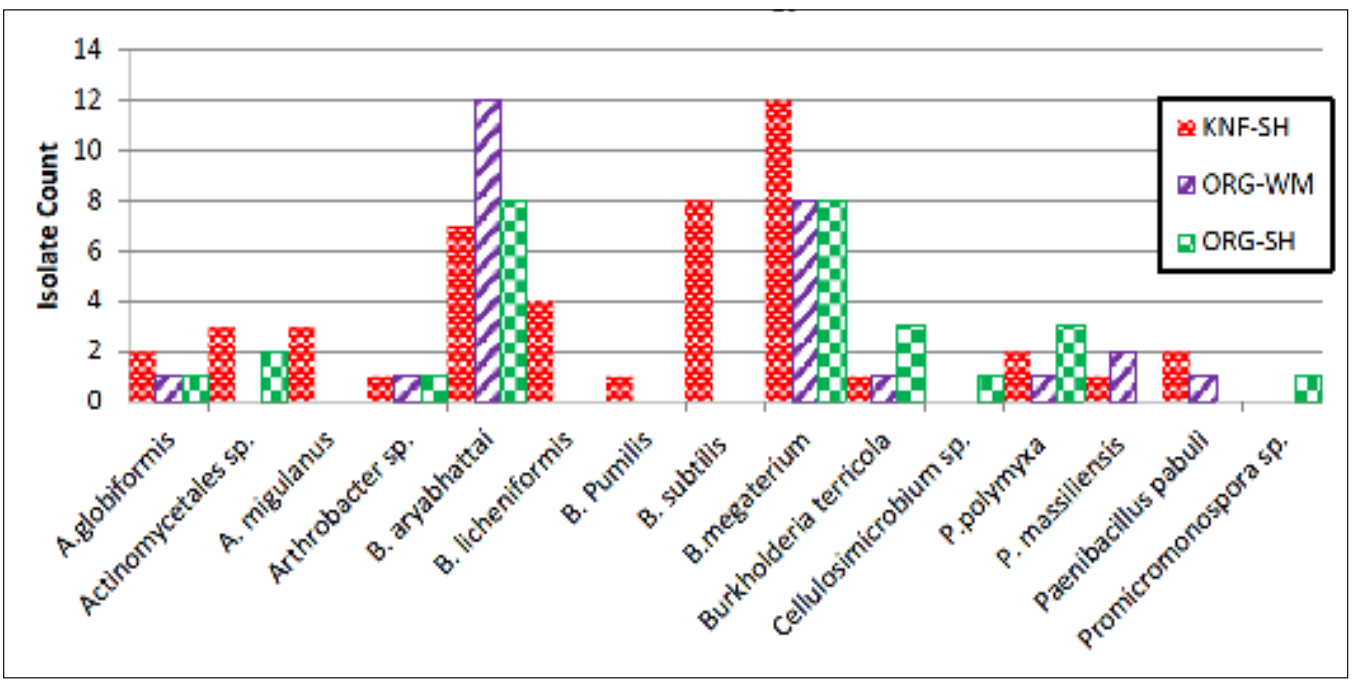


Figure 3. Experiment I, Poamoho Trial. Identification and count of isolated bacteria on day 28 post treatment, T_{28}.



collected on day 28 (T_{28}). The soil samples collected from the KNF-SH plots had the highest CFUs ($P<0.001$; Figures 4 & 5).

In Experiment II, Kula trial, *Arthobacter* sp., *Bacillus aryabhattai*, *Bacillus megaterium*, *S Bacillus simplex*, *Bacillus thuringiensis*, *Burkholderia* sp., *Paenibacillus polymyxa*, *Streptomyces phaeopurpureus*, and *Streptomyces* sp. were present in the soil prior to treatment (T_0). *Bacillus aryabhattai* and *Bacillus megaterium* were the most abundant (Figure 6). Soil samples collected on day 21 post application (T_{21}) showed a greater diversity of bacteria in KNF compared to ORG and CON plots (Figure 7). Bacteria found only in the KNF samples included *Arthrobacter defluvii*, *Bacillus oleronius*, *Bacillus pseudomycoides*, *Bacillus subtilis*, *Bacillus thuringiensis*, *Paenibacillus glucanolyticus*, *Streptomyces djakartensis*, and *Streptomyces galilaeus*. All three farming systems contained *Bacillus aryabhattai, Bacillus megaterium,* and *Bacillus cereus.* Soil samples collected on day 56 post application (T_{56}) showed similar results (Figure 8). Of all the samples (T_0, T_{21}, T_{56}, T_{56r}), the T_{56r} (post-harvest) soil samples, obtained from within the rhizosphere, had the greatest diversity of identified bacteria, particularly in the KNF plots (Figure 9). *Bacillus oleronius*, *Bacillus subtilis*, *Bacillus pumilis*, *Bacillus thuringiensis*, *Paenibacillus terrae*, and *Pseudomonas fluorescens* were present in KNF_r (rhizosphere, T_{56r}) but not ORG_r (T_{56r}) and CON_r (T_{56r}). *Lysinibacillus fusiformis*, *Paenibacillus kobensis*, and *Promicromonospora* sp. were present in ORG_r but not CON_r and KNF_r. *Bacillus megatarium* and *B. aryabhattai* were present in all soil samples collected (T_0, T_{21}, T_{56}, T_{56r}). *B. subtilis*, *B. oleronius*, and *B. thuringiensis* were found only in the KNF soil samples.

Figure 4. Experiment I, Poamoho Trial. Bacterial population (CFU/g, mean ± SE) over a period of 28 days. Data represent the bacterial colonies cultured on phosphorus-solubilizing media. The ORG-SH soil samples collected on days 14 (T_{14}) and 28 (T_{28}) contained significantly higher amounts of bacterial colonies than the ORG-WM samples ($P<0.001$). The KNF-SH soil samples collected on day 14 (T_{14}) and 28 (T_{28}) contained higher amounts of bacterial colonies overall ($P<0.001$) than ORG-WM or ORG-SH. Different superscripts denote significant differences within the day of sampling.

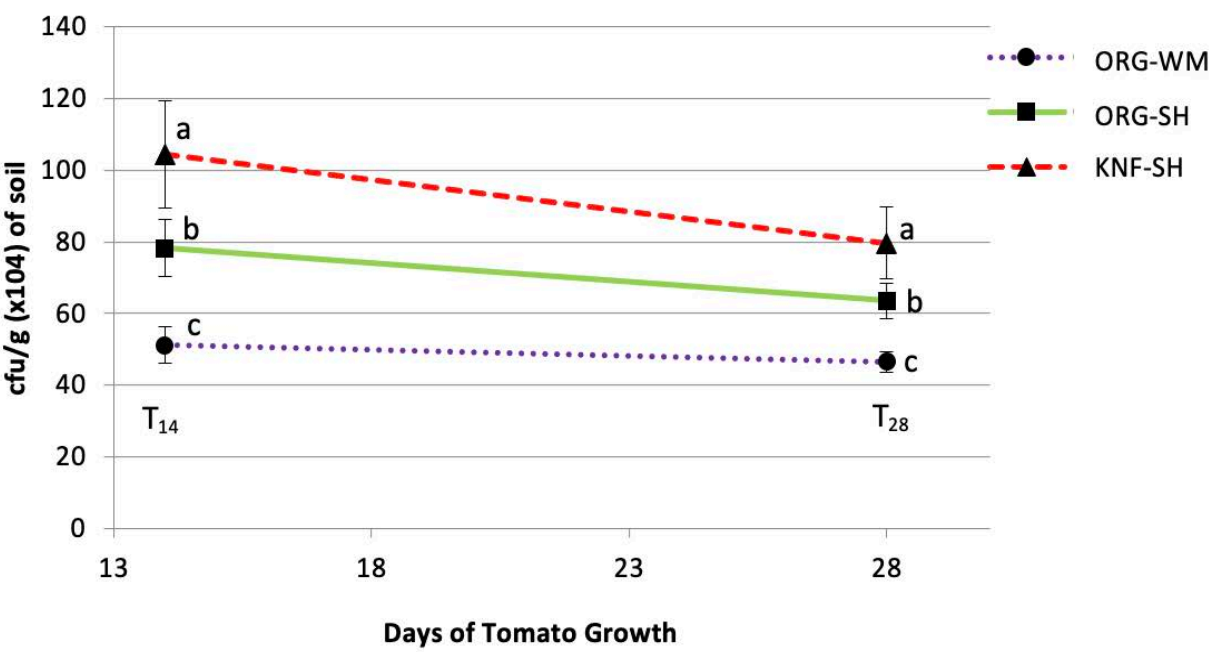


Figure 5. Experiment 1, Poamoho Trial. Bacterial population (CFU/g, mean + SE) over a period of 28 days. Data represent the bacterial counts cultured on Azospirillum media. The KNF-SH and ORG-SH soil samples collected on day 14 (T14) contained higher counts than the ORG-WM. By day 28 (T28), the KNF-SH treatment had higher bacterial counts (CFU) than ORG-SH and ORG-WM. Different superscript numbers within the day of sampling denote significantly dif-ferent levels of CFU/g soil (P<0.001).

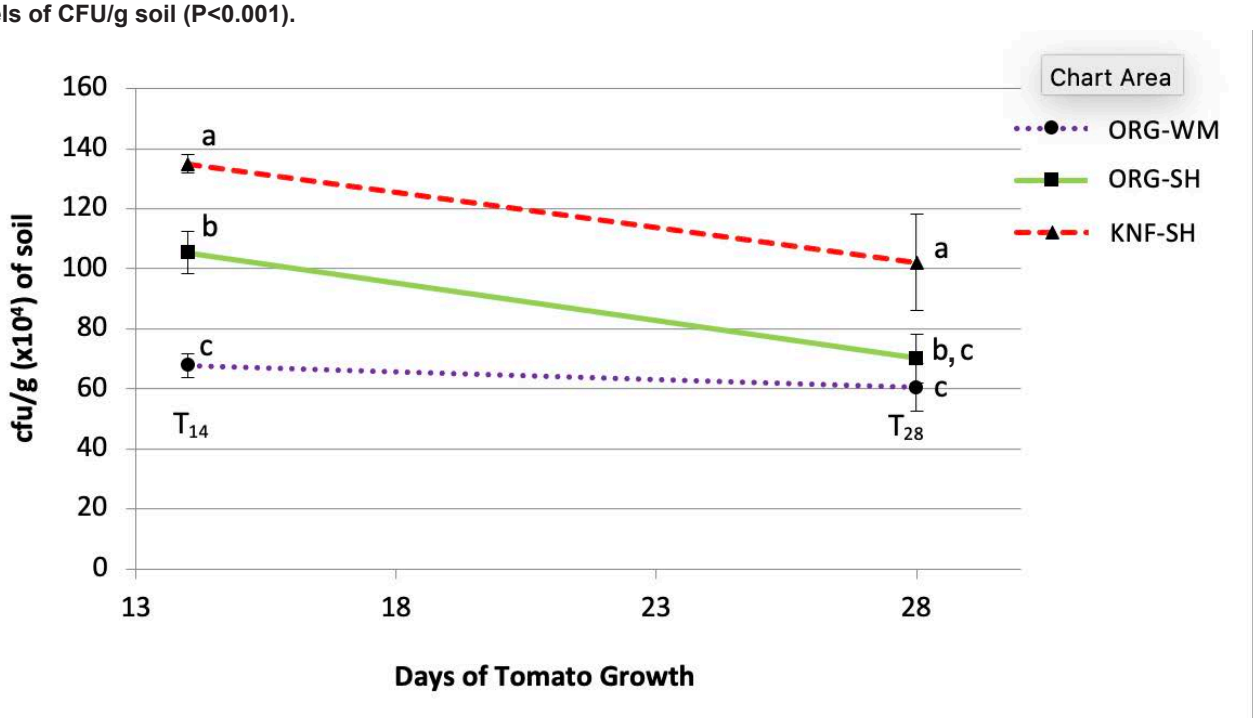


Figure 6. Experiment II, Kula Trial. Identification and count of isolated bacteria (CFU/g) in soil prior to treatment, T0.

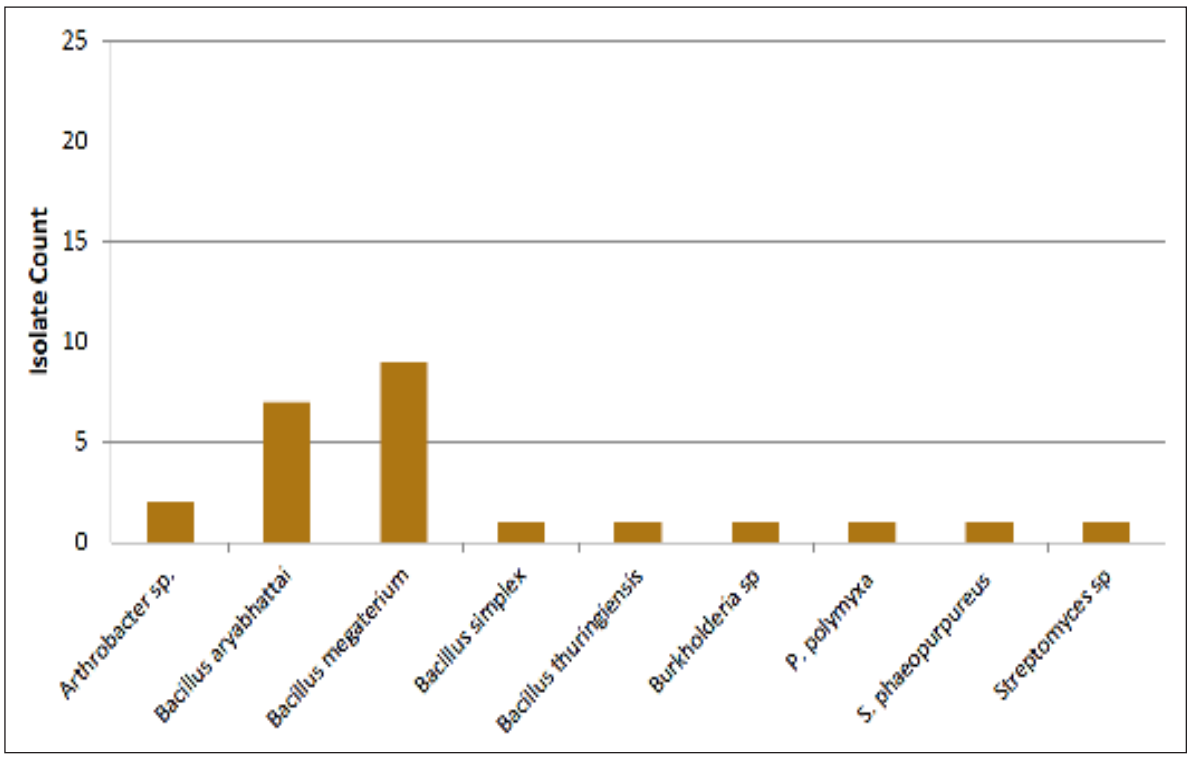


Figure 7. Experiment II, Kula Trial. Identification and count of isolated bacteria (CFU/g) in soil on day 21 post treatment, T_{21}.

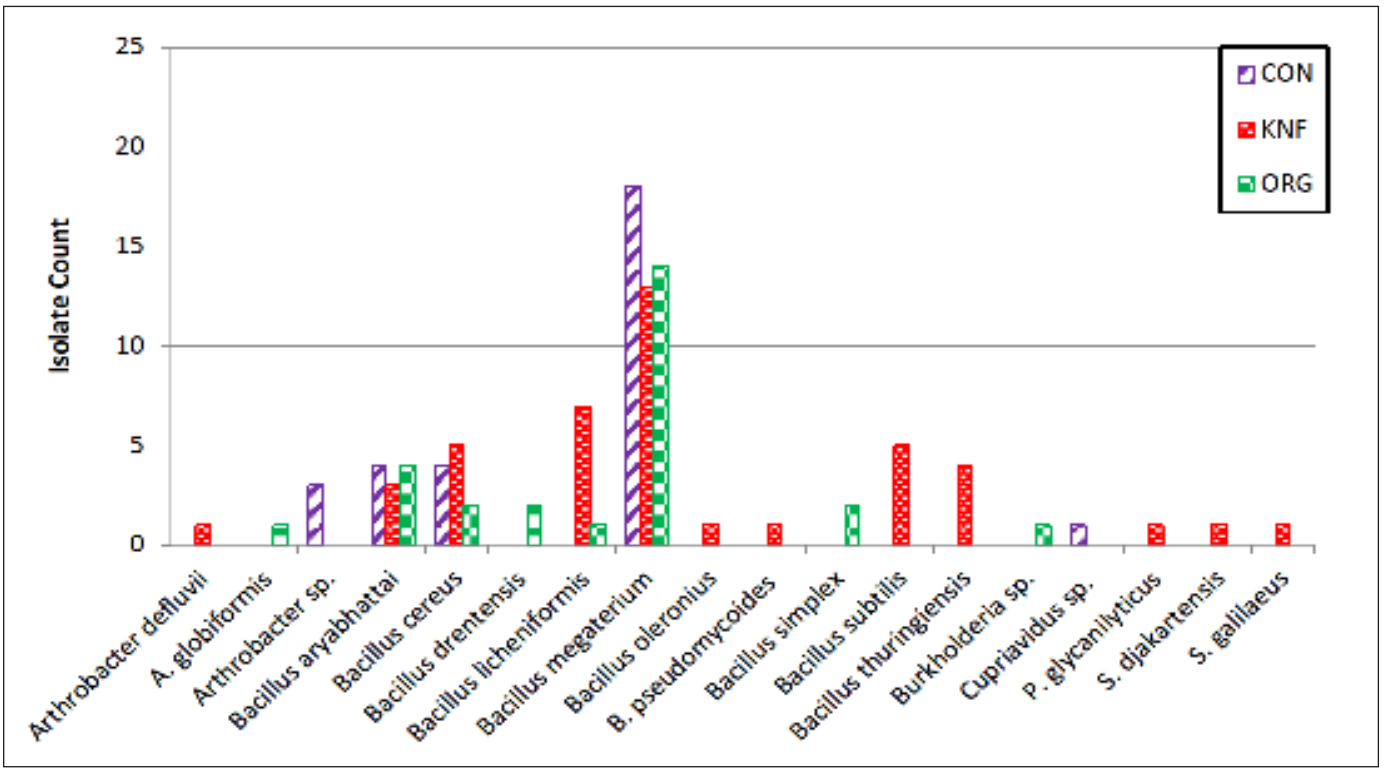


Figure 8. Experiment II, Kula Trial. Identification and count of isolated bacteria (CFU/g) in soil on day 56 post treatment, T_{56}.

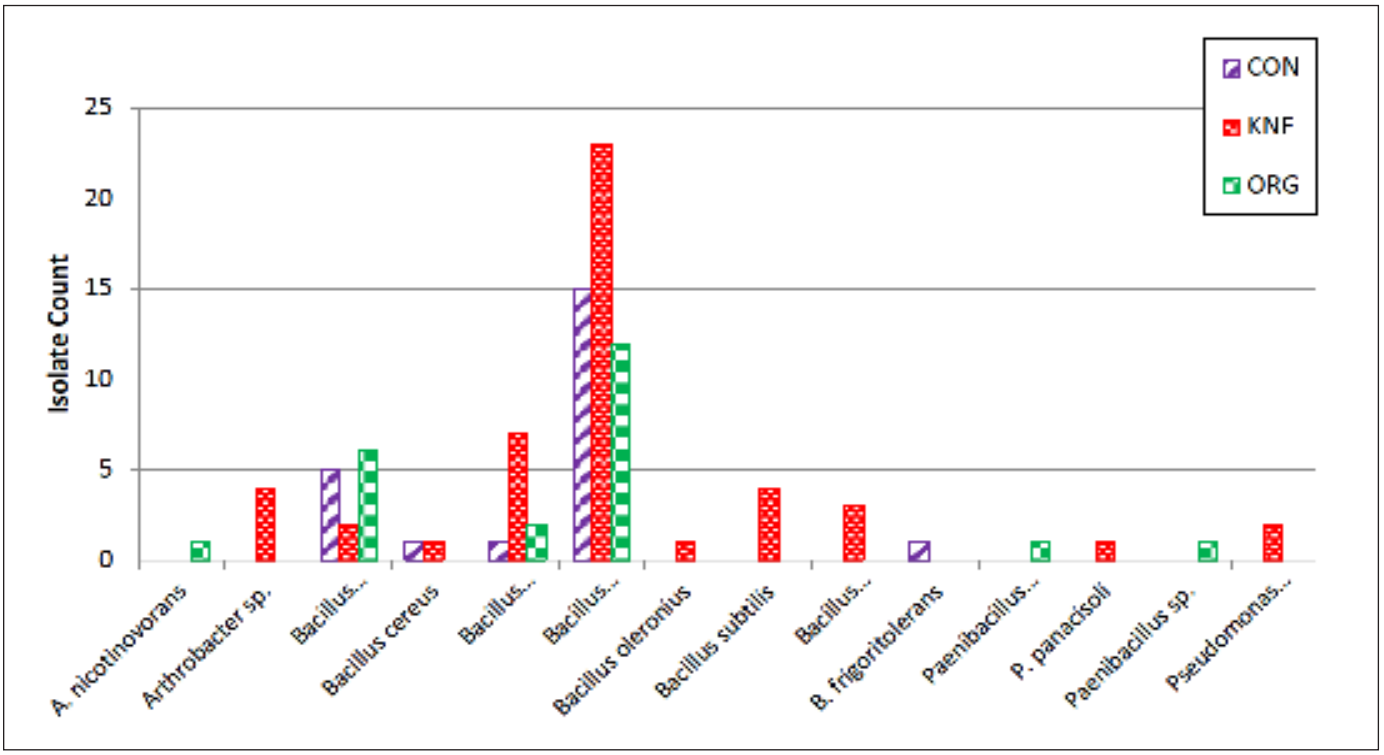
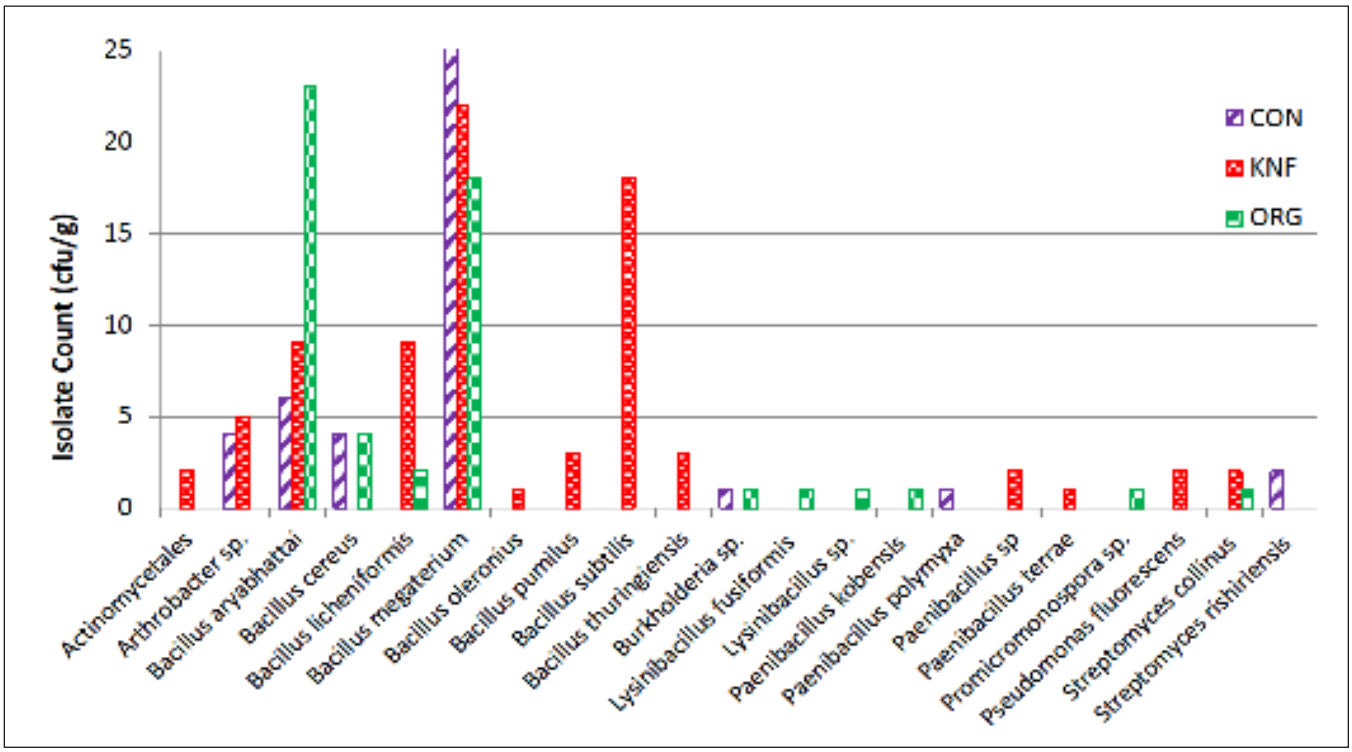


Figure 9. Experiment II, Kula Trial. Identification and count of isolated bacteria (CFU/g) in soil from within the rhizosphere on day 56 post treatment, T_{56r}.

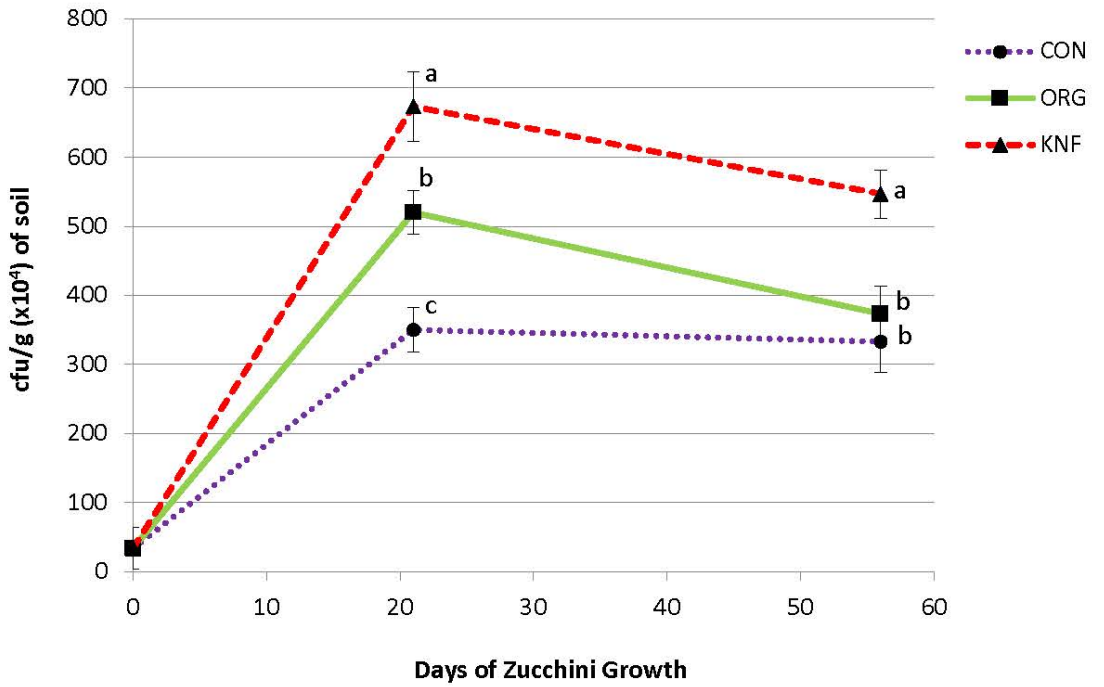


A two-way analysis of variance was performed on the bacterial colony data. KNF soil samples plated on phosphorus-solubilizing media contained higher amounts of bacterial colonies (673.33 x 10^4 CFU/g at T_{21} and 546.66 x 10^4 CFU/g at T_{56}; $P<0.001$) than the CON (356 x 10^4 CFU/g at T_{21} and 333.3 x 10^4 CFU/g at T_{56}) and ORG samples (520 x 10^4 CFU/g at T_{21} and 373.33 x 10^4 CFU/g at T_{56}; Figure 10). The soil samples plated on Azospirillum media showed similar results; at T_{56}, KNF had a higher number of CFUs (506.67 x 10^4 CFU/g; $P<0.001$) than CON (336.6 x 10^4 CFU/g) and ORG (350 x 10^4 CFU/g; Figure 11). At T_{21}, there was a significant difference between KNF (603.3 x 10^4 CFU/g; $P<0.001$) and CON (366.6 x 10^4 CFU/g) but not between KNF and ORG (550 x 10^4 CFU/g). The soil samples plated on MRS media showed that at T_{21}, CFUs were greatest in KNF (134.3 x10^4 CFU/g; $P<0.001$; Figure 12). However, there was no significant difference between the three systems at T_{56}.The bacterial population for all farming systems increased post treatment (T_{21}) but decreased over time. Figure 13 shows a comparison between the bacterial colonies present in within the rhizosphere (T_{56r}) and 12 inches away from the rhizosphere (T_{56}). Excluding CON T_{56} and CON T_{56r} plated on MRS media, all soil samples obtained from within the rhizosphere (T_{56r}) contained significantly higher bacterial colonies than the samples taken from between the plants (T_{56}).

Discussion

The goal of this experiment was to gain a greater understanding of the types of bacteria present within these farming systems. The data suggest that the KNF-treated plots had a higher abundance of bacteria. In addition, the organic and KNF-treated plots had a more diverse group of bacteria compared to the CON plots, which were treated with synthetic fertilizer. In terms of both abundance and diversity, the conventionally treated plots had the least number of bacteria present within the soil. There were also changes in the bacterial population over time. Figure 10 (Experiment II, Kula Trial) clearly shows that the bacterial population increased post application (T_{21}) but decreased over time (T_{56}). In regard to Experiment I, the weed mat used in the organic plots (ORG-WM) seemed to actually suppress bacterial growth, as

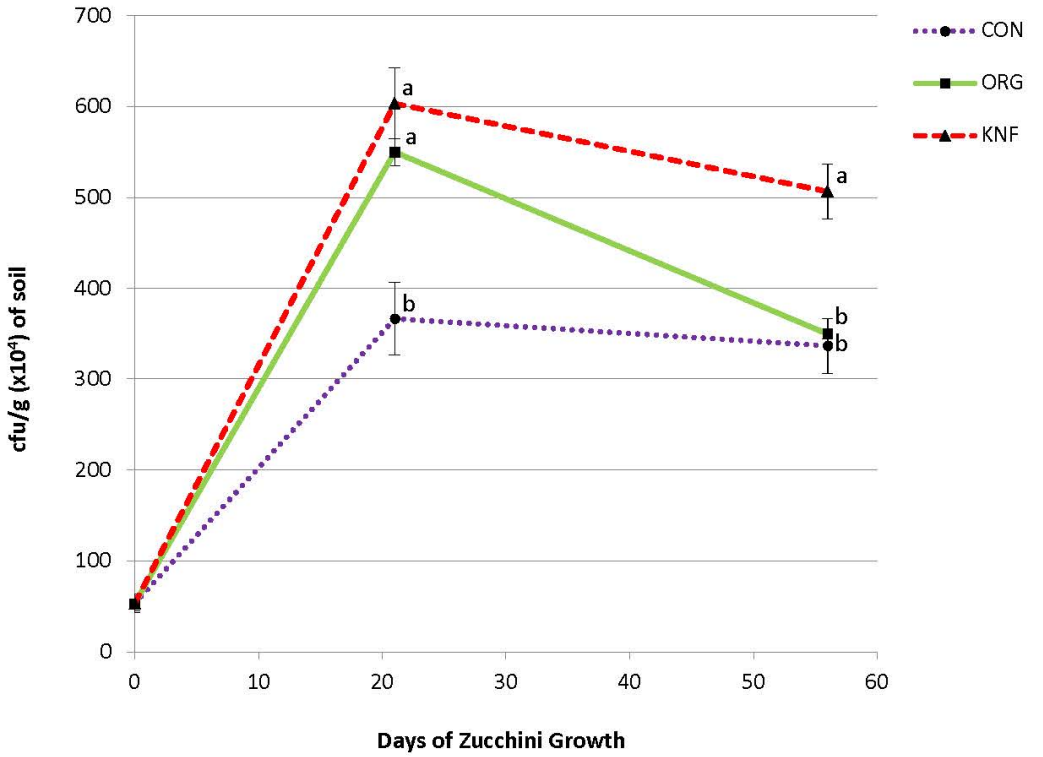
Figure 10. Experiment II, Kula Trial. Bacterial population (CFU/g soil, mean ± SE)) over a period of 56 days. Data represents the bacterial colonies cultured on phosphorus-solubilizing media. KNF samples collected on day 56 contained a significantly higher number of bacterial colonies compared to ORG and CON (P<0.001). Different superscripts within a time period denote significant differences between treatments (P<0.001).



there were fewer bacteria identified and significantly fewer bacterial colonies (CFU/g of soil). Based on the differences between ORG-SH and ORG-WM, sunn hemp mulch seemed to promote bacterial growth.

Both organic and natural farming methods are based on a similar concept: to create a system of natural biodiversity by encouraging the complexity of living organisms to shape each particular ecosystem and thrive along with the plants. Organic farming differs from conventional in that it promotes the growth of bacteria by supplying them with organic material. The decomposition process of organic material is slow; this benefits bacteria as well as plants in that it only releases the necessary amount of nutrients required for both to prosper and grow. This process decreases the risk of over-fertilization (Rauscher 2015). KNF, on the other hand, differs from the other two farming systems in that it involves deliberately increasing the bacterial population in the soil as well as providing natural inputs (i.e., nutrient-rich liquid) as a food source for bacteria and plants (Reddy 2011). Figure 9 shows that KNF and organic farming both promote growth of bacteria within the rhizosphere, just not the same types of bacteria. Unlike KNF and organic, conventional farming relies heavily on inorganic fertilizers that supply essential nutrients to soil immediately. The nutrients are released as soon as the fertilizer dissolves in water. This may be of concern, as any unused portion is at risk of washing away or leaching into the groundwater (Sebilo et al. 2013). It is plausible that the conventional plots contained the least number of bacteria because synthetic fertilizers don't provide the necessary resources bacteria need to survive. According to Nakhro (2010), the addition of organic and natural amendments might

Figure 11. Experiment II, Kula Trial. Bacterial population (CFU/g soil, mean ± SE) over a period of 56 days. Data represents the bacterial colonies cultured on Azospirillum media. KNF-treated samples collected on day 56 contained a significantly higher number of bacterial colonies compared to ORG and CON. On day 21, KNF and ORG soil samples contained higher bacterial colonies than the CON samples. Different superscripts within the time period denote significant differences (P<0.001).



have a large impact on the size and activity of the microbial population.

Both trials showed that the CFU count was higher within the rhizosphere (T_{56r}) than it was 12 inches away from the zucchini plants (T_{56}) (Figure 13). The data also showed that *B. subtilis*, *B. pumilis*, and *B. licheniformis* were present only in the KNF-treated soils sampled. These particular bacteria, amongst others, are commonly referred to as plant growth-promoting rhizobacteria (PGPR). PGPR have the potential to contribute to sustainable plant growth promotion and induce disease resistance (Li et al. 2016). Plant-growth promotion and development can be facilitated both directly and indirectly. Generally, they function in three different ways: synthesizing particular compounds for the plants, facilitating the uptake of certain nutrients from the soil, and lessening or preventing the plants' susceptibility to diseases (Gullap et al. 2014). One PGPR, *B. subtilis*, has been shown to restrict pathogens such as *Pseudomonas syringae* from entering through the stomata by signaling the guard cells to close (Kumar et al. 2012). In a more recent study, when applied to the leaves of broad bean *B. subtilis* enhanced plant photosynthetic activities by increasing leaf photosynthetic efficiency and chlorophyll content (Li et al. 2016). Han et al. (2014) conducted a study to determine whether *B. subtilis* augments salt tolerance of white clover. Their data showed that the presence of this specific bacterium promotes plant growth under both non-saline and saline conditions by direct or indirect regulation of plant chlorophyll content, leaf osmotic potential, cell membrane integrity, and ion accumulation. Other PGPRs such as *B. pumilis* and *P. fluorescens* protect the plant's root system by reducing galling caused by pathogenic nematodes (Almaghrabi et al. 2013). Lim and Kim (2013) found that when inoculated with *B. licheniformis*, pepper plants have the ability to tolerate drought stress and survive longer compared to non-inoculated pepper plants. In another study, *P. poly-*

Figure 12. Experiment II, Kula Trial. Bacterial population (CFU/g soil, mean ± SE) over a period of 56 days. Data represents the bacterial colonies cultured on MRS media. KNF samples collected on day 28 contained a significantly higher number of bacterial colonies compared to ORG and CON (P<0.001). There was no significant difference between the soil samples collected on day 56. Different superscripts within the time period denote significant differences (P<0.001).

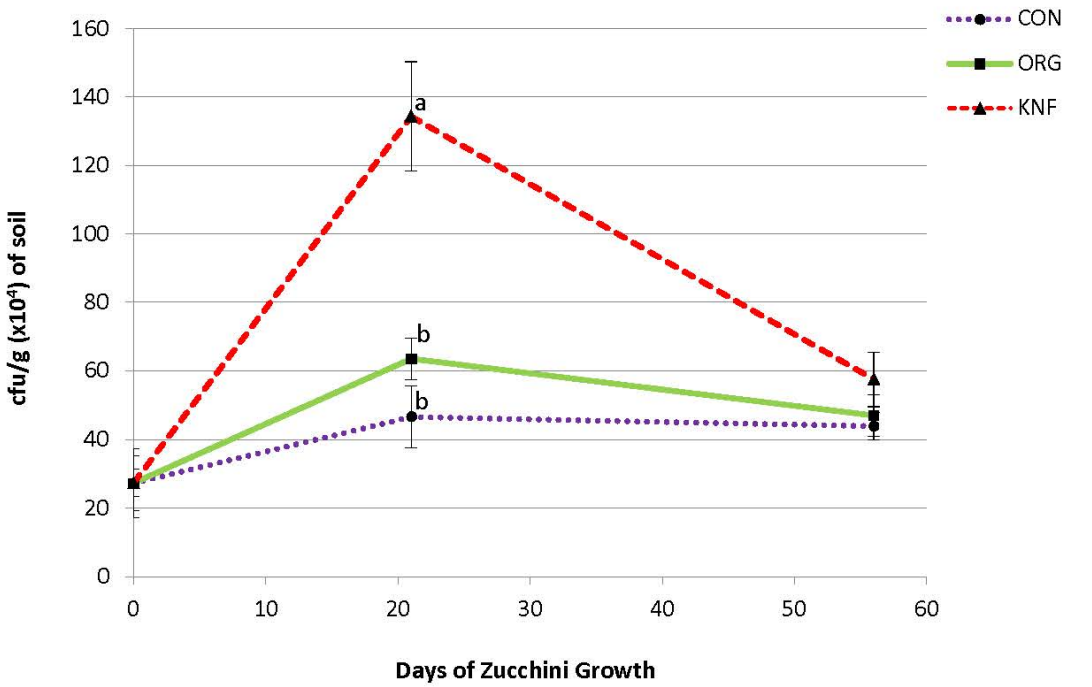
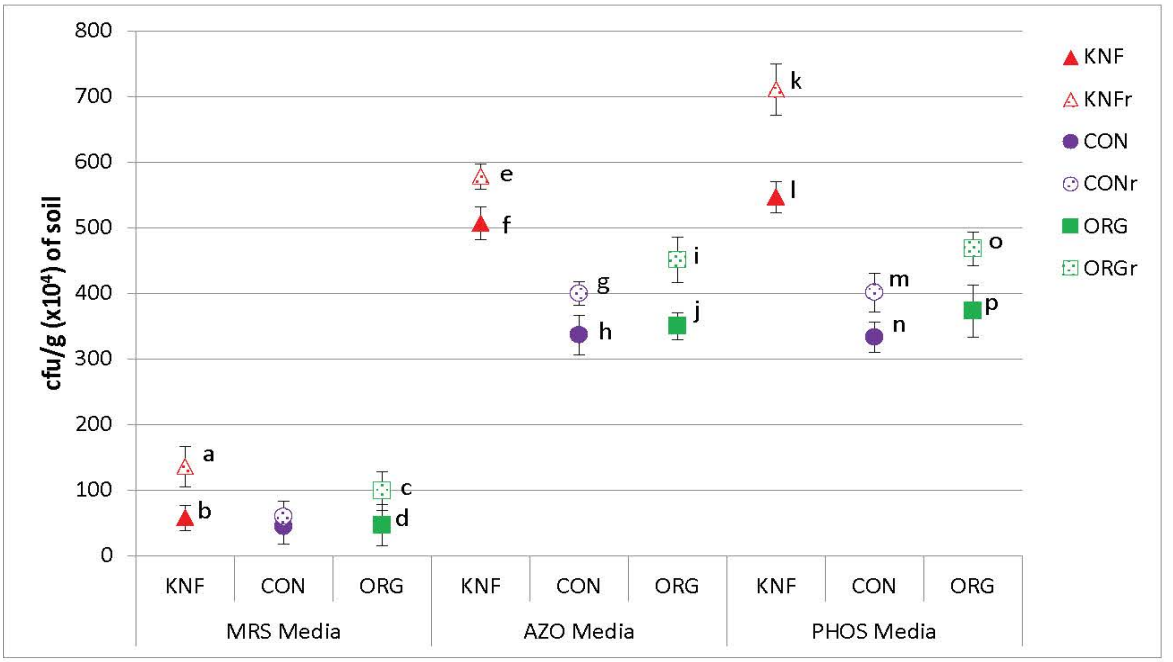


Figure 13. Experiment 3, Kula Trial. Tukey's HSD statistical analysis performed between KNF-T_{56} and KNF_{r-T56}, CON -T_{56} and $CON_{r\_}T_{56r}$, and ORG – T_{56} and $ORG_{r\_}T_{56r}$. All soil samples obtained within the rhizosphere (T_{56r}), except for CON T_{56r} plated on MRS media, contained significantly higher bacterial colonies (CFU/g soil, mean ± SE) than their counterparts collected between the plants. Different superscripts within the time period denote significant differences (P<0.001).



myxa and *B. licheniformis* promoted faster growth and seed germination rate in *Arabidopsis thaliana* (Kefela et al. 2015). Numerous studies have also been conducted on *B. aryabhattai* and *B. megaterium*, the only two bacteria identified in all soils sampled. Both are also considered PGPRs, as they have proven to promote growth and disease resistance in plants (Ramesh et al. 2014, Hu et al. 2014). In addition, Xie et al. (1998) reported that the following species were nitrogen-fixing bacteria, based on nitrogenase activity: *B. megaterium*, *B. cereus*, *B. pumilus*, *B. circulans*, *B. licheniformis*, and *B. subtilis*. These studies suggest that bacteria have multiple beneficial properties. For example, *B. aryabhattai* solubilizes insoluble (calcium) phosphate and zinc and produces gibberellins (Ramesh et al. 2014).

The increased presence of the Bacillus species may be the reason some farmers are seeing a positive response when implementing the KNF system. Many of the bacteria present in the KNF system aid in plant defense. Based on this knowledge, one could hypothesize that plants treated with KNF inputs may excel in growth in areas harboring certain pathogens, as compared to those grown using conventional farming methods. However, environmental conditions vary with geography, so while sustainable agriculture may be most efficient on one farm, it may not be entirely feasible in another.

As previously stated, the primary goal of this research project was to identify the types of bacteria present in the soil samples obtained from two separate experiments conducted by Dr. Wang. Although no formal paper has been published regarding yields in these two experiments at the time of this paper preparation, a progress report can be found on Wang's website "Evaluating the Benefits of Korean Natural Farming Practice on Tropical Vegetable Crop Production in Hawaii" (2012).

In conclusion, this study has established that KNF increases bacterial population in the soil, where the concentration of bacteria was highest within the rhizosphere. In contrast, the bacterial population was lowest in soil treated using conventional farming methods. As previously mentioned, KNF involves the culturing of naturally occurring indigenous microorganisms (IMO) such as fungi, bacteria, and protozoa. This study focused on identifying phosphorus-solubilizing and nitrogen-fixing bacteria. In order to see the whole picture, an analysis of microbial diversity should be performed on the soil samples. This can be accomplished via next-generation DNA-sequencing techniques. It would also be informative to conduct a KNF field study to record plant yield and collect soil samples for microbial testing.

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