# UNRAVELING THE MYSTERY OF THE NATURAL FARMING SYSTEM (KOREAN): ISOLATION OF BACTERIA AND DETERMINING THE EFFECTS ON GROWTH

A THESIS SUBMITTED TO THE GRADUATE DIVISION OF THE UNIVERSITY OF HAWAIʻI AT MĀNOA
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

IN

ANIMAL SCIENCE

JULY 2018

By

Ana Keli'ikuli

Thesis Committee:

Chin Lee, Chairperson
Yong Li
Yong-Soo Kim

Keywords: Korean natural farming, sustainable agriculture, KNF, IMO, indigenous microorganisms

**Acknowledgements**

This project, 294R, was funded by CTAHR's HATCH and Smith-Lever funds for Supplemental Research award; thank you for believing in this project. Additionally, I'd like to thank my committee members, CN Lee, Yong Li, and Yong Soo Kim for their guidance and support - without them, this project would not have been possible. A very special thanks to Hoa Aina O Makaha for allowing us to use their land to carry-out our experiment and CTAHR research stations for their collaborative support. Thank you to Michael Duponte and Koon Hui Wang for collecting soil samples and Dr. Cheah for supplying me with tissue culture equipment and supplies. Thank you Dr. Lee for the life lessons; for inspiring me; driving me to be the best version of myself; and for making me think outside the box. Lastly, thanks to my lab mates and friends for their encouragement and support.

**Abstract**

KNF is a self-sufficient farming system that involves the culturing of indigenous microorganisms (IMO) – fungi, bacteria, and protozoa. It enhances soil microorganism activity and improves soil fertility. This farming approach maximizes the use of on-farm resources, recycles farm waste, and minimizes external inputs while fostering soil health. However, scientific evidence of the benefits of KNF has been limited; little is known as to how this system works, what type of indigenous microorganisms are present in the soil treated under KNF conditions, or whether the collection site plays an integral role in soil fertility. In addition, there is no information on the rate and frequency at which IMO should be re-applied.

There were three studies conducted with 4 overall goals: 1) identify the bacteria present in KNF, specifically phosphorus-solubilizing and nitrogen-fixing bacteria, 2) determine whether or not the collection site plays an integral part in plant growth, 3) determine how often to re-apply IMO 4 to the soil, and 4) inoculate seeds with bacteria isolated from KNF in the hopes of providing a better understanding as to the role it may play in plant growth. The first two studies showed that *Bacillus megaterium* and *Bacillus aryabhattai* were present in all soil samples. *Bacillus subtilis* and *Bacillus licheniformis* were dominant only in the KNF system. In the 2$^{nd}$ study, it was determined that the collection site of IMO plays an integral role and that applying a 2$^{nd}$ IMO application 14 days after the initial treatment increases plant yield. In the 3$^{rd}$ study (seed inoculation), the results showed that *B. subtilis* promoted plant growth in terms of germination rate, lateral root formation, root length, and stem elongation. Inoculating seeds with *P. aeruginosa* on the other hand proved to have little to no effect on plant growth.

Knowing where to collect/cultivate IMO and how often to apply it to the soil will be of great use to farmers who currently practice KNF. This study also provided statistical data that shows KNF to be more effective than conventional farming methods when sufficient bacteria are applied to the soil in a regular schedule. Natural farming is the key to a sustainable future.

**Table of Contents**

# Chapter 1

# Literature Review: Plant Growth Promoting Rhizobacteria

## 1.1 Introduction

Photosynthetic plants play a crucial role in regulating the life cycles of living organisms and nutrient cycling (Wright and Jones, 2006; Hartmann *et al*., 2009). They maintain the atmosphere by converting carbon dioxide into oxygen which is essential for cellular respiration for all aerobic microorganisms (Costa *et al.*, 2006). Roots play a significant role in the growth and development of plants as they provide support (anchorage), absorb water and minerals, and store nutrients (Berg and Smalla, 2009). In addition, roots provide shelter and nutrients to microorganisms such as protozoa, fungi, and bacteria. In turn, these microorganisms, specifically bacteria, aid the root system in nutrient uptake, nitrogen fixation, and defense against pathogens. This type of symbiotic relationship that occurs between bacteria and plant roots takes place within the rhizosphere. The rhizosphere is the narrow zone of soil specifically influenced by the root system (Dobbelaere *et al.,* 2003). This zone is rich in nutrients due in part to the accumulation of varying plant exudates that include sugars, amino acids, polysaccharides, and ectoenzymes (Gray and Smith, 2005). The relationship between bacteria and plants can be positive, negative, or neutral (Dobbelaere *et al.,* 2003). Beneficial bacteria that colonize plant roots and promote plant growth are referred to as plant growth-promoting rhizobacteria (PGPR; Beneduzi *et al.,* 2012).

Much research has been conducted on this phenomenon known as PGPR and has led to some very intriguing connotations in regard to how this physical, biological entity operates. In this review, we will consider the role PGPR plays in regards to plant health and provide a more detailed understanding of how specific strains of PGPR influences the entire eco-system of the planet itself.

Hence, we will delve deeper into the aspects of life that exist at a microscopic level in order to understand larger concepts of living.

## 1.2 Examples of PGPR

There is a myriad of different examples of PGPR and what they actually represent within the confines of plant life. First, however, a clearly defined terminology must be appropriately examined in order to truly understand what PGPR is within the context of the discussion at hand. In technical terms,

PGPR are naturally occurring bacteria found within the soil that colonizes plants' roots whereby promoting plant growth (Saharan & Nehra, 2011).

PGPR has a wide array of applications. For instance, many experts within the field of agriculture have begun to understand the complex nature of PGPR, as well as the full scope of its potential positive influence on overall sustainability in regards to the production of crops (Vejan *et al.,* 2016). PGPR is used to promote growth and counteract the potential damage done due to the harmful effects of insect life on the growth of crops.

Instead of using chemical aids to improve crop yield, scientists can assist farmers in creating richer soil on their farms. PGPR has shown tremendous other benefits for many degraded soil situations by promoting the soil's ability to produce sustainable food sources, as well as its direct correlation to positive growth cycles from the interaction with the plants' roots (Goswami *et al.,* 2016). Thus, the specific applications of this bacterium have only just begun, as the positive correlation between bacteria and plant will only expand as more is learned.

**1.3 Mechanisms of PGPR in enhancing plant growth**

A variety of mechanisms is at constant work while PCPR function to complete their bonding with plants and the creation of strong eco-system modalities. It is the internal processes that allow these microorganisms the ability to transform their surrounding soil into a better and more enriched environment in which plants can grow faster and stronger. The following list covers the primary mechanisms involved in this phenomenon:

- Biofertilization: The primary mechanism that is unique to nearly all PGPR is biofertilization, a process of improving soil fertility with the addition of biofertilizers. (Carvajal and Carmona, 2012; Luterberg & Kamilova, 2009; Schütz *et al*., 2017). Biofertilizers are biological organisms that enrich the soil through the natural processes of nitrogen fixation, solubilizing phosphorus, leading to stimulation of plant growth through the synthesis of growth-promoting substances (Bhardwaj *et al.*, 2014). Biofertilizers are eco-friendly and a great substitute for inorganic fertilizers.

- Stimulation of root growth: it's intricately tied to the final development of the individual plant itself (Luterberg & Kamilova, 2009).

- Rhizoremediation: when rhizoremediation occurs, the surrounding soil is more adapted in handling the ongoing degradation due to the PGPR arresting the soil depletion of vital nutrients due to its counter activity (Luterberg & Kamilova, 2009).

- Plant stress control: this particular mechanism will be thoroughly discussed in a following section and will delve into the details involving how PGPR decrease environmental toxins and other stress-inducing microorganisms (Luterberg & Kamilova, 2009).

- Resource acquisition: this entails the creation or gathering of vital nutrients such as nitrogen, phosphorous, and essential minerals (Ahemad & Kibret, 2014).

- Modulation of plant hormone levels: takes place at the microscopic level in regards to the processes that are ongoing with most PGPR and their accompanying mechanisms (Ahemad & Kibret, 2014).

**1.4 PGPR's Relationship with Plants**

PGPR's symbiotic relationship with plants, as well as their ability to directly align with such relationships, makes for an interesting dynamic. It's important to remember that all plants generally compete with one another in order to develop the means to obtain the necessary components for growth and development. PGPR's are there to aid plants in this process. For example, certain bacteria have the ability to promote growth in plants via nitrogen fixation (Jha & Saraf, 2015). There is also a need to understand the direct relationship with plants in relation to how the soil is impacted. Furthermore, the underlying internal mechanisms are also of interest. In this respect, a study has demonstrated that PGPR has a synergistic impact on the way the surrounding "bulk" soil interacts with other microorganisms (Vejan *et al.,* 2016).

Some of the most valuable resources regarding PGPR have been linked to understanding how and in what ways bacteria stimulates plant growth. Based on numerous studies, PGPR has been proven to:

- Inhibit growth of harmful nematodes (Akhtar *et al.,* 2012).
- Control the biological function of plants by curtailing diseases and pests, thus enriching the soil (Akhtar *et al.,* 2012).
- Create a pathogen-induced system, whereby making plant varieties more resistant to specific pathogens (Beneduzi *et al.,* 2012).
- Decrease the fungal growths around various plants that may inhibit their ultimate growth rates (Prathap, & Kumari, 2015).
- Solubilize nutrients for easy uptake by plants (Usha, 2015)

In addition, these internal relationships are in fact quite reliant on many other external factors involved in the processes taking place at the microscopic level. For instance, climate, previous farming techniques, and crop selection may influence, whether positive or negative, the ongoing mechanisms pertaining to the bacteria cycles within.

**1.5 Species of PGPR**

Within the realm of crop production, PGPR has shown tremendous ability to assist in the maintenance of viable alternatives as opposed to the use of potentially harmful pesticides and other chemicals (Vejan *et al.,* 2016). Another important and valuable component to the process of understanding why and how such soil-bacteria relationships matter in real-world settings has become relevant due to ongoing research in the field of agriculture. For example, some studies have been designed with the specific purpose of discovering how to best implement the process of assisting with potential drought if and when such danger occurs (Sarma, & Saikia, 2013). In effect, these soil and

agricultural experts are trying to determine if their farming and food production processes can help with inhibiting the damage done by droughts.

Agriculture is one of the most important aspects of current civilization needs, due to the prevalence of developing countries and their pressing concern with growing populations and food shortages. To that end, PGPR possesses the ability to stabilize the surrounding soil, stimulate plant growth, and aid in the flourishing of combined microscopic activities in the specific eco-system to which they thrive (Vessey, 2003).

In addition to the symbiotic relationships with other plants, as well as PGPR's direct influence over the entire eco-system of the world in general, understanding the benefits of specific PGPR such as Bacillus *subtilis*, Bacillus *licheniformis*, Bacillus *pumilis*, Bacillus *megatarium*, Bacillus *aryabhattai*, and Pseudomonas *aeruginosa* will provide more insight as to what transpires when these particular bacteria colonize within the rhizosphere. How each of these bacteria promotes plant growth will be highlighted in order to better equate why these living entities are so important.

### 1.6 Bacillus *subtilis*

Bacillus *subtilis* is a PGPR that has been the sole focus of many studies and has shown much promise in promoting plant growth in many ways (Qiao *et al*., 2017). B. *subtilis* has shown that it can in fact accomplish the primary functions of most PGPR (Qiao *et al*., 2017). Such studies have been completed with the intent of discovering what conditions B. *subtilis* will demonstrate its unique abilities.

Foliar application of B. *subtilis* on broad bean 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. Han's 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.

In a more recent study, B. *subtilis* was shown to stimulate growth of tomato plants directly by increasing nutrients through the production of phytohormones, siderophores, organic acids involved in P-solubilization and/or nitrogen fixation and also indirectly by producing antagonistic substances or by inducing the plant resistance against pathogens (Kumar *et al.*, 2015). In addition, B. *subtilis* has been proven to suppress soil-borne disease Fusarium *oxysporum* (Kumar *et al.*, 2015).

Furthermore, this type of PGPR can assist with the process of protection by developing the plants' immune system functioning against various foliar bacterial infections and some fungal infections (Kumar *et al.,* 2012). Strengthening such internal functions can increase plant resistance to not only harmful pests but also increase its ability to grow faster.

**1.7 Bacillus *licheniformis***

B. *licheniformis* is of great interest to researchers for a variety of viable reasons. Lim and Kim (2013) found that pepper plants when inoculated via irrigation with B. *licheniformis* (7.0 × 108 cfu/ml), have the ability to tolerate drought stress and survive longer compared to non-inoculated pepper plants. In another study, P. *polymyxa* and B. *licheniformis* promoted faster growth and seed germination rate in Arabidopsis *thaliana* (Kefele *et al.,* 2015). B. *licheniformis* also has the ability to combat against toxins in the water. For instance, in a study conducted in 2012, B. *licheniformis* acted as a biocontrol agent in combating against heavily contaminated water (runoff/leaching from agricultural fields and industrial companies) in an Indian river called the Hindon, where this particular bacterial strain produced exopolysaccharides (EPS) and siderophore (Dan *et al.*, 2012). EPS help bacteria to inhabit the root surface through specific adhesion, leading to root colonization that eventually results in biofilm formation (Michiels *et al.,* 1991; Matthysse *et al.,* 2005; Ramey *et al*., 2004). This in turn created a positive response in the surrounding soil content. For instance, the bacteria's production of these and other substances decreased the environmental stress of the water supply, which in turn promoted plant growth (Dan *et al.,* 2012). Having the ability to alleviate environmental pressures is one of the most important attributes of PGPRs. Also, in that same study, B. *licheniformis* was shown to inhibit the growth of phytopathogenic fungi, specifically Fusarium *monoliforme*, Fusarium *oxysporum,* Alternaria *solani*, and Sclerotinia *sclerotiorum.*

Conferring positive qualities on surrounding plant life, as well as other microbacterial agents in certain contaminated water supplies, is one of the fundamental aspects of PGPR, in particular B. *licheniformis*. Furthermore, this specific bacterial strain has shown that it can reduce soil stress by conferring the strength and resiliency of its properties onto the surrounding soil through its unique characteristics (Dan *et al.,* 2012). This discovery is powerful due to the potential such bacteria have in helping damaged eco-systems in developing their recovery. Hopefully, future platforms of discovery can further even more research on how to positively impact eco-systems in dire need of rejuvenation and increased plant growth.

### 1.8 **Bacillus *pumilus***

B. *pumilus* is a ubiquitous Gram-positive, aerobic, rod-shaped endospore-forming bacteria that can be isolated from a wide variety of soils, plants, and environmental surfaces (Benardini *et al.,* 2003). B. *pumilus* has shown some tendencies to develop internal chemical mechanisms related to the process of producing antibacterial peptides, which in turn have been seen to increase pro-biotic health in the human digestive system (Thwaite & Atkins, 2012). The relationship between this symbiotic development and the consumption of food, therefore, becomes more perceptive in regard to how the body then digests food, how food grows while in the soil, and ultimately how it is processed through the intestines. This shows how the entire continuum of food production, on through the actual consumption of such items by humans, is dependent upon the microscopic interactions within the study of such PGPR entities.

To that end, other conditional realities of this research have demonstrated clearly how such symbiotic relationships are formed, how they interact with not only the soil but also alongside the internal process of human digestion, and finally the impact on overall health in both plants and humans.

The spores of B. *pumilis* are notoriously resistant to unfavorable conditions such as little to no nutrient availability, extreme desiccation, $H_2O_2$, UV, gamma-radiation, or chemical disinfection (Nicholson *et al.,* 2000; Petrosino, 2017). In a practical sense, B. *pumilis* can actually be placed in the positional mandates of any given farming process and be able to theoretically last longer, survive better, and ultimately improve the soil conditions even when compared to others of a similar build (Petrosino, 2017).

In fact, B. *pumilus* may even be thought of as the single most powerful and resistant PGPR for these given reasons.

In regard to how this bacterium benefits plants, *B. pumilis* aids in plant defense by protecting the plants root system by reducing galling caused by pathogenic nematodes (Almaghrabi *et al.,* 2013). On a side note, it is imperative that we do not categorize all strains of B. *pumilis* as beneficial. There are in fact certain strains considered harmful to both plants and animals (Yuan and Gao, 2015). The same can be said for other bacteria as well.

### 1.9 Bacillus *megatarium*

B. *megaterium* is another plant growth-promoting bacterium. The primary function of this bacteria is to develop and internally create valuable enzymes like amylases and proteases, which in turn can usually be utilized for a variety of industrial uses ("Bacillus megatarium," 2018). In addition, this strain has unique properties and abilities. For instance, it can catalyze the decarboxylation of certain heterocumulenes (a molecule containing a chain of at least three double bonds between consecutive atoms) and thus affect not only the growth cycle of certain plants but also instill a greater sense of cell equilibrium (Trofimov & Nedolya, 2008). In turn, this can increase cell stabilization, decrease certain levels of environmental stress in the surrounding soil compositions, and help to establish a more dynamic and stable planting environment for soil enrichment purposes. Furthermore, this level of complete stabilization could possibly solve the issue with bio-degradation that is experienced with regards to modern farming techniques and soil erosion.

### 1.10 Bacillus *aryabhattai*

Much research has been conducted in connection with how this strain of bacteria is important to the eco-system in which it thrives. One of the most interesting aspects of certain developments within this discussion has been the actual mechanism for increased plant growth rates, which is generally defined by the PGPR being able to directly regulate the phytohormonal activity of the plant by increasing root surface area (Park *et al.,* 2017). In that respect, B. *aryabhattai* thrives due to its individual characteristics. Thus far, however, this ability has been only established under certain environmental conditions.

These conditions have been regulated, examined, and otherwise implemented with the specific purpose of understanding B. *aryabhattai's* ability to accomplish soil improvements and thus increased plant growth rates. To that end, it has been discovered that this PGPR can actually improve the growth rate of wheat by increasing their overall tolerance to oxidative stress due to an increase in antioxidant enzymes like "superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT)" (Park *et al.,* 2017). The primary takeaway from these aspects of how PGPR can improve soil conditions is that under the right circumstances with the correct kind of application, these bacteria can become the missing ingredient in various soil conditions; but only when used correctly.

### 1.11 Pseudomonas *aeruginosa*

As with the bacillus aryabhattai strain, P. *aeruginosa* requires a level of specificity in regards to its overall efficiency at creating the right kind of results when adjusting for particular conditions at hand. For instance, this strain has shown tremendous benefits for the legume plant Pongamia *pinnata* by producing ammonia which in turn increased biomass and major nutrient content (Radhapriya *et al.,* 2015). In addition, P. *aeruginosa* has demonstrated surprising resilience and strength in many ways. For example, in one study, the soil's nitrogen, phosphorus, and potassium uptake was shown to dramatically increase due to the application of this specific strain (Radhapriya *et al.,* 2015). In another study, applying P. *aeruginosa* to the soil increased sugar content, amino acids, and organic acids in plants surrounding the point of application (Radhapriya *et al.*, 2015). Therefore, the entirety of the surrounding soil has thus been improved due to this greater enrichment of the soil's necessary ingredients that have been shown to decrease degradation.

### 1.12 Discussion

All plant and bacterial life on the earth exist in seeming harmony due to the microscopic interactions between them. Although this relationship is often quite difficult to truly understand, it challenges researchers to better equate their methods of discovery within such heavily structured subject matters. This review has created a sizable understanding of the intricacies of plant life, bacteria like the PGPR already reviewed, as well as the overall importance of this constantly evolving microscopic world in

greater detail whenever possible. Studies to improve soil health should be applauded and desired for a variety of reasons as it can enhance food production as well as environmental health conditions.

Researchers might someday further the ability of such microscopic organisms to positively impact greater amounts of individual eco-systems. As the world's population continues to grow at incredible rates, more and more food will need to be produced in order to feed the growing population. As farming techniques rush to fill the gap between production and need, a greater emphasis on soil health, especially as it relates to the maintenance and development of PGPR whenever possible, will be required.

PGPR represent a tiny, yet powerful component of the world's vast amount of available soil. What makes them so vitally important in the modern landscape of massively constructed farming productions is the fact that they can impede and even counteract the deleterious effects of ongoing farming practices that have increased pollution, damaged topsoil, and infected much plant life with dangerous chemical substances (Gupta *et al.,* 2015). In other words, PGPR can possibly bring back nutrients lost in crops due to the negative effects of farming techniques and environmental factors which in turn will affect the health of consumers. In summary, following represents several key points regarding PGPR:

1. PGPR 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 from diseases (Gallup *et al.,* 2014).

2. Plant growth promotion and development can be facilitated both directly and indirectly.

3. The growth of the plants will depend on the type of PGPR, the depth of the inherent relationship, and several other factors.

4. PGPR can and do protect plants by improving their nutrient acquisition and by protecting their cell walls from harm.

5. The ongoing ability of these organisms to influence individual eco-systems in a positive way is not yet fully understood.

6. The future of all agricultural mandates of behavior and operational conditions might be impacted through additional research in this area.

**1.13 Future Applications**

There is still a good deal of research needed to gain a thorough understanding of how these diverse and powerful microorganisms might be properly applied in agriculture. Figuratively speaking, it will most likely come down to the abilities and support of local and international engineers whose tasks involve the development of eco-systems that control and stimulate such PGPR (Vacheron *et al*., 2013). Of course, whatever system that might be put into active practice would never be able to sustain itself without the proper support systems created for such purposes. Without such support mechanisms, no project of any viable size could possibly maintain itself or even be created at all; for it takes a massive amount of support to stimulate any given region or country where farming practices and environmental conditions have become poor.

These support systems might take a variety of forms if they are to solve the current problem of soil erosion, lost crop cycles, and the ongoing chemical treatment of many food products. PGPR has the potential to eliminate poor crop cycles, as well as promote more positive change in regards to pollution and other environmental factors. For instance, since agriculture has long been tied to the economic viability of any given region of the world, especially in lower-income areas, future research and implementation might very well influence many economies of the world in various ways (Singh, 2013). Therefore, if the current methods of farming can be improved in regards to total crop yield, as well as increasing the overall viability of the food produced, it might positively impact the way many underdeveloped countries begin to shape their actual international position and thus future.

There will of course be a number of other barriers to this final realization of a good and sound policy of PGPR and related mandates of action. Some of these barriers include certain bottlenecks at the commercialization level, which in turn means some financial interests are not yet of the mind to actually take action in developing better methods of farming and crop production (Tabassum *et al.,* 2017). This illustrates one of the most important aspects of the future developments of proper PGPR utilization in that

either sizable governing bodies or even industry leaders must take an active role in the creation of better eco-system preservation techniques if the current problems are to be solved. Furthermore, other barriers might entail a lack of technological improvements in existing platforms of usage (Tabassum *et al*., 2017). However, overcoming this barrier will mean greater resources acquisition, i.e. more money, must be gained in order to develop better technology towards this future goal.

### 1.14 Conclusion

The overall goal of this review was to show how PGPR assists in the process of the entire world's eco-system in maintaining a sort of balance. The development of future research methods of a similar type can hopefully bridge the gap between theory and real-world scenarios involving bacteria of this nature and in turn, improve existing plant life. This knowledge could increase crop yield for some farmers and thus improve the quality of food produced. If structured correctly and given the right amount of funding and other types of resources, the future of agriculture in relation to how PGPR can help plants heal and grow might be much more positive.

### 1.15 Utilization of PGPR in Sustainable Farming Systems

Agricultural productivity rests on the foundation of microbial diversity in the soil. Through research and numerous farming trials, PGPR have emerged as an important and promising tool for sustainable agriculture (Tabassum *et al.,* 2017). In addition to the utilization of local and organic resources, PGPR is the driving force behind sustainable farming systems such as organic farming and Korean natural farming (KNF). 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 plants. Organic farming differs from conventional (does not rely on PGPR but rather inorganic fertilizers and pesticides) in that it promotes the growth of PGPR by supplying them with organic material. The decomposition process of organic material is slow; this benefits PGPR 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

organic farming in that it involves increasing the bacterial population (PGPR) in the soil and utilizing natural inputs (i.e. nutrient-rich liquid) as a food source for bacteria and plants (Reddy 2011).

The KNF is a sustainable system developed by Master Han Kyu Cho of the Janong Natural Farming Institute in South Korea, based on generations of sustainable farming methods practiced in Japan, China, and Korea. 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). Recent studies from the University of Hawai'i suggest that there is a correlation between soil fertility and the number of microorganisms present in the soil (Wang *et al.,* 2012). When compared to organic and conventional farming methods, KNF contained more microorganisms. Of the three farming methods, KNF produced healthier plants with visual assessment. However, the specific types of PGPR that are present in this system remain unanswered. Therefore, the overall goal of this MS thesis is to provide a greater understanding of the types of bacteria present within these farming systems.

**1.16 References**


Adesemoye, A., Obini, M., & Ugoji, E. 2007. Comparison of plant growth-promotion. Brazilian Journal of Microbiology, 39, 423-426. doi: 1517-8382

Ahemad, M. & Kibret, M. 2014. Mechanisms and applications of PGPR. Journal of King Saud University, 26(1), 1-20. doi: https://doi.org/10.1016/j.jksus.2013.05.001

Akhtar, A., Hisamuddin, A., Robab, M., Sharf, A., & Sharf, R. 2012. Plant growth promoting Rhizobacteria: An overview. Scholars Research Library. Retrieved from http://www.scholarsresearchlibrary.com/

Almaghrabi, O. A., Massoud, S. I., & Abdelmoneim, T. S. 2013. Influence of inoculation with plant growth promoting rhizobacteria (PGPR) on tomato plant growth and nematode reproduction under greenhouse conditions. Saudi Journal of Biological Sciences, 20(1), 57–61. http://doi.org/10.1016/j.sjbs.2012.10.004

Bacillus megatarium. 2018. NCBI. Retrieved from https://www.ncbi.nlm.nih.gov/genome/?term=Bacillus%20megaterium[Organism]&cmd=DetailsSearch

Beneduzi, A., Ambrosini, A., & Passaglia, L. 2012. Plant growth-promoting rhizobacteria (PGPR): Their potential as antagonists and biocontrol agents. Genetics and Molecular Biology, 35(4), 1044-1051. Retrieved from http://www.sbg.org.br/

Berg G., Smalla K. 2009. Plant species and soil type cooperatively shape the structure and function of microbial communities in the rhizosphere. FEMS Microbiol. Ecol. 68 1–13 10.1111/j.15746941.2009.00654.x

Benardini JN, Sawyer J, Venkateswaran K, & Nicholson WL. 2003. Spore UV and acceleration resistance of endolithic Bacillus pumilus and B. subtilis isolates obtained from Sonoran desert basalt: implications for lithopanspermia. Astrobiology 3: 709-717.

Bhardwaj, D., Ansari, M. W., Sahoo, R. K., & Tuteja, N. 2014. Biofertilizers function as key player in sustainable agriculture by improving soil fertility, plant tolerance and crop productivity. Microbial Cell Factories, 13, 66. http://doi.org/10.1186/1475-2859-13-66

Carvajal-Muñoz J S and Carmona-Garcia C E. 2012. Benefits and limitations of biofertilization in agricultural practices. Livestock Research for Rural Development. Volume 24, Article #43. Retrieved January 5, 2018, from http://www.lrrd.org/lrrd24/3/carv24043.htm

Costa R., Götz M., Mrotzek N., Lottmann J., Berg G., Smalla K. 2006. Effects of site and plant species on rhizosphere community structure as revealed by molecular analysis of microbial guilds. FEMS Microbiol. Ecol. 56 236–249 10.1111/j.1574-6941.2005.00026.x

Dan, V., Mishra, S., Chaudhry, V., Tripathi, S., Singh, P., Yadav, S., Mishra, S., Ijinu, T. P.,

George, V., Varma, A., & Nautiyal, C. 2012. Biocontrol, plant growth promotion and conferring stress tolerance. International Journal of Science and Nature, 3(4), 780783. Retrieved from www.scienceandnature.org

Essoyan, S. 2011. Natural selection: A self-sufficient system of farming is increasing yields across Hawaii. Star Advertiser. Retrieved October 1, 2017, from http://www.staradvertiser.com/news/20110118_Natural_selection.html?id=114074644

Dobbelaere S., Vanderleyden J., Okon Y. 2003. Plant growth-promoting effects of diazotrophs in the rhizosphere. Crit. Rev. Plant Sci. 22 107–149 10.1080/713610853

Gullap, K., Dascı, M., Erkovan, H., Koç, A., & Turan, M. 2014. Plant Growth-Promoting Rhizobacteria (PGPR) and Phosphorus Fertilizer-Assisted Phytoextraction of Toxic Heavy Metals from Contaminated Soils. Communications in Soil Science and Plant Analysis. 45. 10.1080/00103624.2014.929702.

Goswami, D., Thakker, J., Pinakin C., & Dhandhukia, P. (2016). Portraying mechanics of plant growth promoting rhizobacteria (PGPR). Cogent Food & Agriculture, 2. doi: http://dx.doi.org/10.1080/23311932.2015.1127500

Gray EJ, Smith DL. 2005. Intracellular and extracellular PGPR: Commonalities and distinctions in the plant-bacterium signaling processes. Soil Biol Biochem;37:395–412.

Gupta G., Parihar S., Ahirwar, N.K., Snehi S., Singh V. 2015. Plant Growth Promoting Rhizobacteria (PGPR). J Microb Biochem Technol, 7, 096-102. doi:10 .4172/1948-5948.1000188

Han, Q.-Q., Lü, X.-P., Bai, J.-P., Qiao, Y., Paré, P. W., Wang, S.-M., and Wang, Z.-L. 2014. Beneficial soil bacterium Bacillus subtilis (GB03) augments salt tolerance of white clover. Frontiers in Plant Science, 5, 525. http://doi.org/10.3389/fpls.2014.00525

Hartmann A., Schmid M., Tuinen D., Berg G. 2009. Plant-driven selection of microbes. Plant Soil 321 235–257 10.1007/s11104-008-9814-y

Jha, C. & Sraf, M. 2015. Plant growth promoting rhizobacteria (PGPR). Journal of Agricultural Research and Development, 5(2), 0108-0119. Retrieved from http://www.e3journals.org

Kefela T, Gachomo EW, Kotchoni SO. 2015. Paenibacillus polymyxa, Bacillus licheniformis and Bradyrhizobium japonicum IRAT FA3 Promote Faster Seed Germination Rate, Growth and Disease Resistance under Pathogenic Pressure. J Plant Biochem Physiol 3:145. doi: 10.4172/2329-9029.1000145

Kumar, A., Lakshmanan, V., Caplan, J., Powell, D., Czymmek, K., Levia, D., & Bais, H. 2012. Rhizobacteria Bacillus subtilis restricts foliar pathogen entry through stomata. The Plant Journal, 72, 694-702. doi: 10.1111/j.1365-313X.2012.05116.x

Kumar A, Guleria S, Mehta P, Walia A, Chauhan A, Shirkot CK. 2015. Plant growth-promoting traits of phosphate solubilizing bacteria isolated from Hippophae rhamnoides L. (Sea-buckthorn) growing in cold desert Trans-Himalayan Lahul and Spiti regions of India. Acta Physiol Plant. 37(3):1–12. doi: 10.1007/s11738-015-1793-z.

Li, Y., Xu, S., & Gao, J. Plant Growth Regulation 2016. 78: 43. doi:10.1007/s10725-015-0073-7

Lim, J.-H., & Kim, S.-D. 2013. Induction of Drought Stress Resistance by Multi-Functional PGPR Bacillus licheniformis K11 in Pepper. The Plant Pathology Journal, 29(2), 201–208. http://doi.org/10.5423/PPJ.SI.02.2013.0021

Luterberg, B. & Kamilova, F. 2009. Plant growth-promoting rhizobacteria. Annu Rev Microbiol, 63, 541-56. doi: 10.1146/annurev.micro.62.081307.162918.

Matthysse, A.G., Marry, M., Krall, L., Kaye, M., Ramey, B.E., Fuqua, C. and White, A.R. 2005. The effect of cellulose overproduction on binding and biofilm formation on roots by Agrobacterium tumefaciens. Molecular Plant-Microbe Interactions, 18: 1002–1010.

Michiels, K.W., Croes, C.L. and Vanderleyden, J. 1991. Two different modes of attachment of Azospirillum brasilense sp7 to wheat roots. Journal of General Microbiology. 137: 2241–2246.

Nicholson WL, Munakata N, Horneck G, Melosh HJ, & Setlow P. (2000). Resistance of Bacillus endospores to extreme terrestrial and extraterrestrial environments. Microbiol Mol Biol Rev 64: 548-572.

Park, Y., Mun, B., Kang, S., Hussain, A., Shahzad, R., Seo, C., Kim, A., Lee, S., Oh, K., Lee, D., Lee, I., & Yun, B. 2017. Bacillus aryabhattai SRB02 tolerates oxidative and nitrosative stress and promotes the growth of soybean by modulating the production of phytohormones. PLoS ONE 12(3), e0173203. doi:10.1371/journal.pone.0173203

Petrosino, J. 2017. Bacillus pumilus SAFR-032. Human Genome Sequencing Center. Retrieved from https://www.hgsc.bcm.edu/bacillus-pumilus-safr-032

Prathap, M. & Kumari, R. 2015. A critical review on plant growth promoting rhizobacteria. Plant Pathology & Microbiology, 6(4). doi: http://dx.doi.org/10.4172/2157-7471.1000266

Qiao, J., Yu, X., Liang, X., Liu, Y., Borriss, R., Liu, Y. 2017. Addition of plant-growth-promoting Bacillus subtilis PTS-394 on tomato rhizosphere has no durable impact on composition of root microbiome. BMC Microbiology, 17(131). doi: 10.1186/s12866-017-1039-x

Radhapriya, P., Ramachandran, A., Anandham, R., & Mahalingam, S. 2015. Pseudomonas aeruginosa RRALC3 enhances the biomass, nutrient and carbon contents of Pongamia pinnata seedlings in degraded forest soil. PLoS ONE 10(10), e0139881. doi:10.1371/journal.pone.0139881

Ramey, B.E., Koutsoudis, M., Von-Bodman, S.B. and Fuqua C. 2004. Biofilm formation in plant–microbe associations. Current Opinion in Microbiology, 7: 602–609.

Rauscher, F. 2015. Combining Organic & Synthetic Nutrients. Retrieved April 10, 2016 from https://www.maximumyield.com/combining-organic-synthetic-nutrients/2/1456

Reddy, R. 2011. Cho's Global Natural Farming. Retrieved April 10, 2016 from https://ilcasia.files.wordpress.com/2012/02/chos-global-natural-farming-sarra.pdf
Saharan, B. & Nehra, V. 2011. Plant growth promoting rhizobacteria. Life Sciences and Medicine Research. Retrieved from http://astonjournals.com/lsmr

Sarma, R., & Saikia, R. 2014. Alleviation of drought stress in mung bean by strain Pseudomonas aeruginosa GGRJ21. Plant and Soil, 377(1/2), 111-126. Retrieved from http://www.jstor.org/stable/44244611

Schütz, L., Gattinger, A., Meier, M., Müller, A., Boller, T., Mäder, P., & Mathimaran, N. 2017. Improving Crop Yield and Nutrient Use Efficiency via Biofertilization—A Global Meta-analysis. Frontiers in Plant Science, 8, 2204. http://doi.org/10.3389/fpls.2017.02204

Singh, J. 2013. Plant growth promoting rhizobacteria. Resonance. Retrieved from https://www.ias.ac.in/article/fulltext/reso/018/03/0275-0281

Sushanto G, Rout, G. K., Gitishree, D., Spiros P., Han-Seung, S., Jayanta, K. P. 2018. Revitalization of plant growth promoting rhizobacteria for sustainable development in agriculture, Microbiological Research. Volume 206, Pages 131-140. ISSN 0944-5013, https://doi.org/10.1016/j.micres.2017.08.016.

Tabassum, B., Khan, A., Tariq, M., Ramzan, M., Khan, S., Shahid, N., & Aaliya, K. 2017.

Bottlenecks in commercialisation and future prospects of PGPR. Applied Soil Ecology, 121, 102-117. doi: https://doi.org/10.1016/j.apsoil.2017.09.030

Thwaite, J. & Atkins, H. 2012. Bacillus. Science Direct. Retrieved from https://www.sciencedirect.com/topics/agricultural-and-biological-sciences/bacillus-pumilus

Trofimov, B. & Nedolya, N. 2008. Five-membered rings with one heteroatom together with their benzo and other carbocyclic-fused derivatives. Science Direct. Retrieved from https://www.sciencedirect.com/topics/medicine-and-dentistry/bacillus-megaterium

Usha,B. 2015. PGPR Interaction: An Ecofriendly Approach Promoting the SustainableAgriculture System. Advances in Botanical Research, Academic Press, Volume 75, Pages 81-113. ISBN 9780124201163, https://doi.org/10.1016/bs.abr.2015.09.006.

Vacheron, J., Desbrosses, G., Bouffaud, M., Touraine, B., Moënne-Loccoz,Y., Muller, D., Legendre, L., Wisniewski-Dyé, F., & Prigent-Combaret, C. 2013. Plant growth-promoting rhizobacteria and root system functioning. Front Plant Sci. doi: https://doi.org/10.3389/fpls.2013.00356

Vejan, P. Abdullah, R., Khadiran, T., Ismail, S., & Boyce, A. 2016. Role of plant growth promoting rhizobacteria in agricultural sustainability. Molecules, 21, 573. doi:10.3390/molecules21050573

Vessey, J.K. 2003. Plant growth promoting rhizobacteria as biofertilizers. Plant and Soil, 255, 571. doi: https://doi.org/10.1023/A:1026037216893

Wang, K-H., DuPonte M, and Chang K. 2012. Use of Korean Natural Farming for Vegetable Crop Production in Hawai'i. Hanai'AI/The Food Provider.Retrieved September 1, 2016, from http://www.ctahr.hawaii.edu/sustainag/news/articles/V14-Wang-KNF.pdf

Wright J. P., Jones C. G. 2006. The concept of organisms as ecosystem engineers ten years on: progress, limitations, and challenges. BioScience 56 203–209 10.1641/0006-3568(2006)056[0203:TCOOAE]2.0.CO;2

Yuan, Y., & Gao, M. 2015. Genomic analysis of a ginger pathogen Bacillus pumilus providing the understanding to the pathogenesis and the novel control strategy. Scientific Reports, 5, 10259. http://doi.org/10.1038/srep10259

# Chapter 2

# Natural Farming: Comparison of phosphorus-solubilizing and nitrogen-fixing bacteria among Korean Natural Farming, organic, and conventional farming methods

## 2.1 Abstract

Korean natural farming (KNF) is promoted as a self-s farming system that involves culturing indigenous microorganisms (IMO) and reintroducing them into ecosystem disturbed soil to enhance soil microbial activities and fertility. Though there has been a growing interest amongst subsistent farmers, little is known as to how this system 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 (organic treated soil covered with sunn hemp mulch), and 3) ORG-WM (weed mat used in place of sunn hemp mulch). Soil samples were collected in between *Lycopersicon esculentum* (grape tomato) at 14 ($T_{14}$) and 28 ($T_{28}$) days after tomato transplanting. Experiment II was conducted in Kula, Hawai'i. There were three treatments: 1) KNF, 2) ORG, and 3) CON (conventional). All three systems were covered with bamboo mulch. Soil samples were collected from the Kula Experiment Station on 4 dates: three dates ($T_0$, $T_{21}$, and $T_{56)}$ were collected in between *Cucurbita pepo* var. *cylindrica* (zucchini) whereas the fourth ($T_{56r}$) was collected within the rhizosphere at post-harvest (where plants were removed). Soil samples collected from both studies were plated on media 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 was also plated on De Man, Rogosa and Sharpe media (MRS). Each microbial colony was isolated and subjected to polymerase chain reaction (PCR) followed by DNA analysis to identify specific strains of bacteria isolated. The bacterial colony forming units (CFU) was determined. Results showed that *Bacillus megaterium* and *Bacillus aryabhattai* were prevalent in all soil samples collected. KNF had a greater diversity of bacteria overall in both experiments. At $T_{21}$ (Experiment II, Kula trial), KNF contained a significantly higher CFU compared to CON ($6.03 \times 10^6$ CFU/g vs $5.3 \times 10^5$ CFU/g; $P < 0.001$). Additionally, KNF treated soil was the only farming system that contained an abundant amount of *Bacillus subtilis* and *Bacillus licheniformis* in every soil sampled. The bacterial population for all farming systems increased soon after IMO treatment but decreased over time. However, it was greatest in the soil within the rhizosphere compared to those from between plants.

## 2.2 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; moreover, conventional agricultural production in the state also relies on imported inputs such as feed, fertilizer, compost, and pesticides. (“Population and Economic Projections for the State of Hawai’i”, 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 a sustainable farm is to minimize overhead, most of which comes from imported inputs that the farms require to operate successfully. It’s imperative for Hawai’i to implement a solution 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 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 are not dependent on the use of inorganic pesticides, fertilizers, and genetically modified organisms, but rather on ecological processes, biodiversity, and crop cycles adapted to local conditions (Woo, 2010). One such natural farming method that’s practiced in Hawai’i and many parts of Asia is referred to as Korean Natural Farming (KNF), which farmers have found to be an effective and self-sufficient farming method (Essoyan, 2011).

Korean Natural Farming (KNF) is a sustainable system developed by Master Han Kyu Cho of the Janong Natural Farming Institute in South Korea, based on generations of sustainable farming methods practiced in Japan, China, and Korea. 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 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 (i.e. bacteria, fungi, protozoa, nematodes) 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, when compared 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, microorganisms, specifically bacteria, which might be enhanced by KNF practices, have yet to be determined. This research focused on determining common soil bacteria found in KNF across two soil types which were different from CON and ORG farming systems.

The overall goal of this research was to determine the types of bacteria prevalent in KNF that were different from CON and ORG farming systems, and examine the dynamic of bacterial population over time. The objectives for Experiment I was 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 colony forming units (CFU). The objectives for Experiment II were to: 1) identify and quantify the predominant soil bacteria present in KNF, CON, and ORG managed zucchini agroecosystem, 2) compare the identified bacteria between the three farming systems, 3) determine the bacterial population within the rhizosphere and 4) determine the bacterial population over a period of time.

**2.3 Materials and Methods**

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

**Experiment I.** This field trial was conducted in the spring of 2013. Three treatments installed were KNF system followed a sunn hemp no-till cover cropping practice (KNF-SH), organic fertilizer treatment followed a sunn hemp no-till cover cropping practice (ORG-SH), and organic fertilizer treatment followed a bare fallow period and soil was covered by weed mat at transplanting (ORG-WM). Each treatment plot was 2.44 x 9.144 $m^2$ in size, replicated 3 times. Sunn hemp was grown for 2 months and terminated using a roller crimper, with sunn hemp residues left on the soil surface as organic mulch. Polyethylene woven weed mat was used in the ORG-WM (WM = weed mat) treatment. For the KNF treatment, a soil amendment named IMO4 compost was broadcasted at 1.36 kg/30 $m^2$ onto the soil surface followed by sprinkling of soil treatment solution (SOS) at 0.5 L/$m^2$ as described by Cho (2010) prior to roller-crimping of the SH cover crop. Six-week old seedlings of grape tomato 'Felicity' (*Lycopersicon esculentum*) were transplanted at 60-cm between plants within a row with two rows per treatment plot at row spacing of 1.2 m, i.e. a total of 16 plants per treatment plot. For the KNF-SH treatment, the seedlings were drenched with seed treatment solution (SES) in the seedling tray prior to transplanting. Subsequent to transplanting, plants in KNF-SH treatment received Type II and Type III weekly foliar spray following a rotation of 2 weeks of Type II and 1 week of Type III) (Reddy, 2011). Formulations of each of the KNF treatment or spraying solution were described in "How to Prepare Korean Nature Farming Materials" (Wang and Chang, 2012). For the ORG-SH and ORG-WM treatments, plants were fertilized with Sustane 8-2-4 at 67 kg N/ha. At 14 days after transplanting, the first set of soil samples (n=3, $T_{14}$) were collected from the tomato rhizosphere up to 10-cm soil depth with composite of four soil cores from each plot. The 2$^{nd}$ set of samples (n=3, $T_{28}$) was collected on day 28 after transplanting. Upon collection, each soil sample was spread out onto an individual tray and allowed to air dry overnight. Once dried, the soil was sifted through a 2-mm mesh sieve and placed into sterile containers. The soil samples were then subjected to serial dilutions and plating on two selective media: Phosphorus-solubilizing and Nitrogen-fixing media, each in replicates of 3 (n=3).

**Experiment II.** A modification of the Poamoho field trial was conducted at the Kula Research Station in Maui, Hawai'i. Three treatments installed were KNF, organically fertilized (ORG), and conventionally fertilized with synthetic fertilizer as a control (CON) with three replication plots per treatment. Each treatment plot was 2.44 x 3.048 $m^2$ in size. The experimental design was a random complete block design

(RCBD). Ten 2-week old 'Felix' zucchini (*Cucurbita pepo*) seedlings were transplanted per plot at 60-cm plant spacing within row and 120 cm between rows. For the KNF treatment, zucchini seedlings were drenched with SES solution prior to transplanting, followed by weekly Type II and Type III foliar spray rotation (2 weeks of Type II, 1 week of Type III) (Reddy, 2011, Wang and Chang, 2012). IMO4 and SOS solution were prepared and applied to the KNF plots in the same manner as described in Experiment I. Bamboo leaves was applied as surface organic mulch in KNF plots. For the ORG treatment, each seedling was inoculated with 240 ml of Mykos liquid solution (a.i. *Rhizophagus irregularis*, RTI$^{AG}$, Gilroy, CA) prepared from 188 ml Mykos diluted into 38 liter of water. All plants in ORG treatment was fertilized with Sustane 8-2-4 at 180 kg N/ha. For the CON treatment, all plants were fertilized with 16-16-16 N-P-K synthetic fertilizer at 180 kg N/ha rate. The first set of samples (n=3, $T_0$) were collected from each plot prior to soil treatment (day 0). On day 21 after zucchini transplanting, a second set of soil samples (n=3, $T_{21}$) were collected from rhizosphere of zucchini from each plot. The third and fourth sets of samples were collected on day 56. The third set ($T_{56}$) was collected in the same manner as the second set ($T_{21}$). The fourth set ($T_{56r}$) was soil in contact with the roots after removing the plants from the soil. Similar to Experiment I, soil samples were air dried overnight, sifted, and subjected to serial dilutions on 3 selective media: Phosphorus-solubilizing, Nitrogen-fixing, and MRS (De Man, Rogosa, and Sharpe) media.

**Selective Media Preparation** Many variations of selective media could have been used to target certain groups of bacteria present in the soil sample. The primary goal was to specifically target and identify phosphorus-solubilizing and Nitrogen-fixing bacteria. In addition, potassium (K), nitrogen (N) and phosphorus (P) are three 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 requirement 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 $l^{-1}$: Difco Lactobacilli MRS Broth, 55 g and Difco Agar, 15 g (BD™, Franklin Lakes, New Jersey). The azospirillum media contained $l^{-1}$: $K_2HPO_4$, 5 g; $MgSO_4 \cdot 7H20$, 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 $l^{-1}$: Difco Plate Count agar (PCA), 23.2 g; $Ca(PO_4)_2$, 5 g; and Difco agar, 25

g (Atlas, 2010). A nutrient broth of all three selective media was also prepared (devoid of agar) 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 1). 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 media. The techniques used to inoculate the plates were the streak-plate and spread-plate techniques (Mulder and Deinema, 1981). Plates were incubated at 35°C for approximately 16 h.

The colonies appearing on the solid media were counted and recorded to determine the CFU. In the 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 1a).

**Identification of Isolates** Partial sequencing of the 16S rRNA genes of new isolates was carried out as described by Promega, after the 16S rRNA gene was amplified by PCR with oligonucleotide primers 16S1-F (5′-GGAGAGTTTGATCCTGGCTCAG-3′) and 16S1-R (TATTACCGCGGCTGCTGGCAC) (Promega, Madison, Wisconsin). The amplified samples were submitted to the Advanced Studies in Genomics, Proteomics and Bioinformatics (ASGPB) laboratory located at the University of Hawaii at Manoa and subjected to high throughput DNA sequencing. ChromasPro was used to view the DNA sequencing. The sequences were compared with those in the GenBank databases by using the BLAST program (www.ncbi.nlm.nih.gov/blast).

## 2.4 Results

The results for Experiment I, Poamoho trial, show that Bacillus *subtilis,* Streptomyces *collinus*, Bacillus *lichenformis*, and Bacillus *pumilis* were found only in KNF-SH samples obtained on day 14 ($T_{14}$) (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 ($T_{28}$) show that *Bacillus subtilis, Bacillus lichenformis, Bacillus pumilis,* and *Aneurinibacillus migulanus* were found 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 data in Figure 4 represents the bacterial colonies cultured on phosphorus-solubilizing media. Lowercase alphabet indicates a significant difference at $P<0.05$ with each time of collection. The KNF-SH soil samples collected on day 14 ($104.3 \times 10^4 \pm 8.4$ CFU/g) and 28 ($79.6 \times 10^4 \pm 8.1$ CFU/g) contained significantly higher bacterial counts than the ORG-WM samples for each time of collection. Figure 5 represents the bacterial colonies cultured on azospirillum media. The KNF-SH soil samples collected on day 14 ($135 \times 10^4 \pm 10.26$ CFU/g) and 28 ($102 \times 10^4 \pm 15.3$ CFU/g) contained significantly higher bacterial counts than the ORG-WM and ORG-SH samples for each time of collection.

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, $T_{21}$) shows a greater diversity of bacteria in KNF compared to and CON (Figure 7). Bacteria found only in the KNF samples include *Arthrobacter defluvii, Bacillus oleronius, Bacillus pseudomycoides, Bacillus subtilis, Bacillus thuringiensis, Paenibacillus glucanolyticus, Streptomyces djakartensis,* and *Streptomyces galilaeus*. *Bacillus aryabhattai, Bacillus megaterium,* and *Bacillus cereus* were present in all three farming systems. Soil samples collected on day 56 (post application, $T_2$) 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 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 $KNF_r$. All $T_{56r}$ samples contained *Bacillus aryabhattai and Bacillus megaterium*. Additionally, *B. megatarium* and *Bacillus 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 10 represents the bacterial colonies cultured on phosphorus-solubilizing media. Lowercase alphabet indicates a significant difference at $P<0.05$ with each time of collection. The results showed that KNF contained higher amounts of bacterial colonies ($67.3 \times 10^5 \pm 4.33$ CFU/g at $T_{21}$ and $54.6 \times 10^5 \pm 3.48$ CFU/g at $T_{56}$; $P<0.05$) than the CON ($33.3 \times 10^5 \pm 5.89$ CFU/g at $T_{21}$ and $35 \times 10^5 \pm 7.6$ CFU/g at $T_{56}$) and ORG samples ($52 \times 10^5 \pm 4.35$ CFU/g at $T_{21}$ and $37.3 \times 10^5 \pm 5.6$ CFU/g at $T_{56}$). The soil samples plated on Azospirillum media showed similar results; at $T_{56}$, KNF had a higher CFU ($50.6 \times 10^5 \pm 9.27$ CFU/g) than CON ($33.7 \times 10^5 \pm 4.8$ CFU/g) and ORG ($35 \times 10^5 \pm 5$ CFU/g; Figure 11). However, there was no significant difference between the CON ($36.6 \times 10^5 \pm 2.96$ CFU/g) and KNF ($60.3 \times 10^5 \pm 2.6$ CFU/g) samples collected on day 21. Figure 12 represents the bacterial colonies cultured on MRS media. The KNF soil samples collected on day 21 ($13.4 \times 10^5 \pm 0.57$ CFU/g) contained significantly higher bacterial counts than the ORG ($6.36 \times 10^5 \pm 0.56$ CFU/g) and CON ($4.67 \times 10^5 \pm 0.97$ CFU/g) samples. There was no significant difference between the CON ($4.4 \times 10^5 \pm 3.3$ CFU/g), ORG ($4.7 \times 10^5 \pm 1.92$ CFU/g), and KNF ($5.7 \times 10^5 \pm 0.83$ CFU/g) samples collected on day 56. Figure 13 shows a comparison between the bacterial colonies present in within the rhizosphere ($T_{56r}$) and 12 inches away from the rhizosphere ($T_{56}$). The soil samples obtained within the rhizosphere ($T_{56r}$) that were plated on azospirillum contained significantly higher bacterial colonies than the samples that were not ($T_{56}$).

**2.5 Discussion**

The goal of this experiment was to provide a greater understanding of the types of bacteria present within these farming systems. The data suggests that the KNF treated plots had a higher abundance of bacteria and, in addition to the organic treated plots, had a more diverse group of bacteria

when compared to the plots treated with synthetic fertilizer. In terms of both abundance and diversity, the conventional treated plots had the least amount 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 regards to Experiment I, the weed mat used in the organic plots (ORG-WM) seemed to actually suppress bacterial growth as there were less bacteria identified and significantly less bacterial colonies (CFU/g of soil). In addition, 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 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 increasing the bacterial population in the soil and 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 does promote growth of bacteria within the rhizosphere, but just not the same type 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 runs the risk of washing away or leaching into the groundwater (Sebilo *et al*. 2013). It is plausible that the conventional plots contained the least amount of bacteria due to the fact that synthetic fertilizers don't provide the necessary resources bacteria need to survive. According Nakhro (2010), the addition of organic and natural amendments might have large impact on the size and activity of microbial population.

Both trials showed that *B. subtilis,* B. *pumilis and B. licheniformis* were present only in the KNF soils sampled. These particular bacteria, amongst others, are commonly referred to as plant growth promoting rhizobacteria (PGPR). The data also showed that the CFU count was highest within the

rhizosphere $T_{56r}$, as opposed to 12 inches away from the zucchini plant, $T_{56}$ (Figure 13). PGPR have the potential to contribute to sustainable plant growth promotion. Generally, PGPR 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 from diseases (Gullap *et al.,* 2014). Plant growth promotion and development can be facilitated both directly and indirectly. PGPR such as B. subtilis has been proven to promote plant growth and induce disease resistance (Li *et al*., 2016). In one study, *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. Han's 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 PGPR's such as *B. pumilis* and *P. fluorescens* protect the plants 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. *polymyxa* 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 considered PGPR's as they have proven to promote growth and disease resistance in plants (Ramesh *et al.,* 2014; Hu *et al*., 2014). Additionally, 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 unique presence of the bacillus specie may be the reason why 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 may

excel in growth in areas harboring certain pathogens, when compared to conventional farming methods. Additionally, environmental conditions vary with geography so where sustainable agriculture is most efficient on one farm, may not be entirely feasible in another.

As previously stated, the primary goal of this research project is to identify the types of bacteria present in the soil samples obtained from two separate experiments conducted by Dr. Wang. Though no formal paper has been published regarding these two experiments, 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 low in conventional farming methods. As previously mentioned, KNF involves the culturing of naturally occurring indigenous microorganisms (IMO) – 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 Temperature gradient gel electrophoresis (TGGE). It would also be informative to conduct a KNF field study to record plant yield and collect soil samples for microbial testing.

## 2.6 References


Almaghrabi, O. A., Massoud, S. I., & Abdelmoneim, T. S. 2013. Influence of inoculation with plant growth promoting rhizobacteria (PGPR) on tomato plant growth and nematode reproduction under greenhouse conditions. Saudi Journal of Biological Sciences, 20(1), 57–61. http://doi.org/10.1016/j.sjbs.2012.10.004

Atlas, R. M. 2010. "Handbook of Microbiological Media," fourth ed. CRC Press, Boca Raton, FL.

Cho, H.-K., and Cho, J.-Y. 2010. Natural Farming. Han-Kyu Cho. CGNF. Seoul, S. Korea

Essoyan, S. 2011. Natural selection: A self-sufficient system of farming is increasing yields across Hawai'i. Star Advertiser. Retrieved October 1, 2013, from http://www.staradvertiser.com/news/20110118_Natural_selection.html?id=114074644

Ferris, H., Bongers, T., and DeGoede, R. G. M. 2001. A framework for soil food web diagnostics: Extension of the nematode faunal analysis concept. Appl. Soil Ecol. 18:13-29.

Franklin, R.B. and Mills, A.L., 2009. Importance of spatially structured environmental heterogeneity in controlling microbial community composition at small spatial scales in an agricultural field. Soil Biol. Biochem. 41: 1833–1840.

Gullap, K., Dascı, M., Erkovan, H., Koç, A., & Turan, M. 2014. Plant Growth-Promoting Rhizobacteria (PGPR) and Phosphorus Fertilizer-Assisted Phytoextraction of Toxic Heavy Metals from Contaminated Soils. Communications in Soil Science and Plant Analysis. 45. 10.1080/00103624.2014.929702.

Han, Q.-Q., Lü, X.-P., Bai, J.-P., Qiao, Y., Paré, P. W., Wang, S.-M., and Wang, Z.-L. 2014. Beneficial soil bacterium Bacillus subtilis (GB03) augments salt tolerance of white clover. Frontiers in Plant Science, 5, 525. http://doi.org/10.3389/fpls.2014.00525

Hu X, Roberts DP, and Xie L. 2013. Bacillus megaterium A6 suppresses Sclerotinia sclerotiorum on oilseed rape in the field and promotes oilseed rape growth. Crop Protect. 2013;52:151–158.

Hurst C J, Knudsen G R, Mclnerny M J and Stetzenbatch L D. 2000. "Role of Azospiriillum in Nitrogen Fixation", Journal of Manual Envronmental Microbiology, Vol. 6, No. 3, pp. 104-105.

Kefela T, Gachomo EW, Kotchoni SO. 2015. Paenibacillus polymyxa, Bacillus licheniformis and Bradyrhizobium japonicum IRAT FA3 Promote Faster Seed Germination Rate, Growth and Disease Resistance under Pathogenic Pressure. J Plant Biochem Physiol 3:145. doi: 10.4172/2329-9029.1000145

Koorem, K., Gazol, A., Öpik, M., Moora, M., Saks, Ü., Uibopuu, A., and Zobel, M. 2014. Soil Nutrient Content Influences the Abundance of Soil Microbes but Not Plant Biomass at the Small-Scale. PLoS ONE, 9(3), e91998. http://doi.org/10.1371/journal.pone.0091998

Kumar, A. S., Lakshmanan, V., Caplan, J. L., Powell, D., Czymmek, K. J., Levia, D. F. and Bais, H. P. (2012), Rhizobacteria Bacillus subtilis restricts foliar pathogen entry through stomata. The Plant Journal, 72: 694–706. doi:10.1111/j.1365-313X.2012.05116.x

Li, Y., Xu, S., & Gao, J. Plant Growth Regulation 2016. 78: 43. doi:10.1007/s10725-015-0073-7

Lim, J.-H., & Kim, S.-D. 2013. Induction of Drought Stress Resistance by Multi-Functional PGPR Bacillus licheniformis K11 in Pepper. The Plant Pathology Journal, 29(2), 201–208. http://doi.org/10.5423/PPJ.SI.02.2013.0021

Mulder, E.G., & Deinema M.H. 1981. The sheathed bacteria. In: Starr PM, Stolp H, Truper HG, Barlows A and Schlegelhg (eds.) The Prokaryotes: A Handbook on Habitats, Isolation, and Identification of Bacteria. Springer-Verlag, USA. 425-440.

Nakhro, N and M.S. Dkhar, 2010. Impact of Organic and Inorganic Fertilizers on Microbial Populations and Biomass Carbon in Paddy Field Soil. Journal of Agronomy, 9: 102-110.

Olsson P.A., Baath. E., Jakobsen, I., 1997. Phosphorus effects on the mycelium and storage structures of an arbuscular mycorrhizal fungus as studied in the soil and roots by analysis of fatty acid signatures. Appl. Environ. Microbiol. 63: 3531–3538.

Population and Economic Projections for the State of Hawai'i to 2040. March 2012. Retrieved May 10, 2016 from http://files.hawaii.gov/dbedt/economic/data_reports /2040-long-range-forecast/2040-long-range-forecast.pdf

Ramesh, A., Sharma, S., Sharma, M., Yadav, N., & Joshi, O. 2014. Inoculation of zinc solubilizing Bacillus aryabhattai strains for improved growth, mobilization and biofortification of zinc in soybean and wheat cultivated in Vertisols of central India. Applied Soil Ecology. 73. 87–96. 10.1016/j.apsoil.2013.08.009.

Rauscher, F. 2015. Combining Organic & Synthetic Nutrients. Retrieved April 10, 2016 from https://www.maximumyield.com/combining-organic-synthetic-nutrients/2/1456

Reddy, R. 2011. Cho's Global Natural Farming. Retrieved April 10, 2016 from https:// ilcasia.files.wordpress.com/2012/02/chos-global-natural-farming-sarra.pdf

Scholberg, J., B.L. McNeal, K.J. Boote, J.W. Jones, S.J. Locascio, S.M. Olson. 2000 Nitrogen stress effects on growth and nitrogen accumulation by field-grown tomato. Agron. J., 92, pp. 159-167

Sharma, S. B., Sayyed, R. Z., Trivedi, M. H., & Gobi, T. A. (2013). Phosphate solubilizing microbes: sustainable approach for managing phosphorus deficiency in agricultural soils. SpringerPlus, 2, 587. http://doi.org/10.1186/2193-1801-2-587

Singh, M., 2009. Influence of Triacontanol, nitrogen and phosphorus on the growth, yield and quality of ginger (Zingiber officinale Rosc.) and Turmeric (Curcuma longa L.). Ph.D. Thesis, Aligarh Muslim University, Aligarh, India.

Sebilo, M., Mayer, B., Nicolardot, B., Pinay, G., and Mariotti, A. 2013. Long-term fate of nitrate fertilizer in agricultural soils. Retrieved on February 3, 2017 from http://www.pnas.org/citmgr?gca=pnas%3B110%2F45%2F18185

Smith SE, Read DJ., 2008. Mycorrhizal Symbiosis. Third edition. Academic Press, Amsterdam

Wang, K-H., DuPonte, M., and Chang, K. December 2012. Use of Korean Natural Farming for Vegetable Crop Production in Hawai'i. Hanai'Al/The Food Provider. Retrieved September 1, 2015, from http://www.ctahr.hawaii.edu/sustainag/news/articles/V14-Wang-KNF.pdf

Wang, K.-H., and K. Chang. 2012. How to prepare Korean Natural Farming Materials. https://cms.ctahr.hawaii.edu/wangkh/Research-and-Extension/Korean-Natural-Farming.

Xie, G.H., Su, B.L., and Cui, Z.J. 1998. Isolation and identification of N2-fixing strains of Bacillus in rice rhizosphere of the Yangtze River valley. Acta Microbiol Sin 38, 480–483.

2.7 Appendix

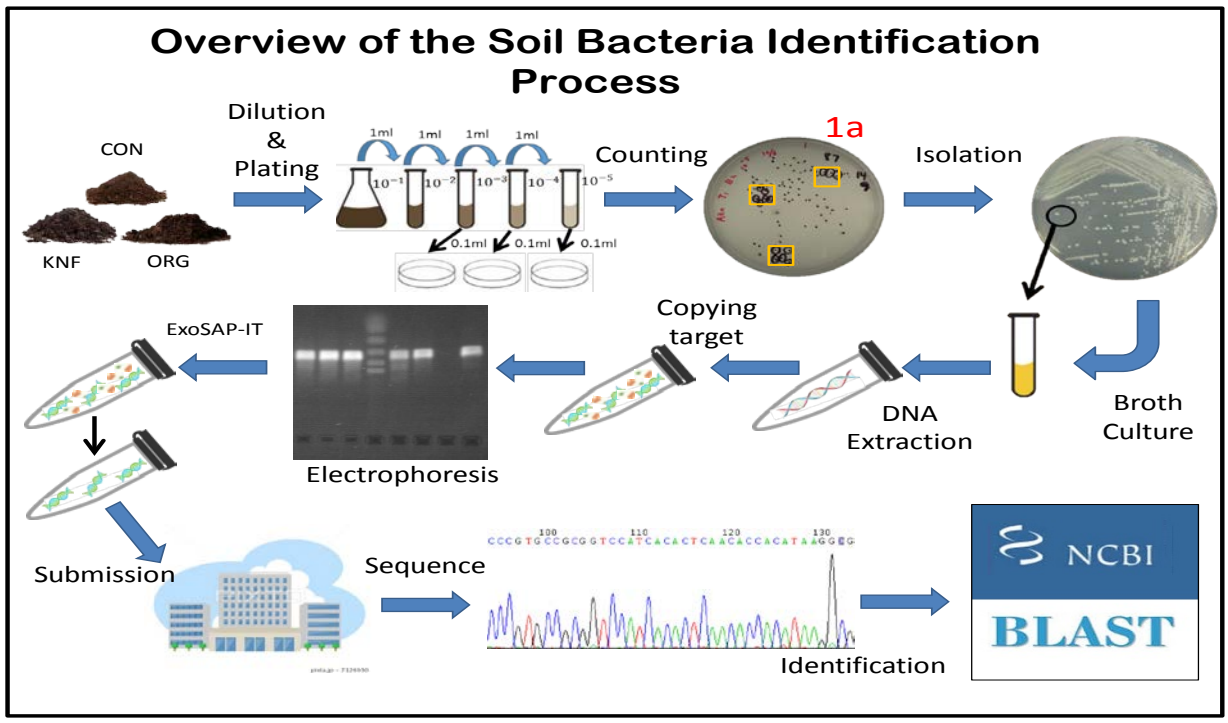



**Figure 1** This is a flow chart showing an overview of the soil bacteria identification process: 1) sample collection, 2) serial dilution and plating, 3) plate count to determine CFU, 4) isolation of selected bacteria to obtain a pure isolate, 5) DNA extraction, and 6) sequencing and identification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

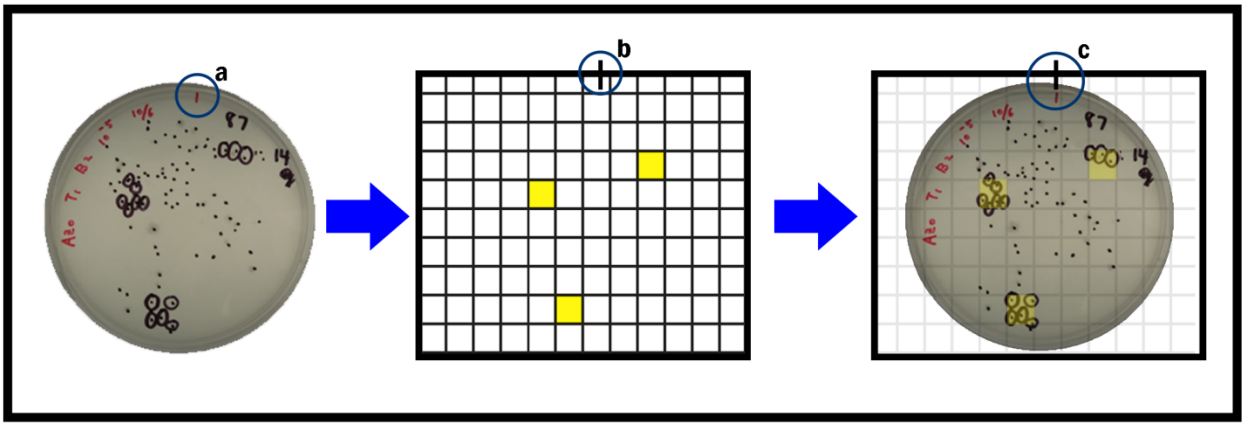



**Figure 1a** In Experiment II, Kula Trial, the underside of each plate was pre-marked (a) with a red line prior to plating/culturing of soil samples. A piece of transparency film containing 1 cm x 1cm squares and a black mark (b) was constructed. Three squares located within the film were selected at 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.

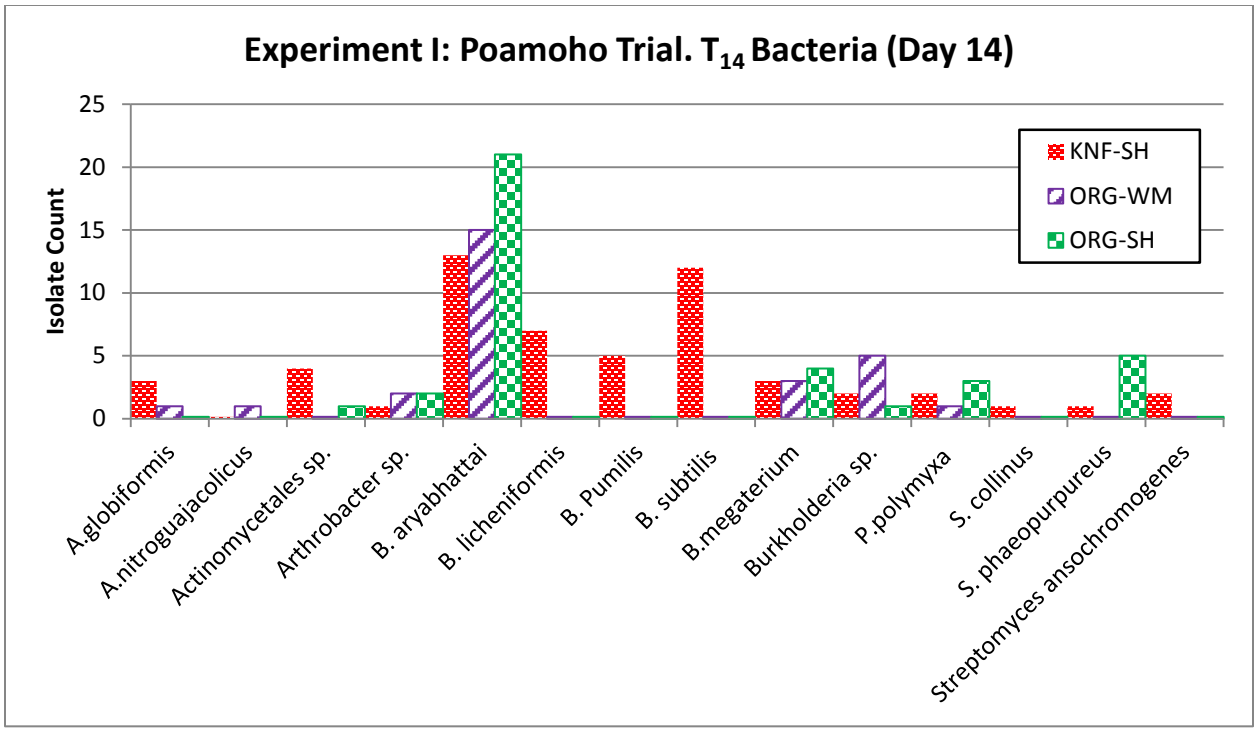



**Figure 2.** Experiment I, Poamoho Trial. Identification and count of isolated bacteria post treatment on day 14, $T_{14}$

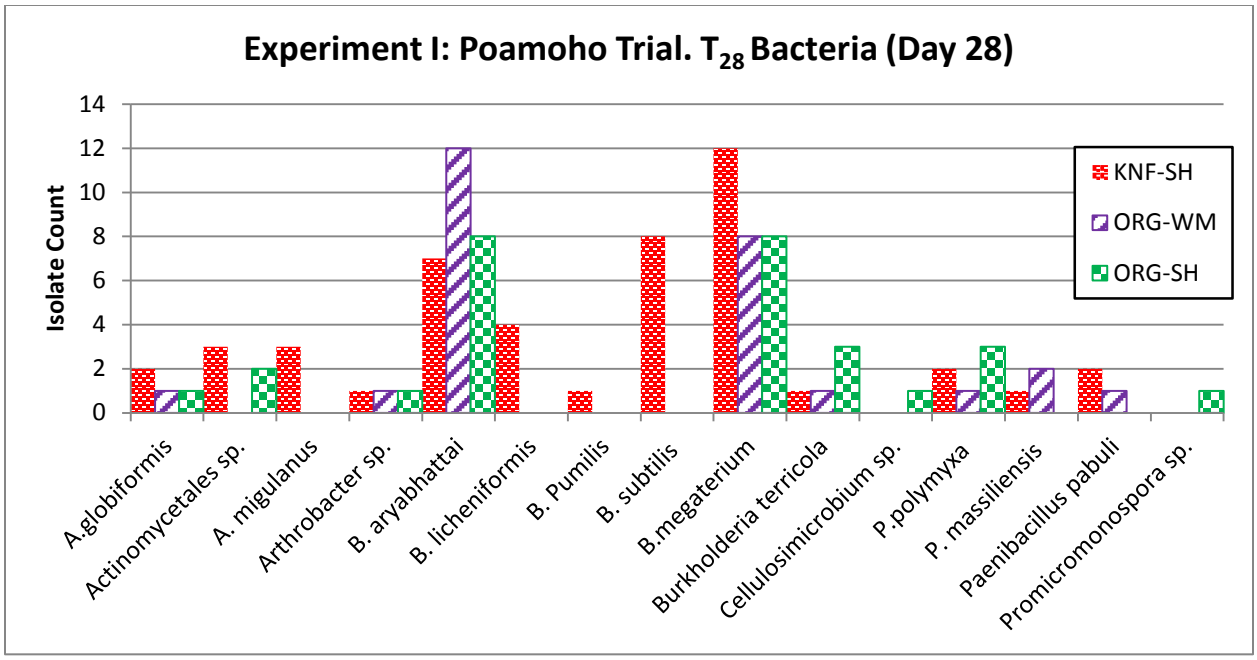



**Figure 3.** Experiment I, Poamoho Trial. Identification and count of isolated bacteria post treatment on day 28, $T_{28}$

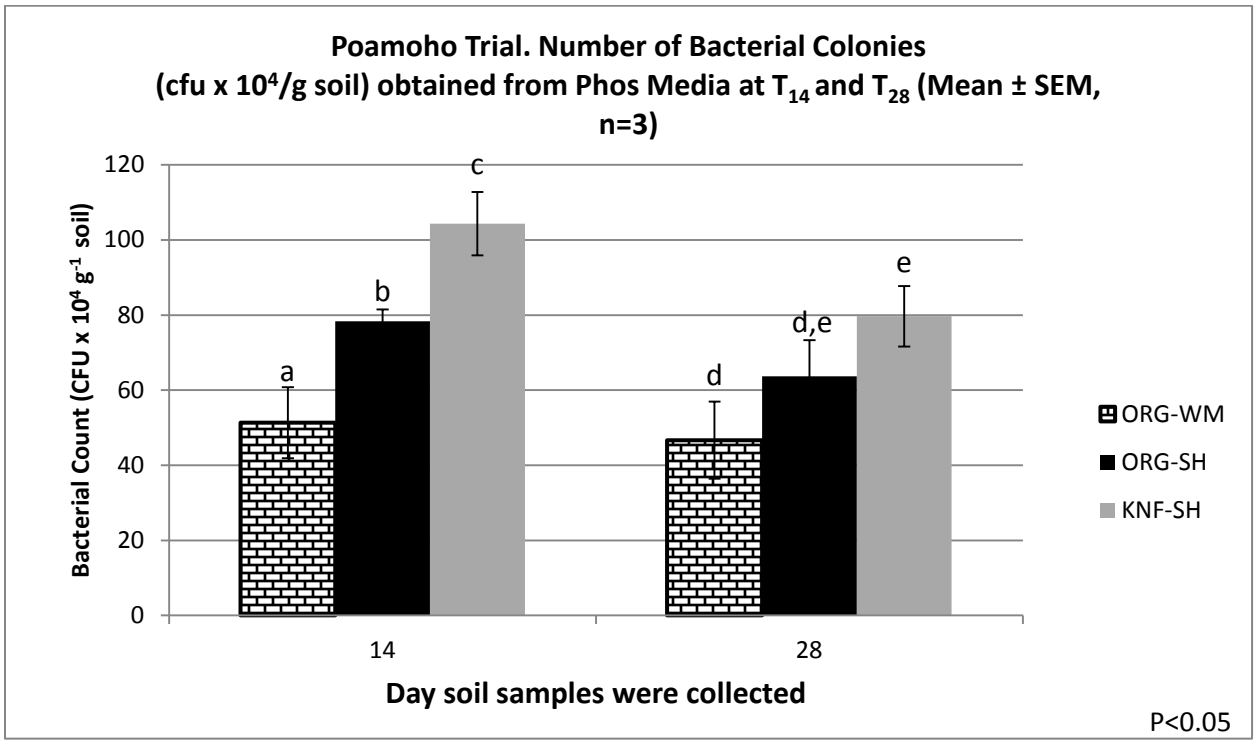



**Figure 4.** Experiment I, Poamoho Trial. Bacterial population (cfu/g) over a period of 28 days. Data represents the bacterial colonies cultured on phosphorus-solubilizing media. Lowercase alphabet indicates a significant difference at $P<0.05$ with each time of collection. The KNF-SH soil samples collected on day 14 ($104.3 \times 10^4 \pm 8.4$ CFU/g) and 28 ($79.6 \times 10^4 \pm 8.1$ CFU/g) contained significantly higher bacterial counts than the ORG-WM samples for each time of collection.

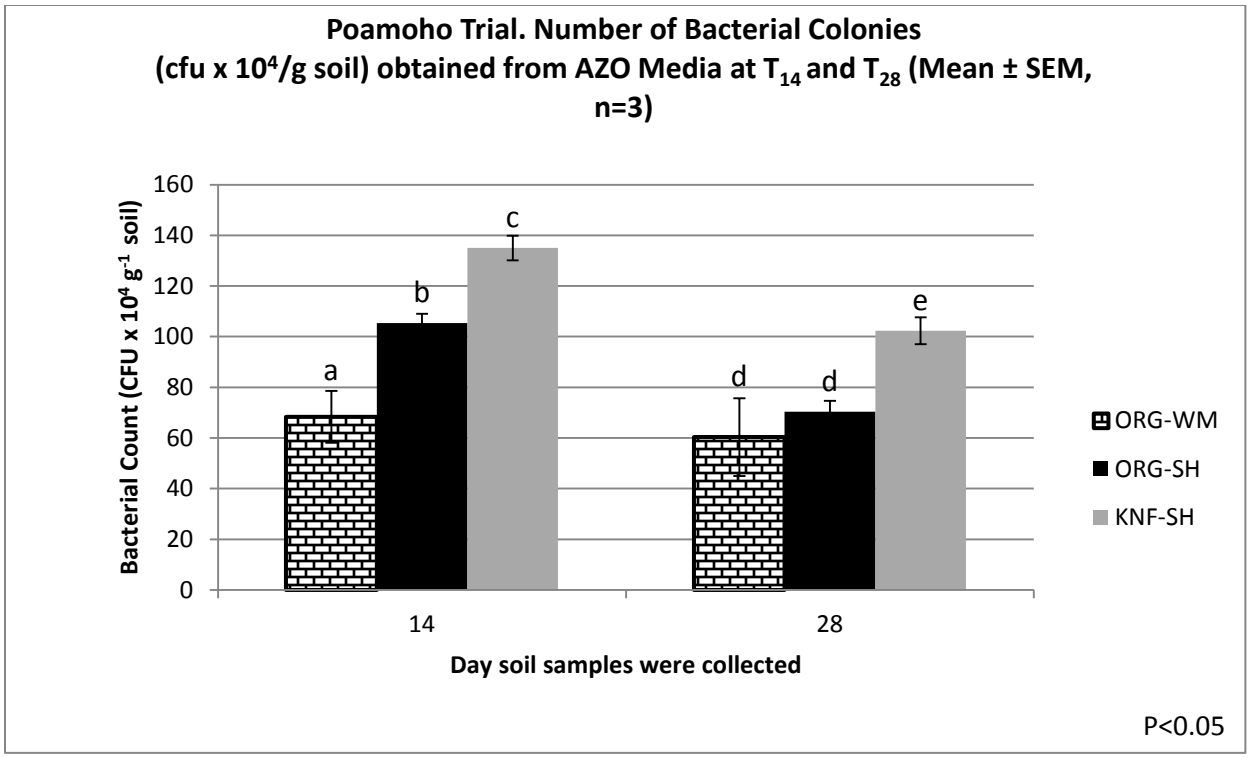



**Figure 5.** Experiment I, Poamoho Trial. Bacterial population (cfu/g) on day 14 and day 28. Data represents the bacterial colonies cultured on Azospirillum media. Lowercase alphabet indicates a significant difference at P<0.05 with each time of collection. The KNF-SH soil samples collected on day 14 (135 x $10^4$ ± 10.26 CFU/g) and 28 (102 x $10^4$ ± 15.3 CFU/g) contained significantly higher bacterial counts than the ORG-WM and ORG-SH samples for each time of collection.

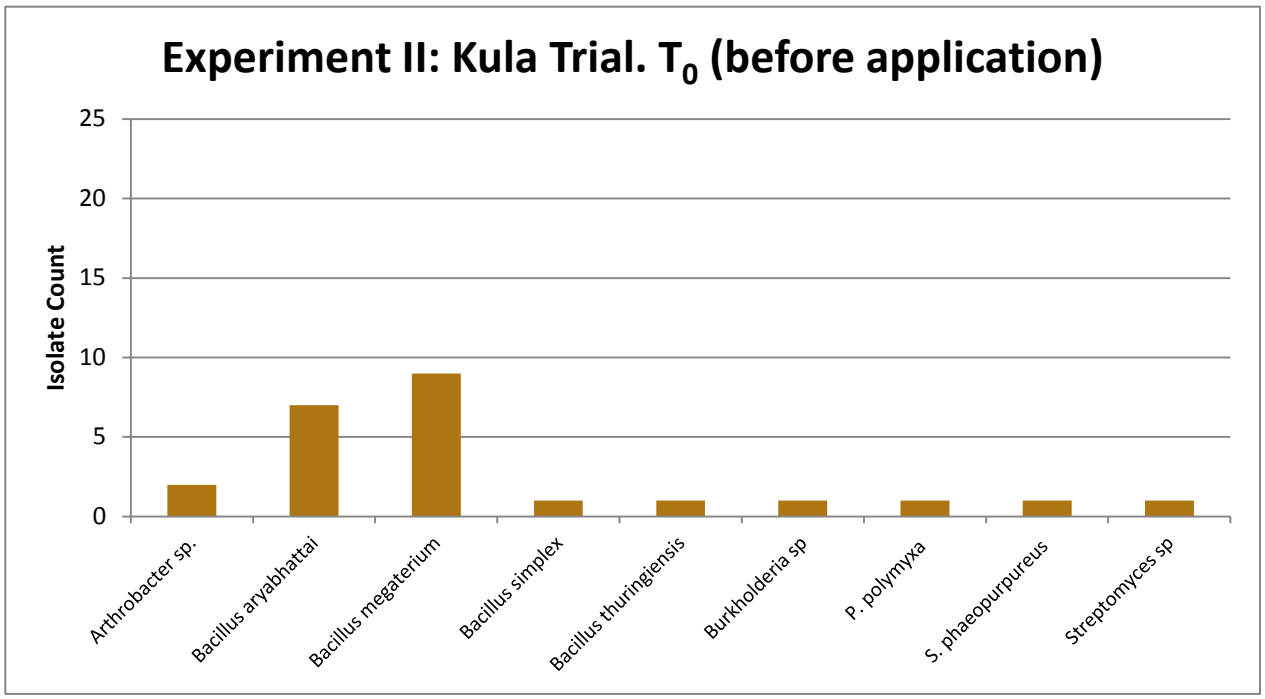



**Figure 6.** Experiment II, Kula Trial. Identification and count of isolated bacteria prior to treatment, $T_0$

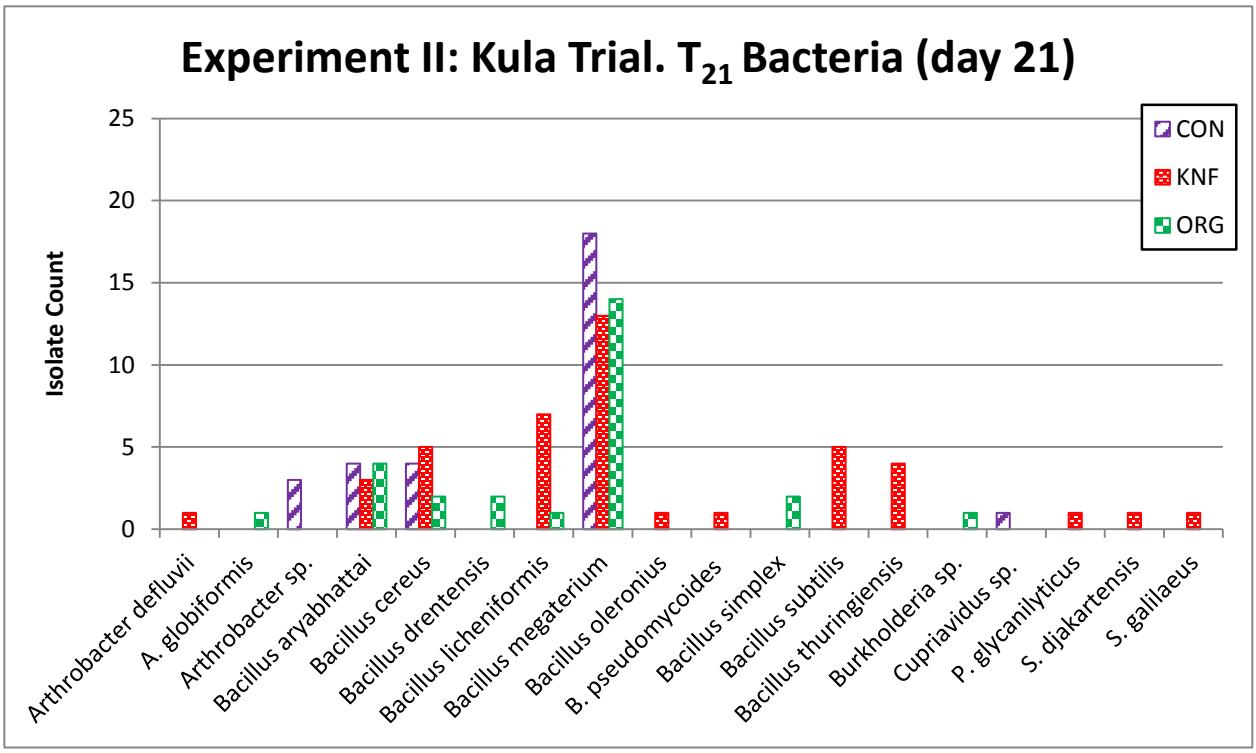



**Figure 7.** Experiment II, Kula Trial. Identification and count of isolated bacteria post application on day 21, $T_{21}$

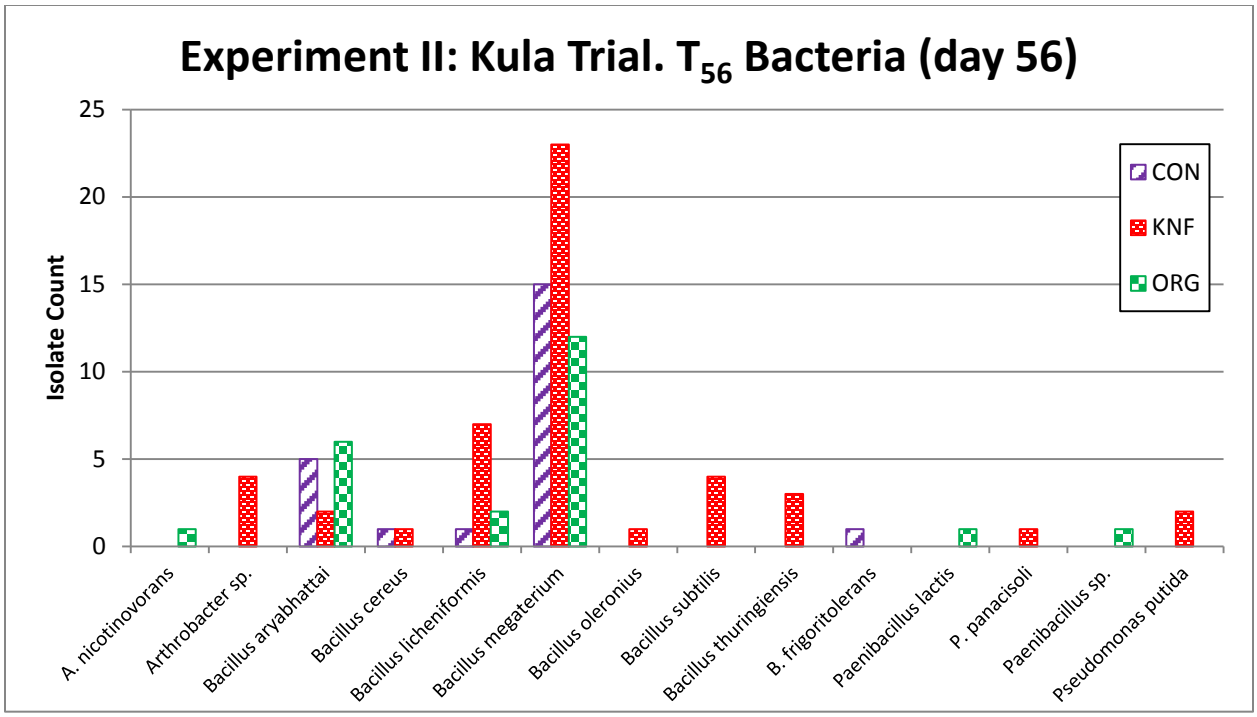



**Figure 8.** Experiment II, Kula Trial. Identification and count of isolated bacteria post application on day 56, $T_{56}$

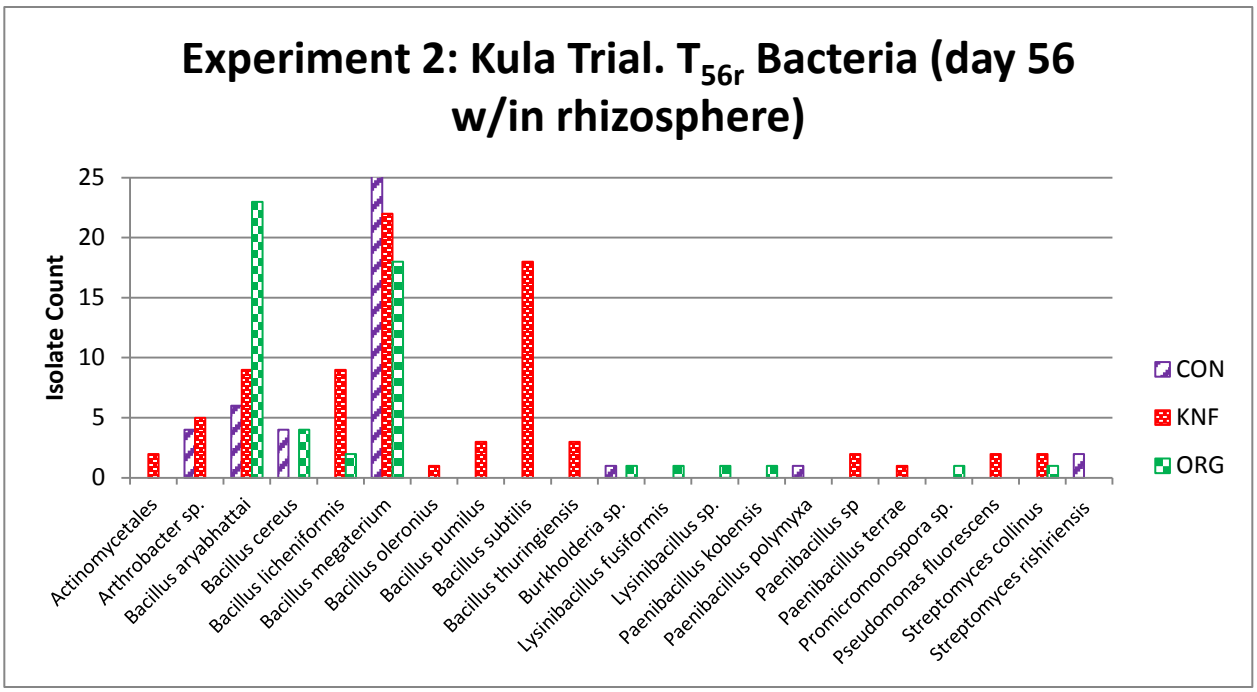



**Figure 9.** Experiment II, Kula Trial. Identification and count of isolated bacteria post-harvest on day 56, $T_{56r}$ (within the rhizosphere)

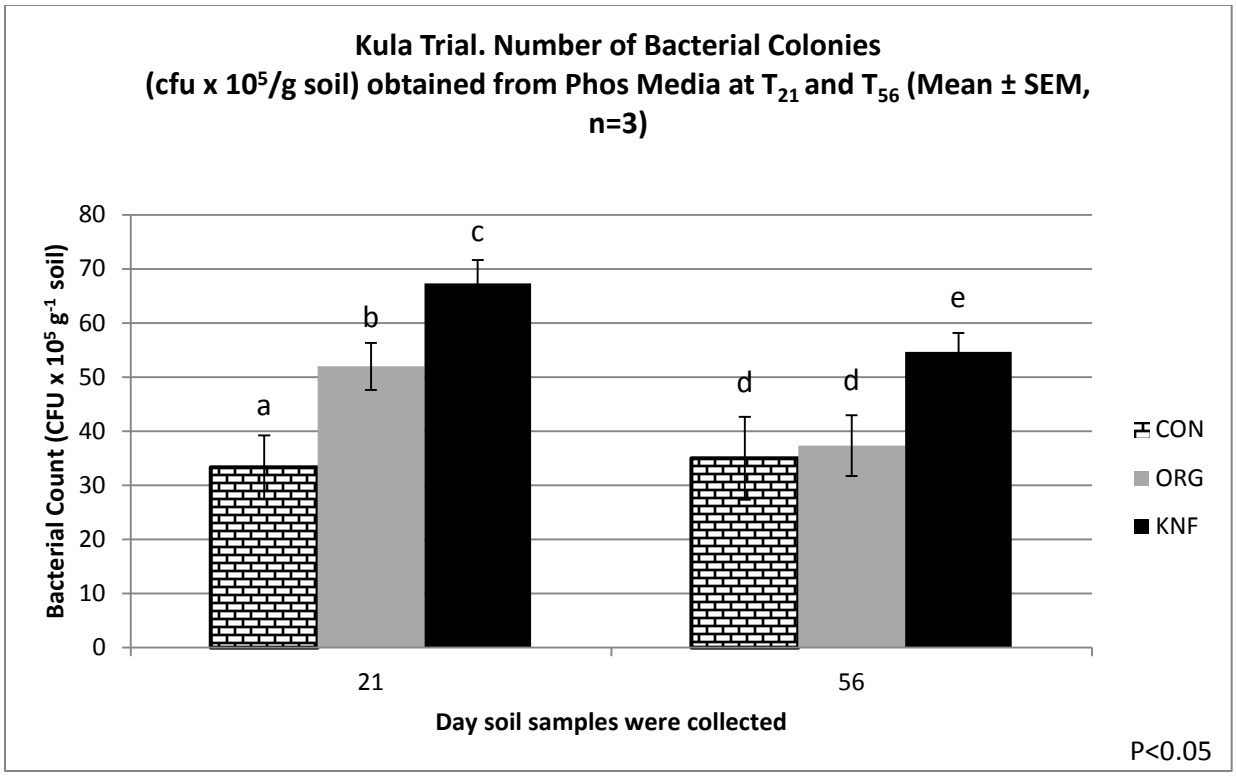



**Figure 10**. Experiment II, Kula Trial. Bacterial population (cfu/g) on day 21 and 56. Data represents the bacterial colonies cultured on phosphorus-solubilizing media. Lowercase alphabet indicates a significant difference at P<0.05 with each time of collection. The KNF soil samples collected on day 21 ($67.3 \times 10^5 \pm 4.33$ CFU/g) and 56 ($54.6 \times 10^5 \pm 3.48$ CFU/g) contained significantly higher bacterial counts than the ORG and CON samples for each time of collection.

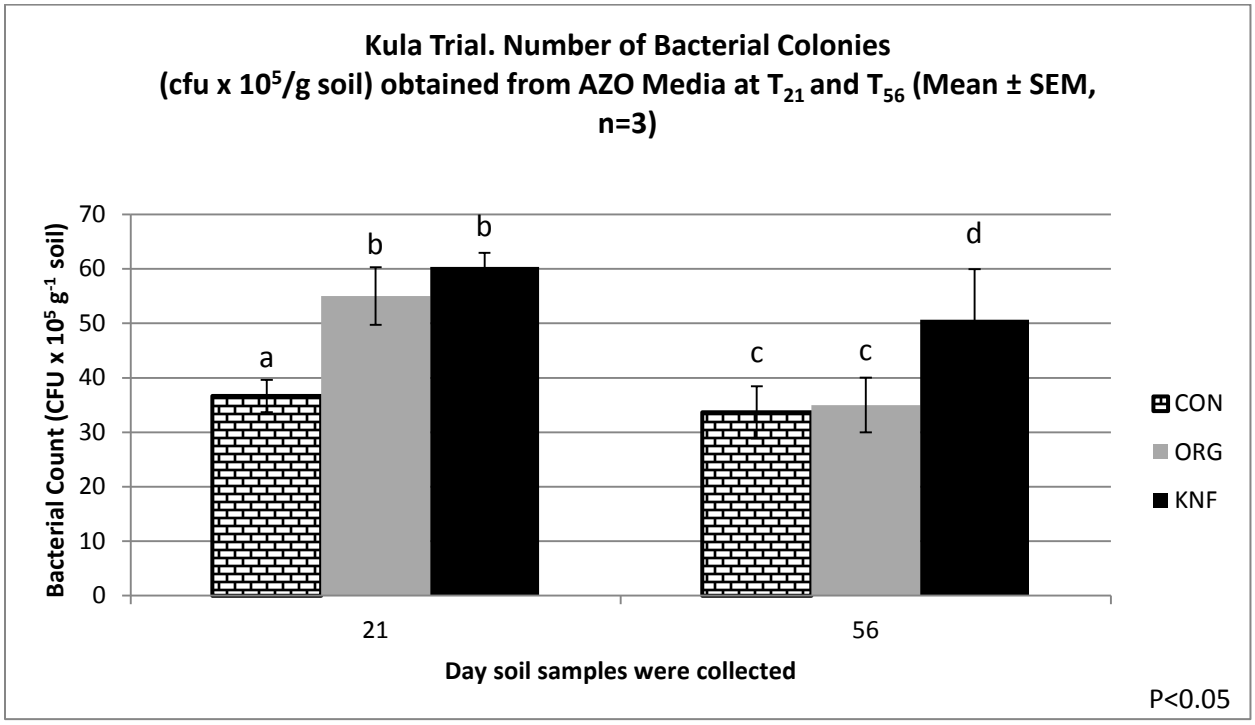



**Figure 11.** Experiment II, Kula Trial. Bacterial population (cfu/g) on day 21 and 56. Data represents the bacterial colonies cultured on azospirillum (AZO) media. Lowercase alphabet indicates a significant difference at P<0.05 with each time of collection. The KNF soil samples collected on day 56 (50.6 x $10^5$ ± 9.27 CFU/g) contained significantly higher bacterial counts than the ORG and CON samples. There was no significant difference between the CON (36.6 x $10^5$ ± 2.96 CFU/g) and KNF (60.3 x $10^5$ ± 2.6 CFU/g) samples collected on day 21.

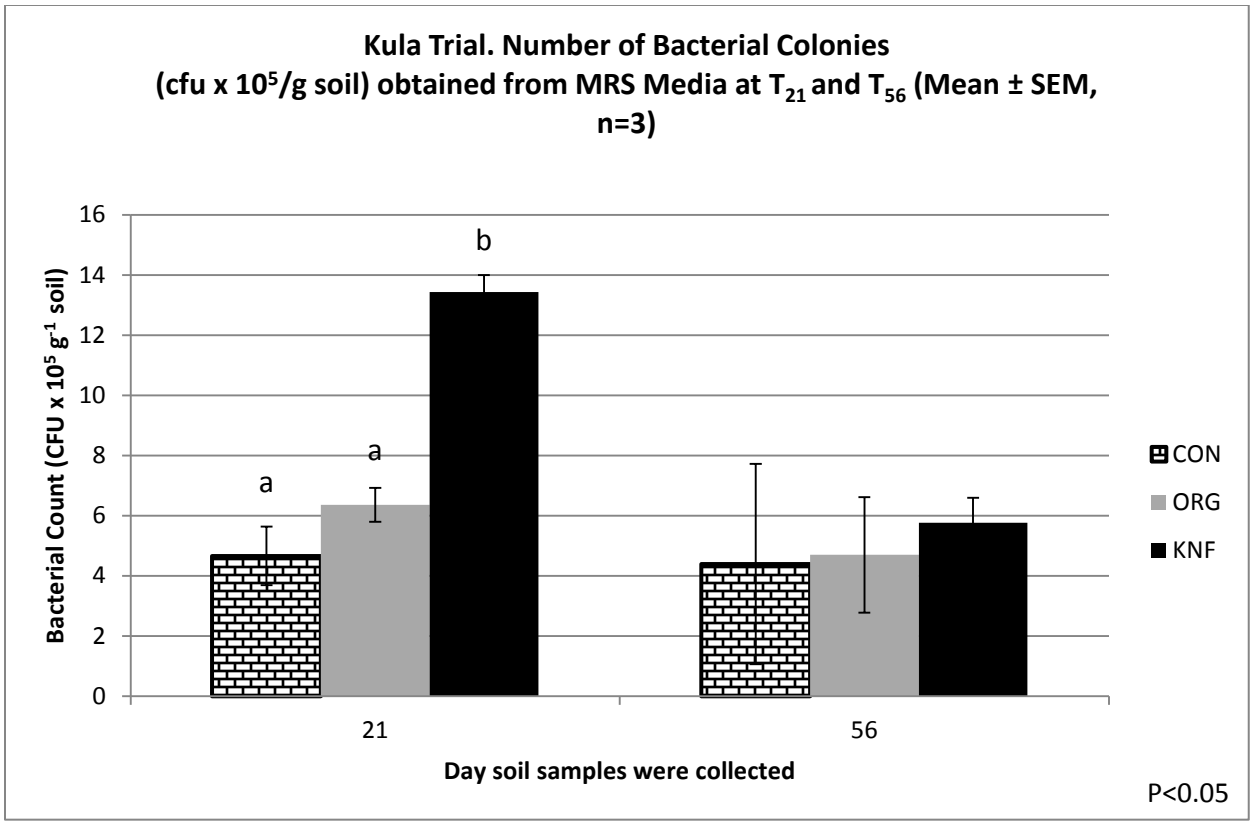



**Figure 12.** Experiment II, Kula Trial. Bacterial population (cfu/g) on day 21 and 56. Data represents the bacterial colonies cultured on MRS media. Lowercase alphabet indicates a significant difference at P<0.05 with each time of collection. The KNF soil samples collected on day 21 (13.4 x $10^5$ ± 0.57 CFU/g) contained significantly higher bacterial counts than the ORG (6.36 x $10^5$ ± 0.56 CFU/g) and CON (4.67 x $10^5$ ± 0.97 CFU/g) samples. There was no significant difference between the CON (4.4 x $10^5$ ± 3.3 CFU/g), ORG (4.7 x $10^5$ ± 1.92 CFU/g), and KNF (5.7 x $10^5$ ± 0.83 CFU/g) samples collected on day 56.

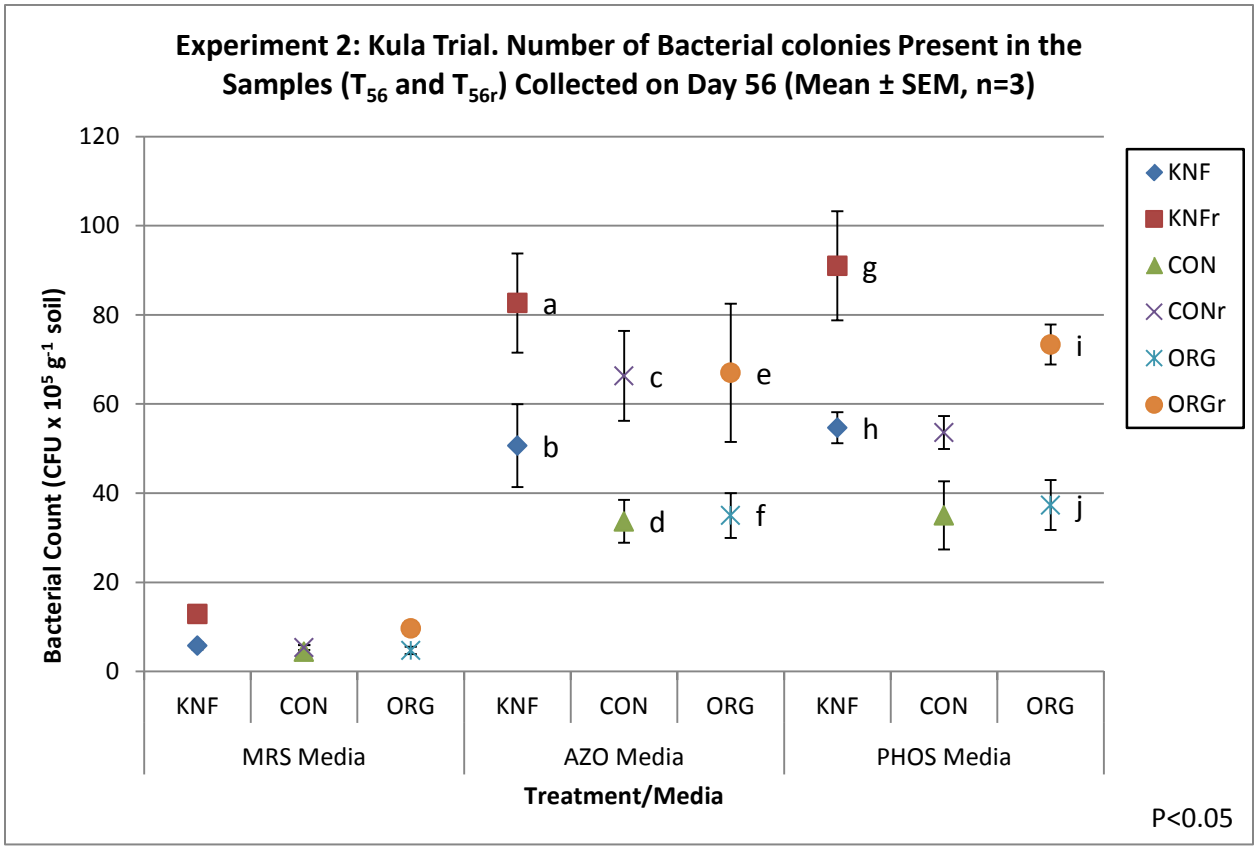



**Figure 13.** Experiment II, Kula Trial. The graph shows a comparison between the bacterial population present in the soil collected in-between plants and the soil collected in within the rhizosphere, for each farming system/media.

# Chapter 3

# Natural Farming: The development of indigenous microorganisms using Korean natural farming methods

## 3.1 Abstract

Korean natural farming is a self-sufficient farming system that involves the culturing of indigenous microorganisms (IMO) – bacteria, fungi, nematodes, and protozoa in place of inorganic fertilizers to produce fertile soil. The culturing and nurturing of indigenous microorganisms is discussed. The materials, methods, and collection site used to culture IMO may vary from farm to farm due in part to locality and availability of materials. However, the overall concept of culturing IMO remains the same. Following the guidelines documented in Cho's 'Natural Farming', this chapter outlined the protocol, the materials and methods used to culture IMO on a 5-acre farm located in Makaha, Hawaii (2010). In addition, an attempt to quantify the related costs was made to provide some guidance for interested parties.

## 3.2 Introduction

Soil plays a vital role in our ecosystem; without it, life for many multicellular organisms would cease to exist. In addition, food shortages are driven by soil degradation, as poor farming practices lead to loss of nutrients through erosion and leaching (Tilman *et al*., 2002). To maximize crop yield, it is imperative that farmers maintain a healthy environment for plants to grow as the quality of the soil can change the outcome of the harvest. The most common way to replenish the soil is by adding fertilizer to it. However, the maintenance of fertile soil does not come cheap. Fertilizers and other soil additives can be expensive, especially in Hawai'i due to the higher costs of shipping. It is vital that Hawaii's farmers minimize costs wherever possible. Korean natural farming (KNF) has been proposed as an attractive alternative for farmers who wish to become less dependent on external inputs without sacrificing crop yield (Hoon and Park 2010).

KNF is a sustainable farming system developed by a practitioner, Master Han Kyu Cho from Janong Natural Farming Institute in South Korea. It has been practiced for over 40 years throughout Asia. KNF is a self-sufficient system that involves culturing indigenous microorganisms (IMO) – fungi, bacteria, and protozoa – and reintroducing them into nutrient depleted soil, thus enhancing soil microbial activity and fertility (Essoyan, 2011). There is a symbiotic relationship that occurs between plants and beneficial

IMO's; the microorganisms convert nutrients into a form that the plant is able to absorb. In turn, the plants provide food to these microorganisms. This type of closed-loop farming system maximizes the use of on-farm resources and recycles farm waste while at the same time minimizing external inputs. KNF is a multifaceted system that adapts to local conditions (Cho, 2010). Introduced to Hawai'i in 1999, KNF is gaining popularity among farmers in Hawai'i who are interested in sustainable agriculture (Wang *et al*., 2012). Farmers who practice this farming method believe that it minimizes their dependence on inorganic fertilizers and pesticides. However, little scientific documentation of the benefits of these practices exists (Park and Duponte, 2010).

### 3.3 Objectives

The objectives for this study were to : a) identify some of the resources readily available on a farm site that can be used for the development of IMO, b) demonstrate the chronicle development of the IMO, and c) provide some estimates of cost and time for the process of developing the IMO. This publication outlines the steps used to collect IMO from a farm in Makaha, Hawai'i. For a detailed guide on how to collect and cultivate indigenous microorganisms using traditional methods, refer to Cho's book titled "Natural Farming" (2010).

### 3.4 Materials and Methods

KNF involves a 4 step process of capturing, cultivating, and preserving indigenous microorganisms (IMO). These 4 steps are often referred to as IMO 1, IMO 2, IMO 3, and IMO 4. As previously mentioned, the process of collecting and culturing microorganisms may vary farm to farm due to locality and availability of materials and supplies. However, the principle remains the same. The following materials were used for the collection and culturing of IMO's.

- 1 x 1 x 0.5 ft. lauhala box with cover (cedar box, plastic container, ceramic bowl are alternatives)
- 340 g (2 cups) of steamed white rice (preferably on the dry side)
- 20 L bucket (with holes drilled on the sides, refer to Figure 1)
- Small mixing container
- 1 large (4 L) glass jar

- twine
- semipermeable material (paper towel, cloth, canvas)
- brown sugar (570 g)
- 22.68 kg bag of Wheat mill run
- shovel
- mixing container
- 20 L of fish pond water
- measuring spoon (15 ml)
- natural inputs (refer to Table 1)

**3.5 Making IMO 1 – Collecting IMO**

The first step to making IMO is to locate an area to collect the microbes. The preferred site to collect IMO is near the rhizosphere of plants whose roots contain sugar. According to Master Cho and other Korean natural farmer's, Bambusoideae (bamboo) is the ideal choice as their roots contain a high amount of sugar (Cho and Cho, 2010). These sugars attract bacterial-dominated microbes and nematodes. In addition to bamboo, broadleaf trees and leaf molds are other commonly used sites (Cho and Cho, 2010). Three specific sites on the farm were selected to collect microbes from: 1) bamboo, 2) Artocarpus *altilis* (breadfruit tree), and 3) Leucaena *leucocephala* (haole koa tree). Hence, 3 separate batches of IMO were made by utilizing 3 different collection sites. The following procedure outlines the collection and cultivation of microbes from bamboo (Figure 1a).

The lauhala box was filled 1/3 full with hard-cooked rice (use less water to cut back on the amount of moisture). The main goal here is to provide the microbes with a food source. The lauhala cover was placed back onto the box. This allows the microbes to enter while at the same time keeping leaf litter and insects out. The container was then placed close to the root system of the bamboo (Figure 1b) and covered with leaf litter and a bucket containing holes. This acts as a second line of defense to keep rain water and animals away. The container was left undisturbed for 5 days (Cho, 2010).

After 5 days had passed, the container was removed from the site (Figure 1c). A successful collection of microbes would present a white cloudy covering of microbes with little to no presence of red and blue molds (Figure 2a). However, if the rice is covered entirely with a green layer (Figure 2b), anaerobic microbes have accumulated (Cho, 2010). Discard the contents and repeat the process.

**3.6 Making IMO 2 – Cultivation of IMO**

This step involves the cultivation of IMO. Materials needed for this process included a small mixing container, jar (i.e. glass, ceramic, or clay), porous material (i.e. paper towel, Korean paper, and cloth), a piece of twine, and brown sugar. The inoculated rice obtained from the previous process (IMO 1) was placed into a container (Figure 3a) followed by 570 g (1:1 ratio) of brown sugar (Figure 3b). The contents were then mixed for approximately 5 minutes (Figure 3c). Once homogenized, the mixture was placed into a jar (Figure 3d). A piece of canvas was placed over the opening and secured with twine. The jar was placed in a cool shaded (out of direct sunlight) area for 7 days. The product of this process is called foundation stock, or more commonly IMO 2.

**3.7 Making IMO 3 – Multiplying the IMO**

Materials and tools needed to make IMO 3 include 22.68 kg of wheat mill run (or a carbohydrate source), 20 L of fish pond water, a 20 L bucket, natural inputs/nutrient liquids (refer to Table 1), a measuring cup, and 15 ml of IMO #2. The wheat mill run was placed in a large container (i.e. wheel barrel, large bucket, tractor dump bucket, etc.) under a cool shaded area (Figure 4a). 15 ml of IMO #2 was added to 20 L of fish pond water and the mixture was stirred thoroughly (Figure 4b). The inputs from Table 1 were added to this mixture.

The contents within the bucket were poured onto the wheat mill run and mixed thoroughly (Figure 4c). The moisture level of this mixture should be between 65-75 % (Reddy, 2011). Soil moisture was determined using the 'feel and appearance method' (Klocke, Norman L., P. E. Fischbach, 1998). The wheat mill run mixture was then placed directly onto the soil (13.7-15.7 in. high) in an area partially shaded (70% shade and 30% light). It was then covered with leaves and allowed to sit for 8 days (Figure 4d).

As the mixture (IMO 3) fermented, the core temperature of the pile increased - microbes present within/near the core may die as a result. To prevent this from occurring, the heat must be evenly distributed throughout the pile. Thus, the pile was turned over every two days to bring down the temperature and to mix the microbes. Visibility of white fungi (mycorrhizae) covering the top layer of the IMO is a sign of successful inoculation. At the end of this process, IMO 3 will have formed into clumps (Figure 5a).

### 3.8 Making IMO 4 – Inoculating the Soil

IMO 4 is the final step to making IMO. Materials needed for this process include IMO 3, a large bucket, soil (from the farm), and biochar. In a bucket, break up the IMO 3 (entire inoculated pile of wheat mill run) so that there are no large pieces (Figure 5b). Mix in 10 L of biochar (Figure 5c). Next, pour the contents out of the bucket and mix with soil in a 1:1 ratio. The fermentation process is similar to that of IMO 3 (moisture 65-70%, 13.8-15.7 in. high, temperature 40-50° C). The pile was then covered with leaf litter and allowed to sit for another 5 days. IMO is much more effective when inoculated to the soil (Cho, 2010). The end product of this process is referred to as IMO 4.

### 3.9 Application of IMO

The final product, IMO 4, should be used as a top dressing. Gently mix 150 kg/0.1 ha (minimum) into the top soil. For optimal results, cover the soil with mulch (i.e. bamboo leaf litter, wood chips, etc.). Adding mulch is very effective as it retains moisture, keeps weeds at bay, and also provides the microbes with protection from direct sunlight. It is recommended that IMO 4 be applied to the soil 7 days before seeding or transplanting and 2 to 3 hours prior to sunset (Cho, 2010). Treating the soil late afternoon gives the microbes more time to adjust to the environmental changes, particularly the increase in temperature.

### 3.10 Discussion

This experiment was designed to cultivate IMO using the equipment and materials available on the farm. The lauhala basket used to make IMO 1 was made with the leaves from the hala tree located on

the property. The traditional method recommends the use of a cedar box. However, any type of container (i.e. lauhala basket, plastic container, ceramic bowl, etc.) will do. In regards to cultivating IMO 1, never attempt to collect IMO during rainy seasons as too much moisture promotes the growth of pathogenic microbes (Cho, 2010). Additionally, the time it takes to cultivate IMO 1 is highly dependent on the weather. The collection process takes 4-5 days in cool weather (~20 °C) or 3-5 days under warmer (>20 °C) conditions (Park and DuPonte, 2010).

In reference to the propagation of IMO 3, 22.68 kg of wheat mill run was used as a carbohydrate source for the microbes. As an alternative, macadamia nut shells (preferably ground), spent grains from brewery's, wood chips, rice bran, ulu (greadfruit), or kalo (taro) skins may be used in place of wheat mill run. The type of nutrient inputs added is entirely dependent on availability. However, no studies have been done to compare the efficacy of the source of carbohydrates used. Natural inputs are important as they enhance plant growth and IMO proliferation. Eventually, the nutrients in the soil will diminish. When that occurs, more nutrients will need to be added to the soil in order for both plants and microbes to flourish. KNF relies on the use of bio-organic fertilizers such as calcium from egg shells, nitrogen from fish waste, or potassium from the tips of healthy leaves (fermented fruit juice). For this study, a nutrient analysis was not performed on each of the inputs used in the production of IMO. However, Gaghirang (2011) provided a proximate analysis of some of the natural inputs used to make IMO (Table 2).

Whether a subsistence or commercial farmer, self-sufficiency, labor intensity, and cost are just a few factors farmers take into account when selecting a farming method that best suits their needs. Korean natural farming is a great alternative for farmers looking to become self-sufficient and less dependent on external inputs such as inorganic pesticides and fertilizers. KNF is environmentally friendly in that it possesses a smaller carbon footprint when compared to conventional farming methods. In some cases, farmers are able to obtain all the materials and equipment needed to cultivate IMO directly from their farms or source them locally. Synthetic fertilizers on the other hand, such as Gaviota 16-16-16 (distributed by BEI Hawaii), are imported to the Hawaiian Islands (http://www.beihawaii.com/company_info.html). However, these inorganic fertilizers require no

preparation and can be applied to the soil at any time whereas IMO needs to be cultivated before application. Hence, additional labor is required to make IMO.

Not including the length of time needed to prepare natural inputs, it takes approximately 25 days to make IMO 1 through IMO 4 (Table 3). Repeating the process takes roughly 11-15 days. The 340 g of rice and 570 g of brown sugar used in this experiment made well over 700 ml of IMO 2. Only 15 ml of IMO 2 was used per batch of IMO. Any unused portion of IMO 2 can be stored between 1-15°C and reused at a later day (Cho, 2010). The jar should remain well ventilated (semi-permeable lid/cover) and be monitored over time for presence of bubbles in the medium means the microbial population has declining.

The overall cost associated with the cultivation and propagation of IMO is quite comparable to the cost of inorganic fertilizers (Table 4). All the materials and equipment needed for this experiment were found on site except for three items – 1) Calrose rice, C&H Golden Brown Sugar, and a 22.68 kg of Wheat mill run ($41.76, Paakea Feed and Farm). The calculations showed that it costs roughly $44.31 to make ~45 kg of IMO 4. At an application rate of 150 kg/0.1 ha (150 g/$m^2$), IMO 4 costs $0.14/$m^2$ for the initial batch and only $0.13/$m^2$ thereafter (using the foundation stock, IMO 2). In comparison, a 9 kg bag of Gaviota 16-16-16 costs $23.82. At a recommended application rate of 49 g/$m^2$, it costs $0.13/$m^2$. The cost difference between the two is very minute. However, this estimated cost does not take into account the additional 3 labor hours required to make the IMO. Finding a cheaper alternative (carbohydrate source) could substantially reduce the overall cost.

In conclusion, the cultivation and propagation of IMO may be somewhat labor intensive. The cost for the IMO is analogous to synthetic fertilizers available on the market, when labor cost is excluded. The cost to make IMO can be significantly reduced if all the materials and equipment needed to cultivate and propagate IMO can be sourced directly on the farm.

### 3.11 References


Cho, H.-K., and J.-Y. Cho. 2010. Natural Farming. Han-Kyu Cho. CGNF. Seoul, S. Korea

Cho, J.-Y. 2010. Natural Farming; Agricultural Materials. Han-Kyu Cho. CGNF. Seoul, S. Korea. www.janonglove.com

Essoyan, Susan. 2011. Natural selection: A self-sufficient system of farming is increasing yields across Hawaii. Star Advertiser. Retrieved October 1, 2017; http://www.staradvertiser.com/news/20110118_Natural_selection.html?id=114074644

Klocke, Norman L., P. E. Fischbach. 1998. Estimating Soil Moisture by Appearance and Feel, Publication G84-690-A, Nebraska Cooperative Extension Service.

Maghirang, Rodel G. 2011. Organic Fertilizers from Farm Waste Adopted by Farmers in the Philippines. Institute of Plant Breeding-Crop Science Cluster. Retrieved January 10, 2017; http://www.fftc.agnet.org/library.php?func=view&id=20110804162833

Reddy, Rohini. 2011. Cho's Global Natural Farming. Retrieved April 10, 2016; https://ilcasia.files.wordpress.com/2012/02/chos-global-natural-farming-sarra.pdf

Wang, Koon-Hui, DuPonte M, and Chang K. 2012. Use of Korean Natural Farming for Vegetable Crop Production in Hawai'i. Hanai'Al/The Food Provider. Retrieved April 1, 2017; http://www.ctahr.hawaii.edu/sustainag/news/articles/V14-Wang-KNF.pdf

Tilman, David, Cassman G., Matson P., Naylor R., and Polasky S. 2002. A review article: Agriculture sustainability and intensive production practices. Nature, 418, 671-677. DOI:10.1038/nautre01014

### 3.12 Appendix

**Table 1**. Natural inputs used to make the IMO

| IMO Inputs | | Amount Added per 22.68 kg of wheat mill run |
|---|---|---|
| **L**actic **A**cid **B**acteria | abundant in the whey from yogurt and cheese-making, or it can be cultivated with rice wash water (saved from the first rinse of rice in preparation for cooking) and unpasteurized milk. | 30 ml |
| **O**reintal **H**erbal **N**utrient | Licorice, angelica, ginger, garlic, and cinnamon are the ingredients used in the preparation of OHN. | 30 ml |
| **F**ermented **P**lant **J**uice | FPJ is made by taking the growing tips of healthy plants—whether it's a vegetable, herb, or weed—and mixing it with brown sugar (needs time for fermentation to occur). | 30 ml |
| **F**ish **A**mino **A**cid | a liquid made from fish waste. Similar to Asian fish sauce used in food preparation, but without the added salt, it is made by mixing fish parts with brown sugar and letting it ferment for a few months. | 15 ml |
| **W**ater-soluble **Ca**lcium | made from roasted eggshells soaked in BRV. | 15 ml |
| Seawater | diluted with fresh water to add minerals, for soil treatment before seeding, and to enhance ripening of fruit. | 1 L |
| Biochar | highly porous charcoal. Provides a storehouse for all the nutrients and microbes. | 10 L bucket |

a. collection site (bamboo tree)

b. rice (covered with a bucket) placed close to rhizosphere of a bamboo tree

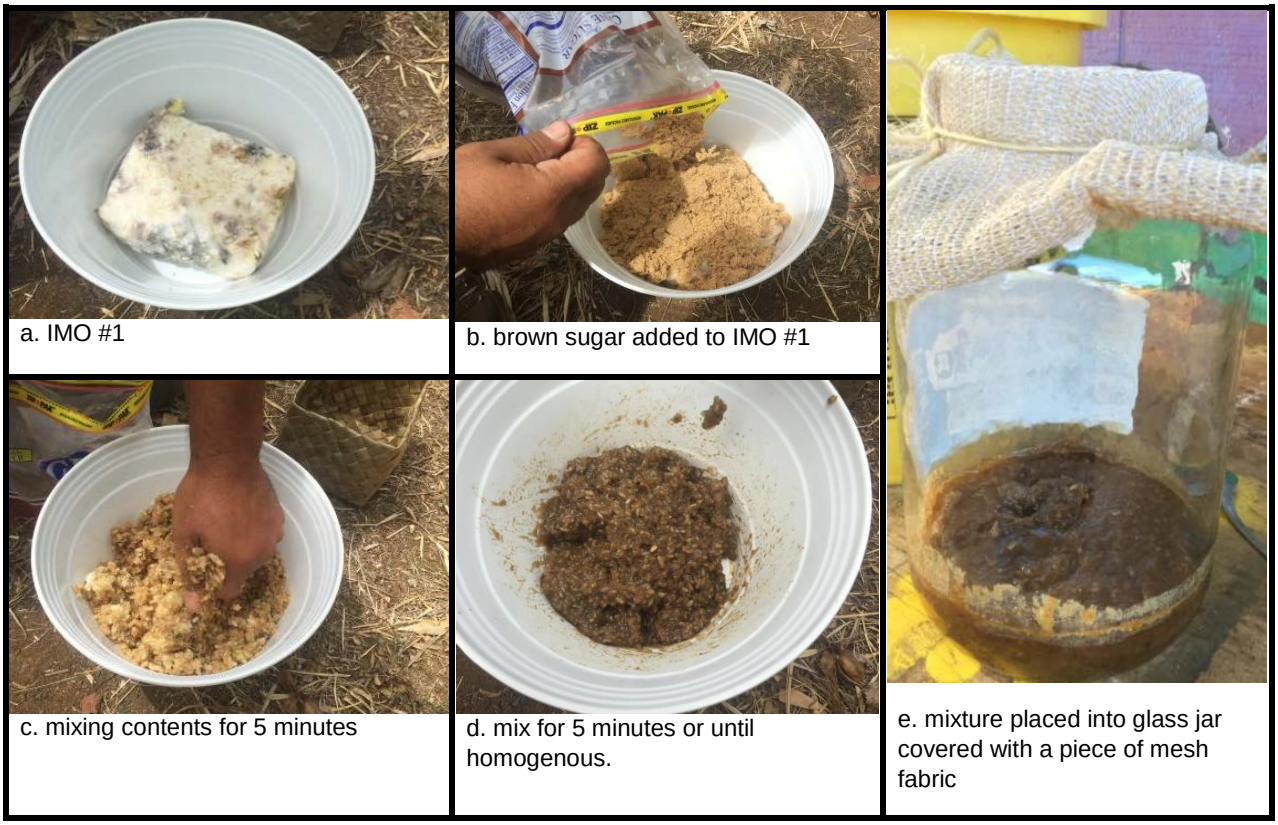
c. removing lauhala basket (containing rice) from collection site 5 days later

**Figure 1.** Collection of IMO 1

a. presence of white mycelium on the surface is representation of a successful collection of microbes

b. presence of a green layer covering the rice represents an unsuccessful collection of microbes.

**Figure 2.** Good collection (A) vs. bad collection (B) of microbes.



a. IMO #1

b. brown sugar added to IMO #1

c. mixing contents for 5 minutes

d. mix for 5 minutes or until homogenous.

e. mixture placed into glass jar covered with a piece of mesh fabric

**Figure 3**. Cultivation of IMO #2

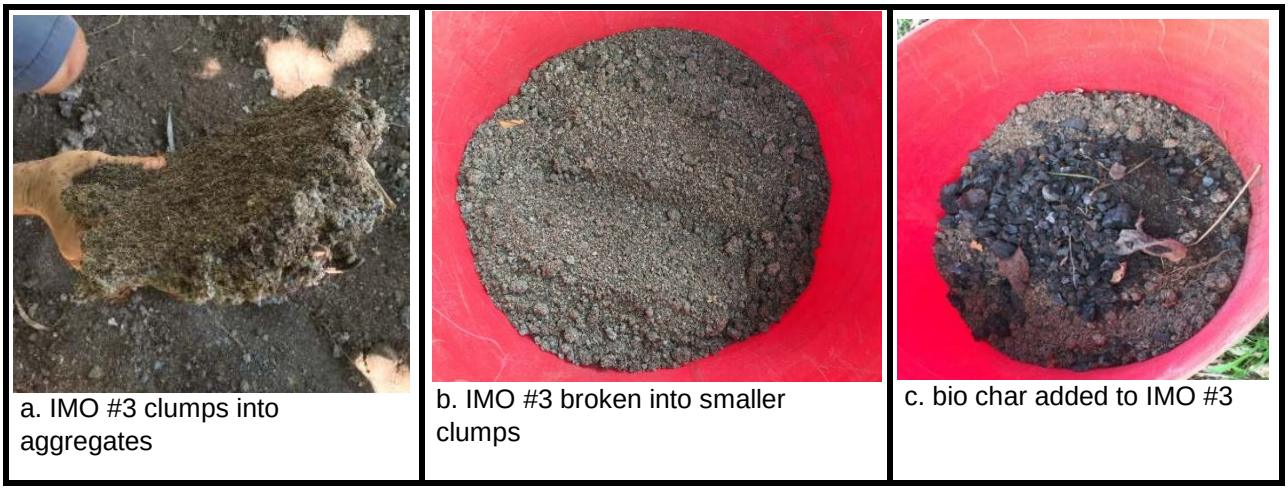
a. wheat mill run (50 lbs.) placed into tractor dump bucket

b. Natural inputs mixed into 20 L of fish pond water

c. pond water mixture added to wheat mill run and mixed till homogenous.

d. wheat mill run placed onto the soil and covered with leaves held in place with rocks.

**Figure 4.** Cultivation of IMO #3



a. IMO #3 clumps into aggregates

b. IMO #3 broken into smaller clumps

c. bio char added to IMO #3

**Figure 5**. Cultivation of IMO #4

**Table 2.** Proximate Analysis of Fermented Inputs (Maghirang, 2011)

| **Nutrient (mg/kg)** | **Fermented Fruit Juice** | **Fermented Plant Juice** | **Fish Amino Acids** | **Oriental Herbal Nutrient** |
|---|---|---|---|---|
| Nitrogen (N) | 429.47 | 855.06 | 1166.34 | 405.16 |
| Phosphorus (P) | 61.87 | 122.72 | 193.44 | 74.84 |
| Potassium (K) | 12017 | 3934.2 | 314.6 | 522.3 |
| Calcium (Ca) | 307.23 | 913.03 | 377.92 | 181.03 |
| Magnesium (Mg) | 119.55 | 333.64 | 80.58 | 111.58 |
| Sodium (Na) | 51.15 | 128.19 | 426.4 | 78.58 |
| Iron (Fe) | 15.07 | 52.24 | 19.73 | 87.19 |
| Copper (Cu) | 0.75 | 0.87 | 0.94 | 0.81 |
| Manganese (Mn) | 2.19 | 4.54 | 1.45 | 4.13 |
| Zinc (Zn) | 1.97 | 3.74 | 5.84 | 2.04 |

**Table 3**. Ingredients and timeline for developing Indigenous Micro-organisms (IMO).

| | Ingredients | Culturing/propagation period (days) | |
|---|---|---|---|
| | | Initial process | Additional process (using IMO 2 foundation stock) |
| IMO #1 | - 340 g of rice | 4-5 | n/a |
| IMO #2* | - 570 g brown sugar | 7 | n/a |
| IMO #3 | - 22.68 kg of wheat mill run<br>- 20 L of pond water<br>- natural inputs*<br>- 15 ml of IMO #2 | 7-10 | 7-10 |
| IMO #4 | - 10 L bucket of bio-char | 4-5 | 4-5 |
| Total preparation time | | 22-26 | 11-15 |

* IMO 2 foundation stock can be stored (1-15°C) for future use

* natural inputs added include: lactic acid bacteria, oriental herbal nutrient, fruit plant juice, fermented fruit juice, fish amino acid, and water soluble calcium

**Table 4**. Cost breakdown between IMO and inorganic fertilizer

| Costs Comparison between IMO and conventional fertilizer (inorganic) | | | | | |
|---|---|---|---|---|---|
| | Cost per whole unit purchased | Amount used to make 1 batch | Adjusted Cost | Application Rate | Cost per $m^2$ |
| IMO | | | | | |
| 6.8 kg bag of Calrose rice | $11.16 | 340 g | $0.56 | 0.15 $kg/m^2$ | |
| C & H Golden Brown sugar 907 g | $3.19 | 570 g | $1.99 | | |
| 22.68 kg bag of Wheat Mill Run | $41.76 | 22.68 kg | $41.76 | | |
| Amount of IMO made per batch | ~45 kg | | | | |
| Total Cost | $56.11 | | $44.31 | | $0.13* - $0.14 |
| Inorganic Fertilizer | | | | | |
| Gaviota 16-16-16 9.07 kg | $23.82 | n/a | n/a | 0.05 $kg/m^2$ | $0.13 |
| *It costs $0.14/$m^2$ to make the 1st batch then $0.13/$m^2$ thereafter (using the IMO 2 foundation stock) | | | | | |

# Chapter 4

# Natural Farming. Growing mustard green (Kai Choi, *Brassica juncea*): a comparison of plant yield and soil bacterial population between Korean natural farming and conventional farming methods.

## 4.1 Abstract

Korean natural farming (KNF) is a self-sufficient farming system that involves the harvesting and culturing of indigenous microorganisms (IMO). It enhances soil microbial activity thus improving soil fertility. The collection site of these IMOs is important and can vary from farm to farm. KNF has been practiced for over 40 years in Asia and is gradually gaining the attention of local farmers in Hawai'i. There is limited scientific evidence in regards to how the benefits of KNF impact crops, and whether the collection site plays an integral role in soil fertility. In addition, there is no information on the rate and frequency at which IMO should be re-applied. The goals of this experiment are to provide a greater understanding of the types of bacteria present in the soil treated under Korean Natural Farming (KNF) conditions and to determine the farming system that provides the greatest plant yield. Two field trials were conducted at Hoa 'Āina O Mākaha, Hawai'i. The objectives for Trial 1 were to: 1) compare plant yield (kai choi; Brassica *juncea*) between farming systems and application rate: a) KNF-$B_1$, IMO cultivated from bamboo (Bambusoideae), 1 application, b) KNF-$U_1$, IMO cultivated from breadfruit/ulu (Artocarpus *altilis*), 1 application, c) $CON_1$ (conventional; use of inorganic fertilizer), 1 application, d) KNF-$B_2$, 2 applications, e) KNF-$U_2$, 2 applications, and f) $CON_2$, 2 applications; 2) determine the types of bacteria prevalent in: a) KNF-$B_1$, b) KNF-$U_1$, and c) $CON_1$; and 3) to evaluate the bacterial population over the growth period (pre-application, post-application, and post-harvest (day 1, 3, and 28, respectively). The objectives for Trial 2 were to: 1) determine plant weight (g); 2) identify the types of bacteria prevalent in: a) KNF-$B_1$, b) KNF-$U_1$, c) KNF-$H_1$, and d) $CON_1$; and to evaluate the bacterial population over the growth period (pre-application, post-application, and post-harvest (day 1, 3, and 28, respectively)). The first set of soil samples (n=3) were collected at random within the plot prior to soil treatment (day 1; $T_1$). The plot was then divided into 18 sub-plots (3 per row, 6 rows in total), with 3 plots representing a different treatment method. In the KNF subplots, IMO 4 (indigenous microorganisms) was mixed into the soil. For the CON plot, inorganic fertilizer was applied. The kai choi plantlets were then planted (9 per subplot). On day 3 a second set of soil samples (n=3; $T_3$) were randomly collected from in-between the plants in each farming system. On day 14, a second application (IMO and inorganic fertilizer) was randomly applied to half of the plots. The third and fourth sets of samples were collected on day 28 ($T_{28}$ and $T_{28r}$). The third set ($T_{28}$) was collected in the same manner as the second set. However, the fourth set ($T_{28r}$) was obtained within the rhizosphere

(soil in contact with the roots). The samples were then subjected to serial dilutions and plated on selective media – Azospirillum (Azo), phosphorus-solubilizing (Phos), and De Man, Rogosa and Sharpe media (MRS) to favor the growth of bacteria essential to plant growth and health: a) nitrogen-fixing bacteria, b) phosphorus-solubilizing bacteria, and c) bacillus bacteria, respectively. Each microbial colony was isolated and subjected to polymerase chain reaction-temporal temperature gel electrophoresis followed by DNA analysis to identify strains of bacteria isolated. On day 28, the kai choi was harvested and weighed individually to determine plant yield. The plots were allowed to rest for one month prior to the commencement of Trial 2. The objective of trial 2 was to: 1. to compare plant yield between a) KNF-$B_1$, b) KNF-$U_1$, c) KNF-$H_1$, IMO cultivated from Leucaena *leucocephala* (haole koa), 1 application, $CON_1$ d) KNF-$B_2$, e) KNF-$U_2$, f) $CON_2$, and KNF-$H_2$, 2 applications; 2. determine the types of bacteria prevalent in: a) KNF-$H_1$ and b) $CON_1$; and 3. evaluate the bacterial population over growth period in KNF-$H_1$, $CON_1$, $CON_2$, and KNF-$H_2$. Results from Trial 1 show that KNF-$B_2$ (2 applications) had the highest plant yield (372.77g ± 7.13; $P<0.001$) overall. Bacillus *megaterium* and Bacillus *aryabhattai* were prevalent amongst all three farming systems. The Bacillus *subtilis* and Bacillus *licheniformis* were dominant only in the KNF farming systems. The bacterial population increased after the first treatment was applied and decreased over time. Additionally, KNF-B showed a ubiquitous amount of Pseudomonas *aeruginosa*. The bacterial population was greatest in the soil within the rhizosphere. Trial 2 showed similar results; KNF-$B_2$ (2 applications) had the highest plant yield (535.15g ± 9.47; $P<0.001$) overall. Plots that received two applications as opposed to one application produced higher yields. Bacillus *megaterium* and Bacillus *aryabhattai* were prevalent amongst all three farming systems. Bacillus *subtilis* and Bacillus *licheniformis* was prevalent in the KNF-$H_1$ plots but not $CON_1$.

**4.2 Introduction**

Fertile soil is the foundation of our food system. It produces healthy crops which in turn nourish us. Maintaining healthy soil is a must for any farmer. However, the maintenance of fertile soil does not come cheap as fertilizers and other soil additives are expensive. Due to the high cost of agricultural inputs in Hawai'i, it is imperative farmers minimize costs wherever possible (Parcon *et al*., 2011). Korean natural farming (KNF) is a great alternative for farmers who wish to become less dependent on external inputs.

Unlike conventional farming methods which rely on synthetic fertilizers, herbicides, and pesticides, KNF is a self-sufficient farming system that involves the culturing of indigenous microorganisms (IMO) (Cho and Cho, 2010; Kumar and Gopal, 2016).

The IMOs are naturally occurring, organic microbes that are native to the area. The most common type of microorganisms found in soil includes fungi, bacteria, and protozoa; many of which are beneficial to plants (Tölgyessy, 1993). They are essential for maintaining the ecological balance and carrying out chemical processes that make it possible for all other organisms to prosper (Kumar and Gopal, 2016). Most crop fields provide more than enough minerals and nutrients for plants to grow. However, plants have trouble absorbing some of these nutrients directly as they lack the proper transport mechanism (Rashid *et al*., 2016). In order for plants to absorb them, these nutrients need to be converted into another form. There's a symbiotic relationship that occurs between plants and beneficial microorganisms. Microorganisms convert nutrients into a form that plants are able to absorb. In turn, plants provide food, in the form of sugars, to these microorganisms (Ortiz-Castro, 2009; Blagodatskaya and Kuzyakov, 2013). Additionally, some of these microorganisms that feed on the nutrients in the soil become a food source for earthworms, beetles, millipedes, and other small creatures that further break down soil nutrients, thus making them available for plants to feed upon. In KNF treated soils, IMOs are used to create fertile and healthy soil conditions ideal for farming and preventing plant diseases (Cho and Cho, 2010). This farming approach maximizes the use of on-farm resources, recycles farm waste, and minimizes external inputs while fostering soil health.

The culturing of indigenous microorganisms is the single most important aspect of KNF. The process of making IMO is broken down into 4 stages; IMO 1, IMO 2, IMO 3, and IMO 4 (please refer to Chapter 3 for a detailed guide on how to make IMO). The first step, IMO 1, involves selecting a site within your farm to collect these microbes. Once a site has been selected, a box (bamboo, cedar, lauhala) containing hard-cooked rice covered over with a paper towel is then placed at the intended site and left undisturbed for 4-5 days (Park and DuPont, 2008). During this time, the microbes will begin to colonize within the box. Collection of IMO near the rhizosphere of bamboo is commonly practiced; however, IMO can be collected from other areas such as a field or a site where decomposed leaf litter is in abundance

(Reddy, 2011). There has been no studies have been published on determining whether or not plant growth is affected by specific sites chosen for collection of IMOs or how often IMO should be reapplied to the system. Furthermore, there have been numerous publications on the cultivation of these microorganisms but limited scientific research as to how KNF compares to conventional farming methods in terms of plant growth or what type of microbes are present in soil treated under KNF conditions (Cho and Cho, 2010; Park and DuPont, 2008; Reddy, 2011; Wang *et al*., 2012).

As previously determined in Chapter 2, phosphorus-solubilizing and Nitrogen-fixing bacteria were identified in KNF, organic, and conventional treated soil samples collected from Poamoho and Kula. The results showed that Bacillus *megaterium* and Bacillus *aryabhattai* were prevalent amongst all three farming systems and that Bacillus *subtilis* was present only in the KNF treated soil. In addition, the bacterial population for all farming systems increased post-treatment and declined over time. These experiments gave us some insight as to what types of bacteria are present in KNF. However, more studies need to further unravel the mysteries of the benefits of these bacteria in crop production.

Hence, the overall goal of this study is to provide more insight into KNF and how it fares in terms of plant yield. Two field trials were conducted on a farm located in Makaha, Hawai'i. Experiment 1 was designed to: 1. compare plant yield (Brassica *juncea*) between farming systems and application rate: a) KNF-$B_1$, IMO cultivated from Bambusoideae (bamboo), 1 application, b) KNF-$U_1$, IMO cultivated from Artocarpus *altilis* (breadfruit/ulu), 1 application, c) $CON_1$ (conventional; use of inorganic fertilizer), 1 application, d) KNF-$B_2$, 2 applications, e) KNF-$U_2$, 2 applications, and f) $CON_2$, 2 applications; 2. determine the types of bacteria prevalent in: a) KNF-$B_1$, b) KNF-$U_1$, and c) $CON_1$; and 3. evaluate the bacterial population over growth period (pre-application, post-application, and post-harvest (day 1, 3, and 28, respectively). In Experiment 2, the objectives were to 1: to compare plant yield between a) KNF-$B_1$, b) KNF-$U_1$, c) KNF-$H_1$, IMO cultivated from Leucaena *leucocephala* (haole koa), 1 application, $CON_1$ d) KNF-$B_2$, e) KNF-$U_2$, f) $CON_2$, and KNF-$H_2$, 2 applications; 2. determine the types of bacteria prevalent in: a) KNF-$H_1$ and b) $CON_1$; and 3. evaluate the bacterial population over growth period in KNF-$H_1$, $CON_1$, $CON_2$, and KNF-$H_2$.

### 4.3 Materials and Methods

**Collection of IMO** Three IMO collection sites were carefully selected within the farm where this experiment was conducted. The sites chosen were near the base of the 1) bamboo (Bambusoideae), 2) breadfruit (ulu, Artocarpus *altilis*), and 3) Leucaena *leucocephala* (haole koa). The first collection site, near the base of the bamboo, was selected due in part that it's common practice in KNF systems and is referenced in IMO cultivation processes (Cho and Cho, 2010; Park and DuPont, 2008; Reddy, 2011). The second site, near the base of the ulu tree, was chosen specifically because ulu can be found all throughout Hawai'i and was once considered a staple food in Hawai'ian culture. Lastly, haole koa was chosen as it is an invasive plant that grows everywhere and it is leguminous plant. Note that three separate batches of IMO were made from these sites; the IMO collected from each site was not combined. In this study, there were four different farming systems: 1) KNF-B (IMO cultivated from bamboo), 2) KNF-U (IMO cultivated from ulu), 3) KNF-H (IMO cultivated from haole koa), and 4) conventional (use of synthetic fertilizers). Refer to chapter 3 for a detailed guide on how the IMO 4 used in this experiment was prepared.

**Plot Set-Up** Both field trials in this study took place between the months of August – November 2016 at Hoa ʻĀina O Mākaha; located on the west side of Oahu, Hawai'i. This farm practices both conventional and natural farming methods. Site selection within the farm was based primarily on where the plants would receive the most amount of sunlight. A total of 18 (0.91 x 0.91 x 0.3 m) redwood boxes with an open base were constructed. The boxes were placed next to one another in rows of three (0.91 x 2.7 m), creating 6 rows in total spaced 0.61 m away from one another. Additionally, 6 PVC frames measuring 3 x 1.2 x 1.1 m was assembled and covered with insect mesh. Each frame covered the row of boxes for the entire trial period and was only taken off for re-application, soil collection, weed removal, and harvesting. For irrigation, PVC pipes were installed 0.91 m above the ground (just below the screen), running directly across the center of the row. Fogger misters (0.91 m diameter coverage, DIG Corporation, Vista, California) were fitted onto the PVC pipe (pointing down, toward the plant) directly in the center above each box (3 misters per row, 18 misters in total). Using a tractor, soil (from the farm, uniformly mixed) was placed into each box, filling 80% of the space.

**Trial 1** Kai choi (Brassica *juncea*) seedlings were planted in a tray containing Black Gold Seedling mix (SunGro, Aagawam, Massachusetts). The trays of seedlings were then placed in the nursery for a period of 2 weeks. The plantlets were checked for uniformity (height, number of leaves); plantlets that lacked uniformity were discarded. The first set of soil samples (n=3) were collected at random within the entire plot prior to soil treatment (day 1, $T_1$). Following the collection of $T_1$ samples, the first treatment was applied to the soil. There were 6 plots in total (2.7 x 0.91 m), each containing 3 subplots (0.91 x 0.91 m), and 6 treatments – one treatment per 3 boxes – KNF breadfruit (ulu; Artocarpus *altilis*) with one application (KNF-$U_1$), KNF bamboo (Bambusoideae) with one application (KNF-$B_1$), conventional with 1 application ($CON_1$), KNF ulu with 2 applications (KNF-$U_2$), KNF bamboo with 2 applications (KNF-$B_2$), and conventional with 2 applications ($CON_2$). The IMO 4 cultivated from the ulu tree (0.15 kg/$m^2$) was applied to 6 subplots on day 1. Of the 6, 3 were randomly selected to receive a second application on day 14. The same process was repeated with the KNF bamboo and conventional subplots except the KNF bamboo subplots were treated with IMO 4 cultivated from bamboo and the conventional subplots were treated with a 16-16-16 NPK (Nitrogen-Phosphorus-Potassium) complete fertilizer (0.5 kg/$m^2$, Gaviota; distributed by BEI Hawaii), respectively. Refer to chapter 3 for a detailed guide on how the IMO 4 used in this experiment was prepared.

On day 2, the 2 week old kai choi plantlets were transplanted; 9 plantlets per subplot, evenly spaced from one another. There were 162 plantlets in total (9 x 18). Mulch (tree trimmings, Menehune Magic, Kapolei, Hawai'i) was applied to all subplots. An irrigation timer was installed and set to turn on every day at 5 am for 10 minutes. On day 3, a second set of soil samples (n=3; $T_3$) were randomly collected from in-between the plants in each treatment. The kai choi was harvested on day 28 after trans-planting. During the harvest, the third (n=3) and fourth (n=3) sets of soil samples were collected. The third set ($T_{28}$) was collected in the same manner as the second set. However, the fourth set ($T_{28r}$) was obtained within the rhizosphere (soil in direct contact with the roots). The soils samples were immediately spread out onto a tray and allowed to air dry for 3 days. Once dry, the soil was sifted through a 2-mm mesh sieve and placed into sterile containers. The soil samples were then subjected to serial dilutions and plating on selective media.

**Trial 2** Field trial 1 was replicated once more (one month after the initial harvest) using the same subplots. KNF-U and KNF-B subplots were treated exactly the same as in Trial 1. However this time, the conventional subplots (6 in total) consisted of 4 treatments; conventional with one application (1 subplot), conventional with two applications (2 subplots), KNF haole koa with one application (IMO 4 cultivated from haole koa, 1 subplot), and KNF haole koa with two applications (2 subplots). The KNF bamboo and KNF ulu treatments remained the same. In field trial 2, there were 8 treatments total.

**Selective Media Preparation** 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 $l^{-1}$: Difco Lactobacilli MRS Broth, 55 g and Difco Agar, 15 g (BD™, Franklin Lakes, New Jersey). The azospirillum media contained $l^{-1}$: $K_2HPO_4$, 5 g; $MgSO_4 \cdot 7H20$, 0.975 g; NaCl, 1 g; yeast extract, 0.5 g; and Difco Agar, 15 g; the pH was adjusted to 6.8 prior to autoclaving (Hurst *et al.*, 2000). The phosphorus-solubilizing medium contained $l^{-1}$: Difco Plate Count agar (PCA), 23.2 g; $Ca(PO_4)_2$, 5 g; and Difco agar, 25 g (Atlas, 2010). A nutrient broth of all three selective media was also prepared (devoid of agar) for growth of purified isolates. Many variations of selective media could have been used to target certain groups of bacteria present in the soil sample. In this occurrence, the primary goal was to specifically target and identify phosphorus-solubilizing and Nitrogen-fixing bacteria. In addition to potassium (K), nitrogen (N) and phosphorus (P) are three 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 requirement followed by phosphorus.

**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. 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 media. The techniques used to inoculate the plates were the streak-plate and spread-plate techniques (Mulder and Deinema,1981). Plates were incubated at 35 °C for approximately 16 h.

The colonies appearing on the solid media were counted and recorded to determine the CFU. The cultured plates were then 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.

**Selective Media Preparation** 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 $l^{-1}$: Difco Lactobacilli MRS Broth, 55 g and Difco Agar, 15 g (BD™, Franklin Lakes, New Jersey). The azospirillum media contained $l^{-1}$: $K_2HPO_4$, 5 g; $MgSO_4 \cdot 7H20$, 0.975 g; NaCl, 1 g; yeast extract, 0.5 g; and Difco Agar, 15 g; the pH was adjusted to 6.8 prior to autoclaving (Hurst *et al.*, 2000). The phosphorus-solubilizing medium contained $l^{-1}$: Difco Plate Count agar (PCA), 23.2 g; $Ca(PO_4)_2$, 5 g; and Difco agar, 25 g (Atlas, 2010). A nutrient broth of all three selective media was also prepared (devoid of agar) for growth of purified isolates. Many variations of selective media could have been used to target certain groups of bacteria present in the soil sample. In this occurrence, the primary goal was to specifically target and identify phosphorus-solubilizing and Nitrogen-fixing bacteria. In addition, potassium (K), nitrogen (N) and phosphorus (P) are three 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 requirement followed by phosphorus.

**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. 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 media. The techniques used to inoculate the plates were the streak-plate and spread-plate techniques (Mulder and Deinema, 1981). Plates were incubated at 35°C for approximately 16 h.

The colonies appearing on the solid media were counted and recorded to determine the CFU. The cultured plates were then 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.

**Identification of Isolates** Partial sequencing of the 16S rRNA genes of new isolates was carried out as described by Promega, after the 16S rRNA gene was amplified by PCR with oligonucleotide primers 16S1-F (5′-GGAGAGTTTGATCCTGGCTCAG-3′) and 16S1-R (TATTACCGCGGCTGCTGGCAC) (Promega, Madison, Wisconsin). The amplified samples were submitted to the Advanced Studies in Genomics, Proteomics and Bioinformatics (ASGPB) laboratory located at the University of Hawaii at Manoa and subjected to high throughput DNA sequencing. ChromasPro was used to view the DNA sequencing. The sequences were compared with those in the GenBank databases by using the BLAST program (www.ncbi.nlm.nih.gov/blast).

### 4.4 Experimental Design and Data Analysis

Treatments were arranged in a complete randomized design (CRD) with six treatments (described above) and 9 plants per box. CRD was performed only once; this random design remained the same for both trials (except for the CON plots in Trial 2, KNF-H treatments were applied to the three CON plots that received only 1 treatment). Data were analyzed using a fit model (JMP 13 data analysis software; SAS Institute, Cary, NC). One-way ANOVA was performed on the colony forming units data (cfu). Weighted means were calculated using LSMEANS with mean comparisons using all pairwise comparisons (Tukey HSD).

### 4.5 Results

**Trial 1.** A multiple comparisons (Tukey HSD) test was performed on plant yield. Figure 1 shows a box-plot of Kai Choi plant yield. In the plots treated with one application, KNF-$B_1$ (Bamboo) had a significantly higher plant weight (mean=416.65g SE=8.51; $P<0.001$) than KNF-$U_1$ (Ulu; mean=372.77g SE=7.13; $P<0.001$) but not $Con_1$ (Conventional; mean=391.77g SE=4.43; $P<0.001$). In the plots treated with two applications, KNF-$B_2$ (mean=528g SE=14.5; $P<0.001$) plant weight was significantly higher than $CON_2$

(mean=471g SE=6.95; P<0.001) and KNF-$U_2$ (mean=453.31g SE=8.43; P<0.001). Bacillus *megaterium*, Bacillus *aryabhattai*, Bacillus *subtilis*, Bacillus *velezensi*, Bacillus *circulans*, Arthrobacter *globiformis*, Bacillus *aerius*, Bacillus *barbaricus,* and Bacillus *drentensis* were present in the soil prior to treatment ($T_1$; Figure 2). B. *aryabhattai* and B. *megaterium* were most abundant in the soil samples collected from the CON plots at $T_3$ (Figure 3), $T_{28}$ (Figure 6), and $T_{28r}$ (Figure 9). Soil samples collected from KNF-B at $T_3$ (Figure 4), $T_{28}$ (Figure 7), and $T_{28r}$ (Figure 10) contained an abundance of B. *subtilis* and B. *licheniformis.* A ubiquitous amount of Pseudomonas *aeruginosa* was also present in the KNF-B plots ($T_{28r}$). More than 50% of the bacteria in the soil samples collected from KNF-U plots at $T_3$ (Figure 5), $T_{28}$ (Figure 8), and $T_{28r}$ (Figure 11) were identified as B. *subtilis*, B. *licheniformis* and B. *aryabhattai*. There was a greater variety of identified bacteria present in the soil collected within the rhizosphere as opposed to soil collected in-between the plants. Figure 12 represents the bacterial colonies cultured on MRS media. Lowercase alphabet indicates a significant difference at P<0.05 with each time of collection. KNF-B and KNF-U samples collected on day 3 ($T_3$; 136 x $10^5$ ± 6.4 CFU/g and 120.6 x $10^5$ ± 9.1 CFU/g, respectively) and 28 ($T_{28}$; 69.3 x $10^5$ ± 15.8 CFU/g and 57.3 x $10^5$ ± 7.9 CFU/g, respectively) contained a significantly higher amount of bacterial colonies compared to CON ($T_3$ = 55 x $10^5$ ± 4.1; $T_{28}$ = 16.1 x $10^5$ ± 22.7 CFU/g; P<0.05). Figure 13 shows the bacterial population over time cultured on Phosphorus-solubilizing media. KNF-B and KNF-U samples collected on day 3 ($T_3$; 164.3 x $10^5$ ± 5.7 CFU/g and 171 x $10^5$ ± 5.2 CFU/g, respectively) contained a significantly higher amount of bacterial colonies compared to CON (116.6 x $10^5$ ± 8.1 CFU/g; P<0.05). There was no significant difference between the soils collected at $T_{28}$ (CON = 95.3 x $10^5$ ± 33.9 CFU/g; KNF-B, 107.3 x $10^5$ ± 19.2 CFU/g; KNF-U, 110.3 x $10^5$ ± 9.0 CFU/g). As for the bacterial colonies cultured on Azospirillum media, KNF-B and KNF-U samples collected on day 3 ($T_3$; 211.6 x $10^5$ ± 6.5 CFU/g and 201.7 x $10^5$ ± 7.5 CFU/g, respectively) and 28 ($T_{28}$; 132.3 x $10^5$ ± 29.7 CFU/g and 129.3 x $10^5$ ± 27.4 CFU/g, respectively) contained a significantly higher amount of bacterial colonies compared to CON ($T_3$ = 124 x $10^5$ ± 11.3 CFU/g; $T_{28}$ = 71.3 x $10^5$ ± 4.95 CFU/g; P<0.05; Figure 14). Figure 15 represents the CFU count (CFU x $10^5$ $g^{-1}$ soil) obtained from the soil samples collected on day 28. Three sets of soil samples were compared within each farming system; $T_{28}$ (soil collected from in-between the plants with one application applied on day 1; $CON_1$, KNF-$B_1$, and KNF-$U_1$), $T_{28r}$ (soil collected within the rhizosphere, post-harvest, with one application applied on day 1; KNF-$B_{1r}$, KNF-$U_{1r}$,

and $CON_{1r}$), and $T_{28(2r)}$ (soil collected within the rhizosphere, post-harvest, from the plots that received 2 applications, the first on day 1 (plantlets planted on day 2) and the second on day 14; $CON_{2r}$, KNF-$B_{2r}$, and KNF-$U_{2r}$). Three different culturing media was used to obtain the CFU/g of soil; MRS media, Phosphorus-solubilizing media (PHOS), and Azospirillum media (AZO). In regards to the CON samples plated on the MRS and PHOS media, there was no significant difference between $CON_1$, $CON_{1r}$, and $CON_{2r}$. However, KNF-$B_{2r}$ contained significantly higher bacterial counts overall.

**Trial 2.** Figure 16 shows a box-plot of the plant yield from Trial 2. In plots that received 1 application, KNF-$B_1$ (IMO cultivated with Bamboo) had a significantly higher yield (mean=411.33g ± 8.3; P<0.001) than $CON_1$ (Conventional; mean=358g ± 12.8; P<0.001), KNF-$U_1$ (IMO cultivated with Ulu; mean=377.4g ± 8.54; P<0.001), and KNF-$H_1$ (IMO cultivated with Haole koa; mean=392g ± 9.7; P<0.001). In the plots that received two applications, KNF-$B_2$ (mean=535.15g ± 9.47; P<0.001) was significantly higher than $CON_2$ (mean=428.2g ± 14.9; P<0.001), KNF-$U_2$ (mean=455.4g ± 9; P<0.001) and KNF-$H_2$ (mean=455.4g ±13.4; P<0.001). Bacillus *aryabhattai*, Bacillus *megaterium*, Arthrobacter *globiformis*, Bacillus *drentensis*, Bacillus *aerius*, Bacillus *amyloliquefaciens*, Bacillus *circulans*, Bacillus *nealsonii*, and Bacillus *velezensi* were present in the soil prior to treatment ($T_1$; Figure 17). B. *aryabhattai* and B. *megaterium* were most abundant in the soil samples collected from the CON plots at $T_3$ (Figure 18), and $T_{28}$ (Figure 20). Unlike Trial 1, the isolation and identification of bacteria present in soil samples collected within the rhizosphere ($T_{28r}$) were not determined, only CFU was recorded. As for the KNF-H samples collected at $T_3$ (Figure 19) and $T_{28}$, (Figure 21), close to 50% of the bacteria were identified as B. *licheniformis* and B. *subtilis*. Figure 22 represents the bacterial colonies cultured on Phosphorus-solubilizing media. KNF-H (IMO cultivated with Haole koa) soil samples collected on day 3 ($T_3$) and day 28 ($T_{28}$) contained significantly higher bacterial colonies (111.3 x $10^5$ ± 18.1 CFU/g and 84 x $10^5$ ± 3.8 CFU/g, respectively) compared to CON ($T_3$ = 80.7 x $10^5$ ± 8.8 CFU/g; $T_{28}$ = 51.3 x $10^5$ ± 4.4 CFU/g; P<0.05). Figure 23 represents the bacterial colonies cultured on MRS media. Soil samples obtained from KNF-H (IMO cultivated with Haole koa) plots on day 28 ($T_{28}$) contained significantly higher bacterial colonies (44 x $10^5$ ± 4.3 CFU/g) compared to CON ($T_{28}$ = 19.2 x $10^5$ ± 8.3 CFU/g). There was no significant difference between the soils (KNF-U and CON) collected on day 3, $T_3$. Figure 24 represents the bacterial colonies cultured on Azospirillum media. KNF-H (IMO cultivated with Haole koa) soil samples collected on day 3 ($T_3$) and day 28 ($T_{28}$) contained

significantly higher bacterial colonies (193 x $10^5$ ± 10.1 CFU/g and 136 x $10^5$ ± 11 CFU/g, respectively) compared to CON ($T_3$ = 111.7 x $10^5$ ± 8.6 CFU/g; $T_{28}$ = 73.3 x $10^5$ ± 4.6 CFU/g; P<0.05). Figure 25 represents the CFU count (CFU x $10^5$ $g^{-1}$ soil) obtained from the soil samples collected on day 28. Three sets of soil samples were compared within 2 farming systems (CON and KNF-H); $T_{28}$ (soil collected from in-between the plants with one application; $CON_1$ and KNF-$H_1$), $T_{28r}$ (soil collected within the rhizosphere, post-harvest, with one application; KNF-$H_{1r}$ and $CON_{1r}$), and $T_{28(2r)}$ (soil collected within the rhizosphere, post-harvest with 2 applications, the first on day 1 (plantlets planted on day 2) and the second on day 14; $CON_{2r}$ and KNF-$H_{2r}$). Three different culturing media was used to obtain the CFU/g of soil; MRS media, Phosphorus-solubilizing media (PHOS), and Azospirillum media (AZO). There were no significant difference between any of the treatments/media.

**4.6 Discussion**

The goals of this study were to provide a greater understanding of the types of bacteria present in the soil treated under KNF conditions, to determine whether or not the collection site of microbes (1$^{st}$ step to preparing IMO for KNF treatment) plays an integral role in plant yield, and to compare plant yield between KNF and conventionally (CON) treated plots.

In terms of plant growth, the plots that received two applications (day 1, 14) of IMO cultivated from bamboo (KNF-$B_2$) out performed all other treatments (mean=528g SE=14.5; P<0.001; Figure 16). Additionally, there was no significant difference between KNF-U, KNF-H, and CON (P<0.001; Figure 1, 16). When comparing the KNF treated plots, the collection site of microbes played an integral role in plant growth. As previously mentioned, the only difference between KNF-U, KNF-H, and KNF-B plots was how the IMO had been cultivated; every step that followed was performed in the same manner with no deviations. The data suggests that collecting microbes near the rhizosphere of bamboo, as opposed to breadfruit and haole koa, promotes a higher plant yield. This begs the question as to what makes bamboo the optimal site for collecting and cultivating IMO. The mystery lies within the rhizosphere of bamboo, breadfruit, and haole koa.

The rhizosphere (soil in close/direct proximity to the plants roots) is rich in nutrients due to the accumulation of plant exudates containing amino acids and sugars which provide an optimal environment for colonizing bacteria (Beneduzi *et al.,* 2012) This forces the bacterial community to colonize the rhizoplane and rhizosphere (Bulgarelli *et al.,* 2012).

Bamboo is a multifunctional evergreen plant belonging to the subfamily Bambusoideae of the grass family Gramineae. There are roughly 1,500 identified species in 87 genera around the world with China having the richest diversity (Cao *et al*., 2011; Zhou *et al.,* 2011). Bamboo plants propagate, grow, and regenerate rapidly. Once established, rapid growth and regeneration increases their ability to compete for light and space by forming a dense forest (Silveira, 2005). In fact, the world record for the fastest growing plant belongs to a bamboo species (www.guinnessworldrecords.com). There unique biological characteristics and growth habits enable bamboo forests to serve ecological and environmental functions such as land rehabilitation, water conservation and control of soil erosion (Zhou *et al*. 2005). These, among other reasons, have lead researchers on a path to further understand bamboo and it's characteristics; a great deal of research has been done to better understand the soil microbial communities present within the bamboo's rhizosphere (Han *et al.,* 2009; Yeasmin *et al.,* 2015; Lin *et al.,* 2015; and Lin and Chiu, 2016). Recent studies show that bamboo rhizosphere has a greater microbial (bacteria, fungi, and protozoa) diversity compared to non-bamboo rhizosphere (Han *et al.,* 2009; and Susanti *et al.,* 2015). Additionally, certain bacteria present within the rhizosphere have been proven to increase plant growth as well as suppress phyto-pathogens. These beneficial bacteria are commonly referred to as plant growth promoting rhizobacteria (PGPR). PGPR possess the ability to synthesize particular compounds for the plants, facilitate the uptake of certain nutrients from the soil, and lessen or prevent the plants from diseases (Gullap *et al.,* 2014). Some of the bacteria identified in these studies include *B. subtilis, B. licheniformis, Pseudomonas putida, B. pumilus, B. megaterium, B. thuringiensis, B. circulans, Paenibacillus pabuli, Lysinibacillus sphaericus, Buttiauxella izardii,* and *Burkholderia ubonensis* (Han *et al.,* 2009; Ruangsanka, 2014; and Darma *et al.,* 2016). Many of the aforementioned bacteria, which are PGPR, were also identified in the soil samples collected from KNF-U, KNF-B, and KNF-H (Figures 10, 11, and 21) but there were fewer similarities in the soil collected prior to treatment ($T_1$) simply because there were less bacteria present in the samples (Figure 2). Not including the CON soil samples,

at least 40% of the bacteria isolated and identified (KNF-U, KNF-B, and KNF-H) were that of *B. subtilis* and *B. licheniformis*.

*B. subtilis*, one of most well studied Bacillus species, is known to have roughly 5% of its genome devoted to antibiotic synthesis and has the potential to produce more than two dozen structurally diverse antimicrobial compounds (Stein, 2005). In terms of plant defense, *B. subtilis* has proven to inhibit the growth of pathogens such as *Sclerotium rolfsii, Ganoderma boninense, Fusarium sp., and Curvularia sp.* and restrict *Pseudomonas syringae* from entering through the stomata by signaling the guard cells to close (Kumar *et al.* 2012; and Darma *et al.,* 2016). *B. licheniformis* on the other hand has shown that it can actually reduce soil stress by conferring the strength and resiliency of its own properties onto the surrounding soil through its unique characteristics (Dan *et al*., 2012). ). Additionally, Lim and Kim (2013) found that when inoculated (via irrigation) with B. *licheniformis*, pepper plants have the ability to tolerate drought stress and survive longer compared to non-inoculated pepper plants.

Much research has been done on identifying PGPR associated with Bamboo, however, little is known about the types of bacteria present within the rhizosphere of breadfruit and haole koa. Given that the bacteria identified in KNF-B, KNF-U, and KNF-H plots were somewhat similar, it's safe to assume that the rhizosphere of breadfruit and haole koa contained similar PGPR.

In terms of the bacterial population (CFU x $10^5$ $g^{-1}$ soil), there was no significant difference between KNF-U, KNF-B, and KNF-H plots, however, all three were significantly higher ($P<0.05$) than the CON plots (Figures 12, 14, 23, and 24). This is justifiable as KNF relies on nourishing the soil with organic nutrients and IMO whereas CON relies on the use of synthetic fertilizer. The bacterial population for all farming systems increased post application and decreased over time. Additionally, in comparison to just one treatment, applying a 2$^{nd}$ treatment two weeks after the 1$^{st}$ application resulted in a higher plant yield (Figures 1 and 16).

Though a lot of informative data was collected during this study, there isn't enough tangible evidence to fully grasp an understanding as to why KNF-B produced a higher plant yield than KNF-H and KNF-U. With that said, there is one noticeable difference worth mentioning regarding the bacteria

identified in the KNF-B, KNF-U, and KNF-H soil samples - *Pseudomonas aeruginosa* was present in the KNF-B plots (~11%) but not KNF-H and KNF-U (Figure 10). Certain strains of *P. aeruginosa* have demonstrated surprising resilience and strength in many ways. In one study, the inoculation of *P. aeruginosa* on *Pongamia pinnata* (legume) increased NPK uptake which in turn increased plant growth and biomass (Radhapriya *et al.,* 2015). Another study showed similar results; seeds from *Abelmoschus esculentus L.* (okra), *Lycopersicon esculentum L.* (tomato) and *Amaranthus sp.* (African spinach) were inoculated with a bacterial suspension consisting of *P. aeruginosa* (Adesemoye and Ugoji, 2009). The data showed that the inoculated plants had greater biomass than the control group. On the contrary, some *P. aeruginosa* strains are opportunistic pathogens. Walker *et al.* (2004) showed that *P. aeruginosa* is capable of forming a biofilm on the roots of *Ocimum basilicum* (genovese basil) resulting in black necrotic regions at the root tips whereby inhibiting plant growth. Assuming the *P. aeruginosa* strains identified in this study are PGPR, the presence of *P. aeruginosa* in the KNF-B treated plots could be one of many reasons why bamboo rhizosphere is the optimal site for collecting and cultivating IMO.

There is however many other variables effecting (whether positive or negative) plant growth that have yet to be identified and determined. The primary focus of this study was to identify phosphorus-solubilizing, Nitrogen-fixing and Bacillus sp. using selective media. Unfortunately, using selective media significantly limits the growth of other bacteria present in the soil samples. In addition to bacteria, the IMO used in KNF systems also include the collection and cultivation of fungi, protozoa, and nematodes. Many of these unexplored factors play a role in soil fertility and plant growth.

**4.7 Future Research**

For future KNF studies, an analysis of microbial diversity, specifically fungi and bacteria, should be performed on the soil samples. This process would allow us to identify more bacteria and fungi without the use of selective media. A microbial diversity analysis can be accomplished via Temperature gradient gel electrophoresis (TGGE) or Denaturing gradient gel electrophoresis (DGGE). It would also be beneficial to analyze soil samples from the collection site (IMO 1 process) and IMO 4 (final product that is applied to the soil in a KNF farming system). This would allow us to identify bacteria and fungi present within the collection site (IMO 1) and compare those findings to the microbes present in IMO 4. Some of

the bacterial isolates collected in this study, which include *P. aeruginosa,* were stored for future use. Further research needs to be conducted on *P. aeruginosa* in order to determine whether or not this specific isolate promotes or inhibits plant growth. Inoculating plants with this particular bacteria and possibly others, will give us more insight into this present study.

### 4.8 Conclusion

From a sustainability perspective, KNF is a viable alternative to conventional farming. KNF-B outperformed all other farming systems in terms of plant growth. KNF-H and KNF-U promoted plant growth just as well as the conventionally treated plots (control). It has also been determined that the collection site of microbes effects plant development and that applying 2 treatments (14 days apart) as opposed to one increases plant yield. Additionally, this study showed that $\geq$ 40% of the bacteria isolated and identified from the KNF plots (KNF-U, KNF-B, and KNF-H) were *B. subtilis* and *B. licheniformis* and that a ubiquitous amount of *P. aeruginosa* was present only in KNF-B. This study has provided some answers, and perhaps more questions, as to what makes bamboo the optimal site for collecting and cultivating IMO. To fully understand how KNF works, more research needs to be conducted and documented.

### 4.9 References


Adesemoye, A., & Ugoji, E. 2009. Evaluating Pseudomonas aeruginosa as plant growth-promoting rhizobacteria in West Africa, Archives of Phytopathology and Plant Protection, 42:2, 188-200, DOI: 10.1080/03235400601014791

Atlas, Ronald M., 2010. Handbook of Microbiological Media, Fourth Edition. CRC Press, USA 1397-1398

Beneduzi, A., Ambrosini, A., and Passaglia, L.M. 2012. Plant growth-promoting rhizobacteria (PGPR): Their potential as antagonists and biocontrol agents. Genet Mol Biol. 35(4 (suppl):1044–51.

Blagodatskaya, E., Kuzyakov, Y., 2013. Active microorganisms in soil: critical review of estimation criteria and approaches. Soil Biology & Biochemistry 67, 192e211

Bulgarelli, D., Schlaeppi, K., Spaepen, S., Themaat, E., and Schulze-Lefert, P. 2013. Structure and Functions of the Bacterial Microbiota of Plants. Annu Rev Plant Biol. 64(1):807–38.

Cho, H.-K., and J.-Y. Cho. 2010. Natural Farming. Han-Kyu Cho. CGNF. Seoul, S. Korea

Cao, Y., Fu, S., Zou, X., Cao, H., Shao, Y., and Zhou, L. 2010. Soil microbial community composition under Eucalyptus plantations of different age in subtropical China. Eur. J. Soil Biol., 46, 128–135. doi:10.1016/j.ejsobi.2009.12.006

Dan, V., Mishra, S., Chaudhry, V., Tripathi, S., Singh, P., Yadav, S., Mishra, S., Ijinu, T. P., George, V., Varma, A., & Nautiyal, C. (2012). Biocontrol, plant growth promotion and conferring stress tolerance. International Journal of Science and Nature, 3(4), 780783.

Darma, R., Purnamasari, I.M., Agustina, D., Pramudito, T.E., and Sugiharti, M. 2016. A Strong Antifungal-producing Bacteria from Bamboo Powder for Biocontrol of Sclerotium rolfsii in Melon (Cucumis melo var. amanta). J Plant Pathol Microbiol 7:334. doi:10.4172/2157-7471.1000334

Gullap, K., Dascı, M., Erkovan, H., Koç, A., & Turan, M. 2014. Plant Growth-Promoting Rhizobacteria (PGPR) and Phosphorus Fertilizer-Assisted Phytoextraction of Toxic Heavy Metals from Contaminated Soils. Communications in Soil Science and Plant Analysis. 45. 10.1080/00103624.2014.929702.

Guinness. 2000. Guinness world records - Fastest Growing Plant. Guinness World Records London. Retrieved April 2, 2018; http://www.guinnessworldrecords.com/world-records/fastest-growing-plant/

Han, J., Xia, D., Li, L., Sun, L., and Yang, K. 2009. Diversity of culturable bacteria isolated from root domains of moso bamboo (Phyllostachys edulis). Microb Ecol 58: 363-373.

Hurst C.J., Knudsen G.R., Mclnerny M.J., and Stetzenbatch L.D. 2000. “Role of Azospiriillum in Nitrogen Fixation”, Journal of Manual Environmental Microbiology, Vol. 6, No. 3, pp. 104-105.

Kumar, B. L., & Gopal, D. V. R. S. 2015. Effective role of indigenous microorganisms for sustainable environment. 3 Biotech, 5(6), 867–876. http://doi.org/10.1007/s13205-015-0293-6

Lim, J.-H., & Kim, S.-D. 2013. Induction of Drought Stress Resistance by Multi-Functional PGPR Bacillus licheniformis K11 in Pepper. The Plant Pathology Journal, 29(2), 201–208. http://doi.org/10.5423/PPJ.SI.02.2013.0021

Lin, Y.-T., Whitman, W., Coleman, D., Shi, S.-Y., Tang, S., and Chiu, C.-Y. 2015. Changes of soil bacterial communities in bamboo plantations at different elevations, FEMS Microbiology Ecology, Volume 91, Issue 5, fiv033, https://doi.org/10.1093/femsec/fiv033

Lin, Y.-T., & Chiu, C.-Y. (2016). Elevation gradient of soil bacterial communities in bamboo plantations. Botanical Studies, 57, 8. http://doi.org/10.1186/s40529-016-0123-0

Mulder E.G. and Deinema M.H. 1981. The sheathed bacteria. In: Starr PM, Stolp H, Truper HG, Barlows A and Schlegelhg (eds.) The Prokaryotes: A Handbook on Habitats, Isolation, and Identification of Bacteria. Springer-Verlag, USA. 425-440.

National Center for Biotechnology Information (NCBI). Bethesda (MD): National Library of Medicine (US), National Center for Biotechnology Information; Retrieved November 5, 2017 from: https://www.ncbi.nlm.nih.gov/

Ortiz-Castro, R., Contreras-Cornejo, H. A., Macias-Rodriguez, L., & Lopez-Bucio, J. 2009. The role of microbial signals in plant growth and development. Plant Signaling & Behavior, 4(8), 701–712.

Parcon, H., S. Arita, M. Loke, and P. Leung 2011. A comparison of agricultural input prices: Hawai'i vs. its major Export competitors. CTAHR Cooperative Extension Service Publication EI-20. 23 pp.

Park, H. and M. Duponte. 2008. How to cultivate indigenous microorganisms. CTAHR Cooperative Extension Service Publication. BIO-9. pp.

Radhapriya, P., Ramachandran, A., Anandham, R., & Mahalingam, S. 2015. Pseudomonas aeruginosa RRALC3 enhances the biomass, nutrient and carbon contents of Pongamia pinnata seedlings in degraded forest soil. PLoS ONE 10(10), e0139881. doi:10.1371/ journal.pone.0139881

Rashid, M.I., Mujawar, L.H., Shahzad, T., Almeelbi, T., Ismail, I.M.I., Oves, M., 2016. Bacteria and fungi can contribute to nutrients bioavailability and aggregate formation in degraded soils. Microbiol. Res. 183, 26–41.

Reddy, R. 2011. Cho's Global Natural Farming. Retrieved April 10, 2016; https://ilcasia.files.wordpress.com/ 2012/02/chos-global-natural-farming-sarra.pdf

Ruangsanka, S. 2014. Identification of phosphate-solubilizing bacteria from the bamboo rhizosphere. ScienceAsia 40 (2014): 204–211. doi: 10.2306/scienceasia1513-1874.2014.40.204

Scholberg, J., B.L. McNeal, K.J. Boote, J.W. Jones, S.J. Locascio, S.M. Olson. 2000 Nitrogen stress effects on growth and nitrogen accumulation by field-grown tomato. Agron. J., 92, pp. 159-167

Sharma, S. B., Sayyed, R. Z., Trivedi, M. H., & Gobi, T. A. (2013). Phosphate solubilizing microbes: sustainable approach for managing phosphorus deficiency in agricultural soils. SpringerPlus, 2, 587. http://doi.org/10.1186/2193-1801-2-587

Singh, M., 2009. Influence of Triacontanol, nitrogen and phosphorus on the growth, yield and quality of ginger (Zingiber officinale Rosc.) and Turmeric (Curcuma longa L.). Ph.D. Thesis, Aligarh Muslim University, Aligarh, India.

Silveira, M. 2005. The open bamboo forest in southwest Amazonia: patterns and processes at multiple scales. pp.1–153. EDUFAC, Rio Branco.

Stein, T. 2005. Bacillus subtilis antibiotics: structures, syntheses and specific functions. Mol Microbiol 56: 845-857.

Susanti, W., Widyastuti, R., and Wiyono, S. 2015. The role of bamboo rhizosphere fungi and bacteria in increasing plant growth and the phenomenon of disease suppressive soil. Dissertation, Bogor Agricultural University.

Tölgyessy, J. 1993. 8 Soil biology. Studies in Environmental Science. Elsevier. Volume 53, pp. 699-723, https://doi.org/10.1016/S0166-1116(08)70072-4.

Walker, T. S., Bais, H. P., Déziel, E., Schweizer, H. P., Rahme, L. G., Fall, R., & Vivanco, J. M. 2004. Pseudomonas aeruginosa-Plant Root Interactions. Pathogenicity, Biofilm Formation, and Root Exudation. Plant Physiology, 134(1), 320–331. http://doi.org/10.1104/pp.103.027888

Wang, K-H, DuPonte M, and Chang K. 2012. Use of Korean Natural Farming for Vegetable Crop Production in Hawai'i. Hanai'Al/The Food Provider. Retrieved April 1, 2017; http://www.ctahr.hawaii.edu/sustainag/news/articles/V14-Wang-KNF.pdf

Yeasmin, L., Ali, M. N., Gantait, S., & Chakraborty, S. (2015). Bamboo: an overview on its genetic diversity and characterization. 3 Biotech, 5(1), 1–11. http://doi.org/10.1007/s13205-014-0201-5

Zhou, Z., Fu, M., Xie, J., Yang, X., and Li, Z. 2005. Ecological functions of bamboo forest: research and application. J.For. Res., 16, 143–147. doi:10.1007/BF02857909

Zhou, GM., Meng, CF., Jiang, PK., and Xu, Q. 2011. Review of carbonfixation in bamboo forests in china. Bot. Rev., 77, 262–270. doi:10.1007/s12229-011-9082-z

## 4.10 Appendix

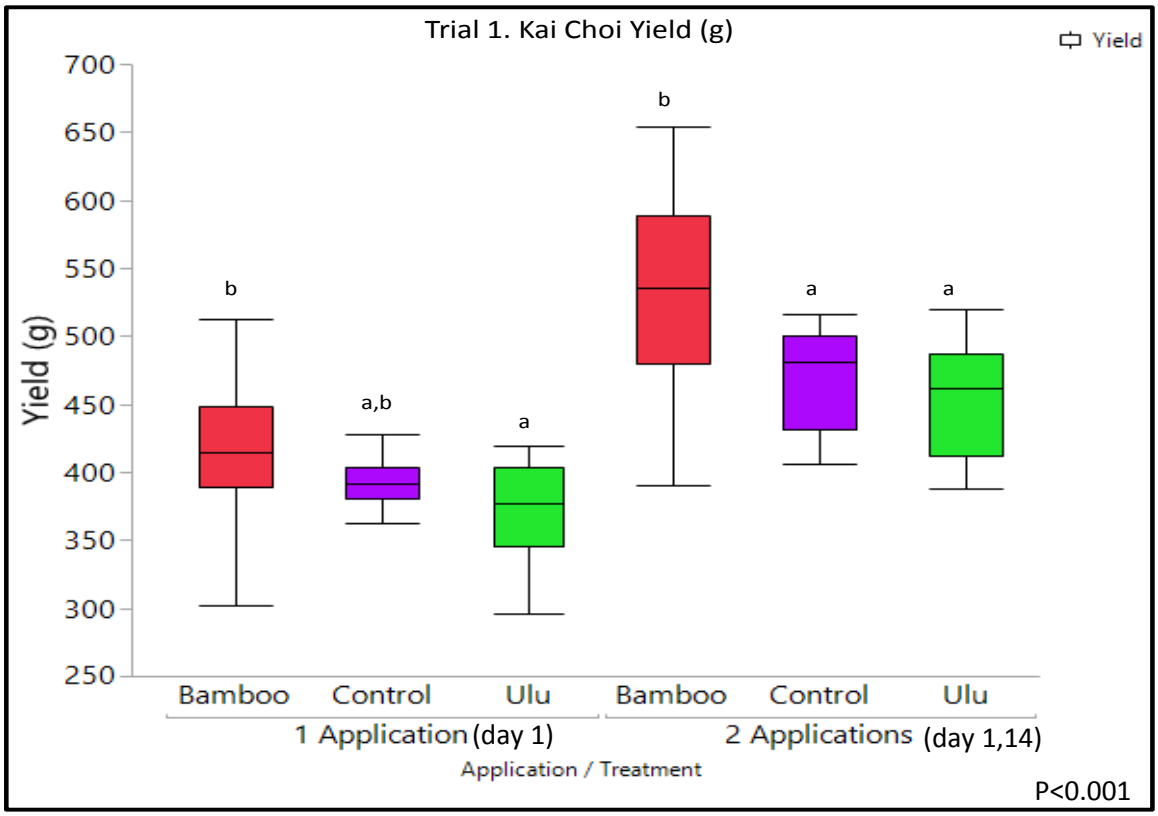



**Figure 1.** Box-plot of Kai Choi plant yield. The band inside the box is the median, the bottom and top of the box are the 25% and 75% quartiles, respectively, and the lower and upper whiskers represent the minimum and maximum values, respectively. The data shows that KNF-B (Bamboo) with 1 application was significantly higher (mean=416.65g SE=8.51; P<0.001) than KNF-U (Ulu) with 1 application (mean=372.77g SE=7.13; P<0.001) but not Con (Control) with 1 application (mean=391.77g SE=4.43; P<0.001). In reference to two applications, KNF-B (mean=528g SE=14.5; P<0.001) was significantly higher than Con (mean=471g SE=6.95; P<0.001) and KNF-U (mean=453.31g SE=8.43; P<0.001).

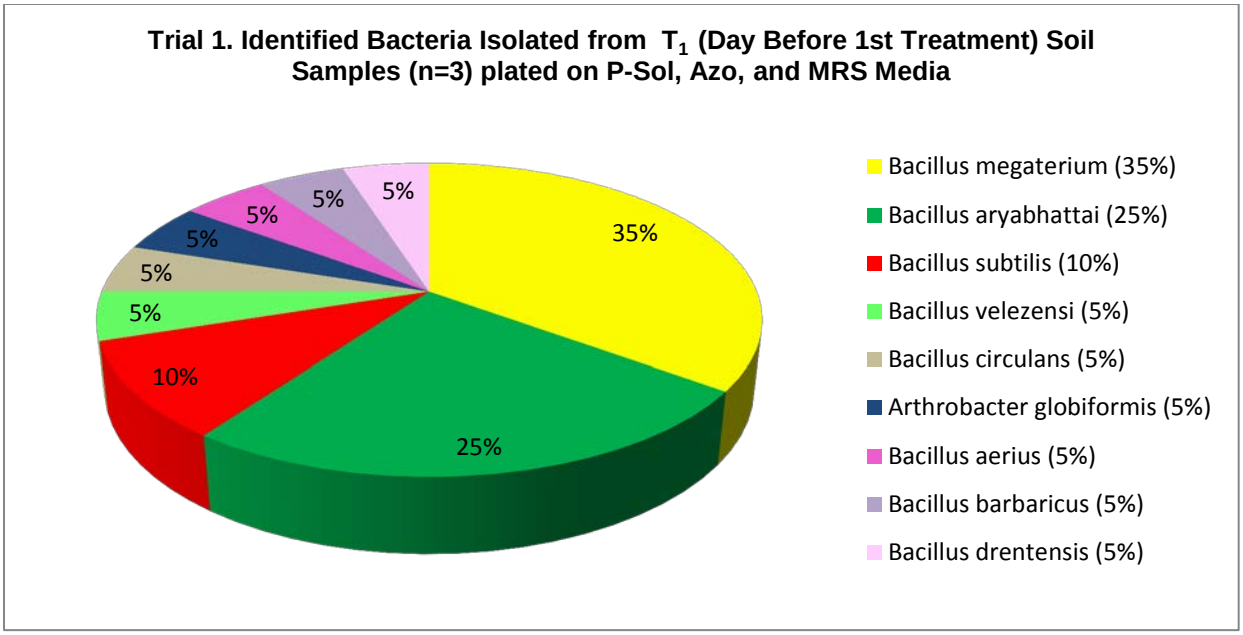



**Figure 2.** Pie chart summarizing the distribution of identified bacteria isolated from soil samples collected (n=3) prior to treatment and plated on P-Sol, Azo, and MRS Media. The soil contained an abundance of Bacillus *megaterium* (35%) and Bacillus *aryabhattai* (25%). 10% of the bacterial isolates were Bacillus *subtilis*.

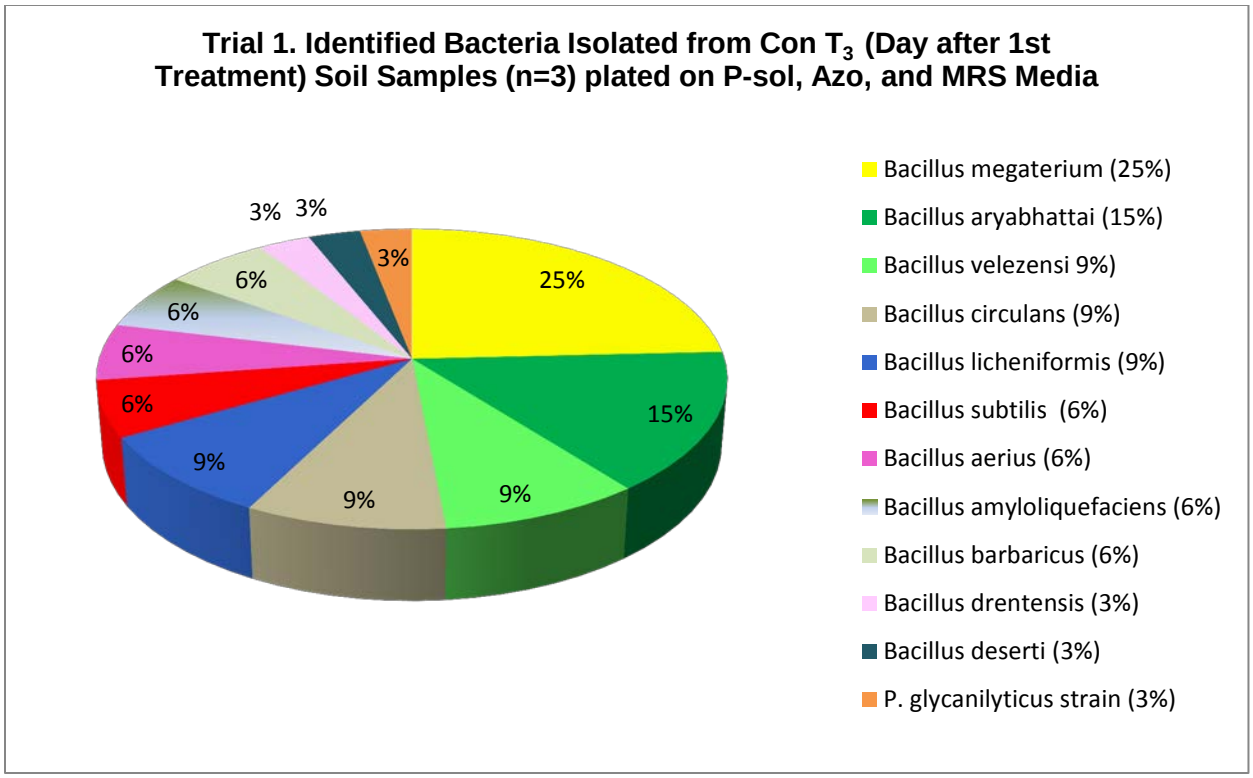



**Figure 3.** Pie chart summarizing the distribution of identified bacteria isolated from soil samples collected (n=3) from the Conventional (Con) plots and plated on P-Sol, Azo, and MRS Media. The soil contained an abundance of Bacillus *megaterium* (25%) and Bacillus *aryabhattai* (15%).

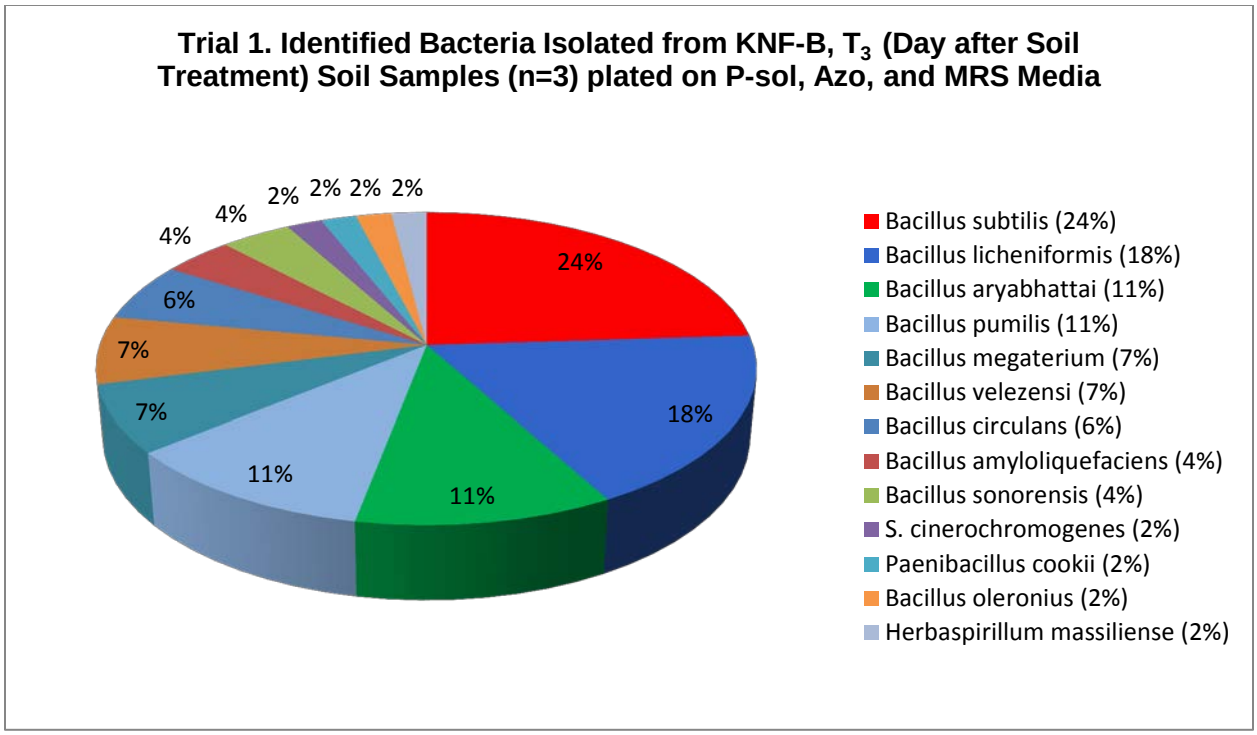



**Figure 4**. Pie chart summarizing the distribution of identified bacteria isolated from soil samples collected the day after soil treatment (n=3) from the KNF-B plots; plated on P-Sol, Azo, and MRS Media. Many of the isolates identified were Bacillus *subtilis* (24%) and Bacillus *licheniformis* (18%).

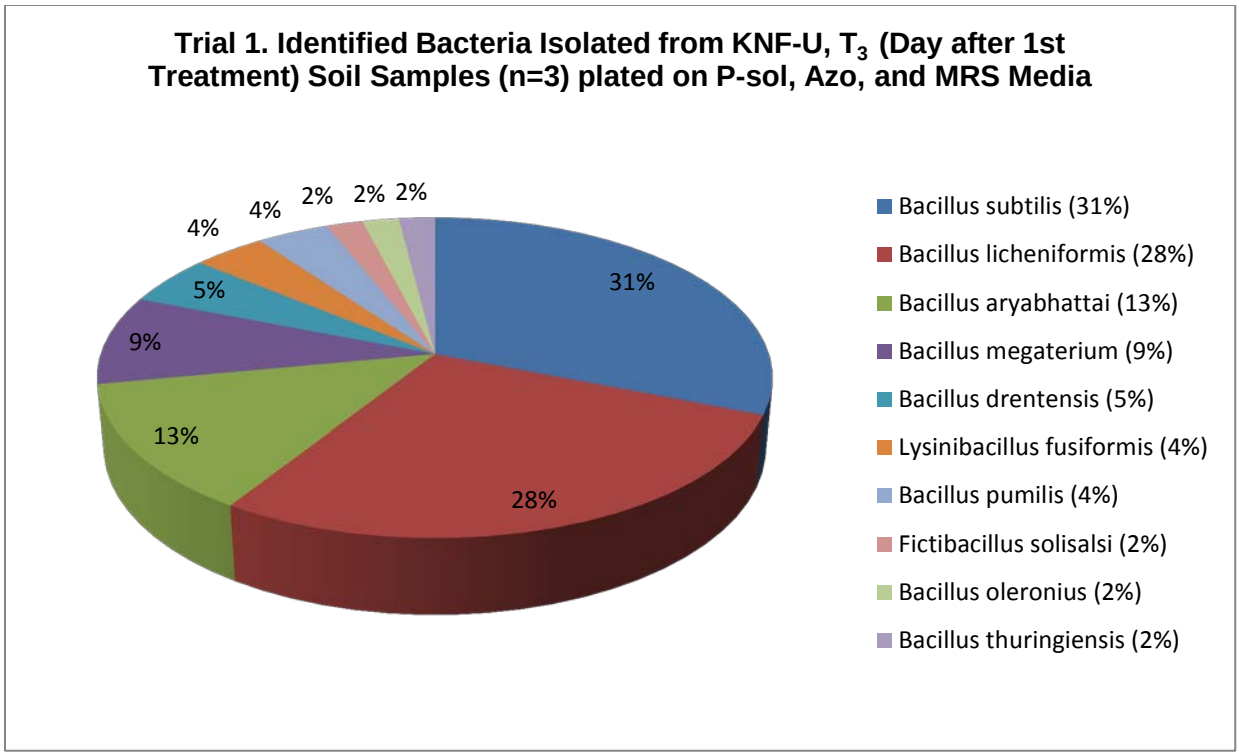



**Figure 5.** Pie chart summarizing the distribution of identified bacteria isolated from soil samples collected (n=3) from the KNF-U plots the day after soil treatment; plated on P-Sol, Azo, and MRS Media. The soil samples contained an abundance of Bacillus *subtilis* (31%) and Bacillus *licheniformis* (28%).

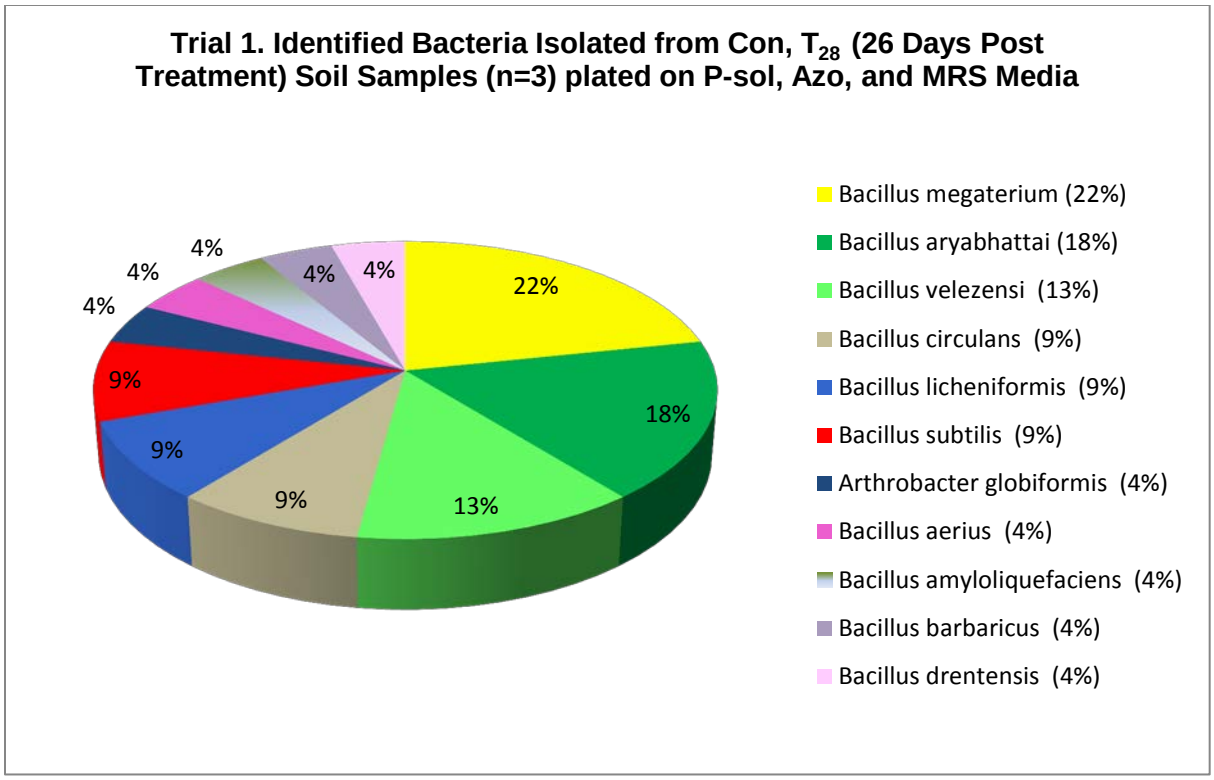



**Figure 6**. Pie chart summarizing the distribution of identified bacteria isolated from soil samples collected (n=3) from the Con plots 26 days after the soil had been treated with synthetic fertilizer; plated on P-Sol, Azo, and MRS Media. Results show that the soil samples contained many bacterial isolates identified as Bacillus *megaterium* (22%) and Bacillus *aryabhattai* (18%).

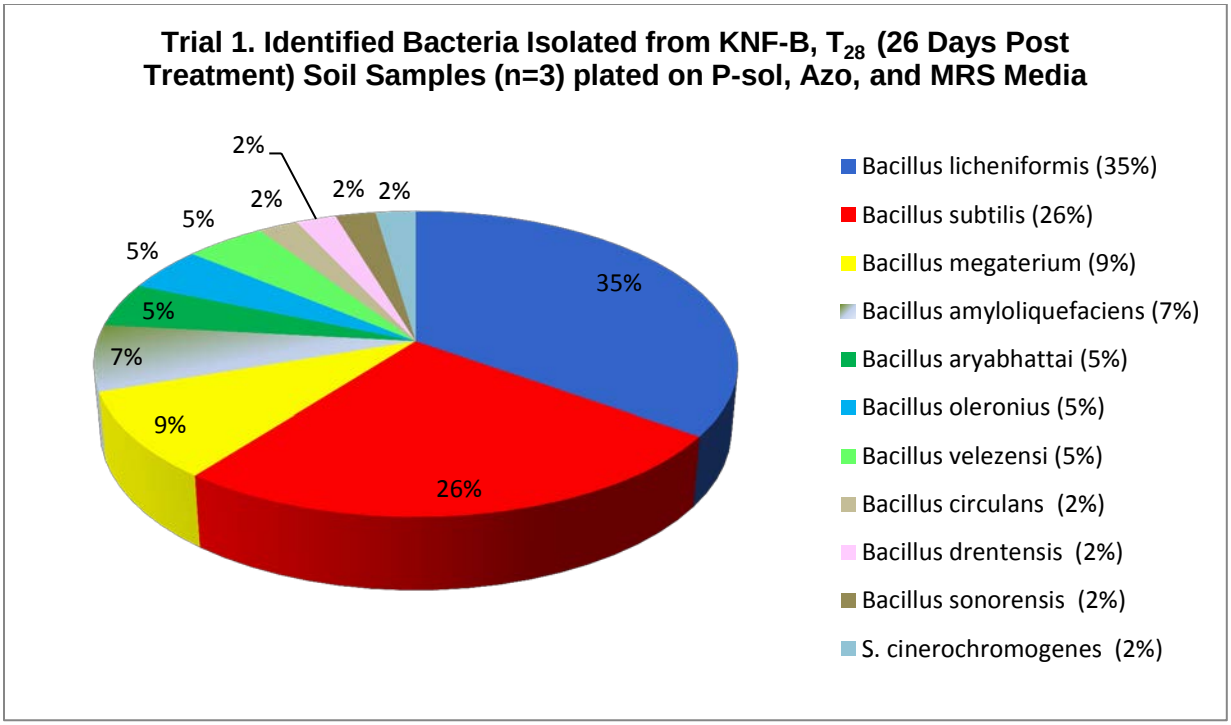



**Figure 7**. Pie chart summarizing the distribution of identified bacteria isolated from soil samples collected (n=3) from the KNF-B plots 26 days post treatment; plated on P-Sol, Azo, and MRS Media. There was an abundance of Bacillus *subtilis* (35%) and Bacillus *licheniformis* (26%) present in the soil sample.

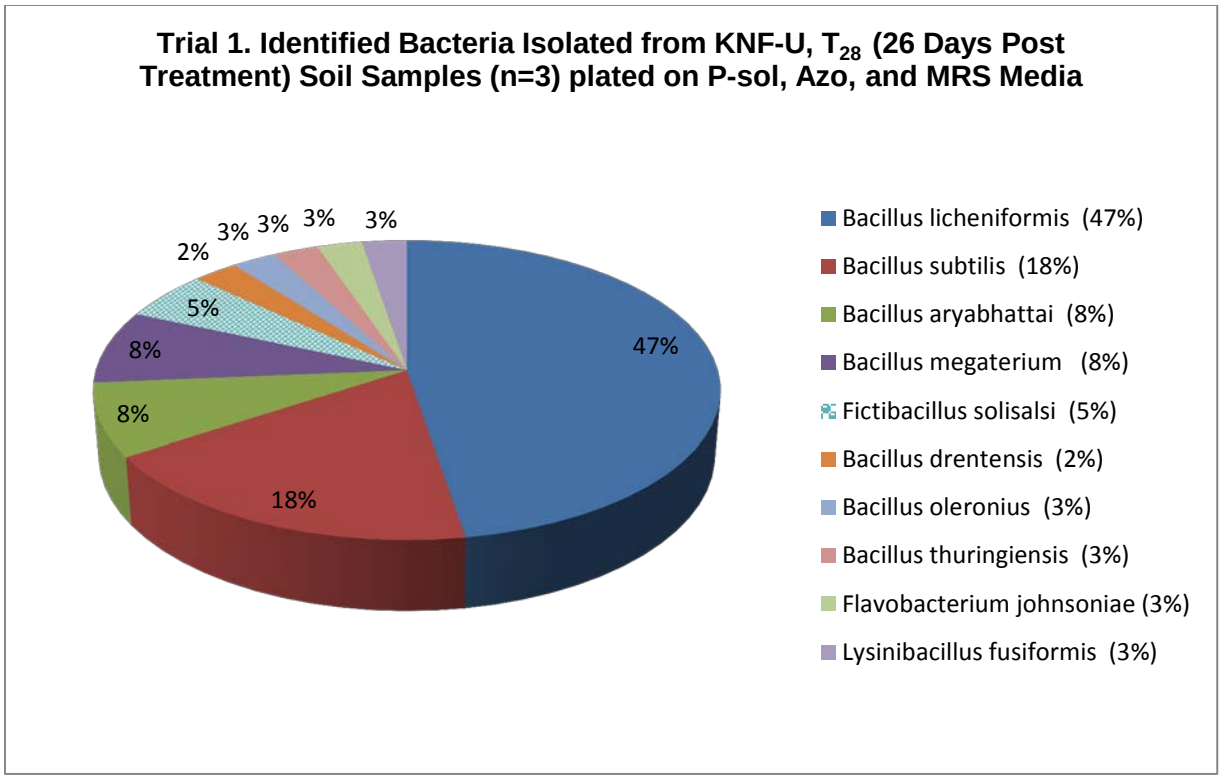



**Figure 8**. Pie chart summarizing the distribution of identified bacteria isolated from soil samples collected (n=3) from the KNF-U plots; plated on P-Sol, Azo, and MRS Media. Nearly half of the isolates were identified as Bacillus *licheniformis* (47%); 18% were Bacillus *subtilis.*

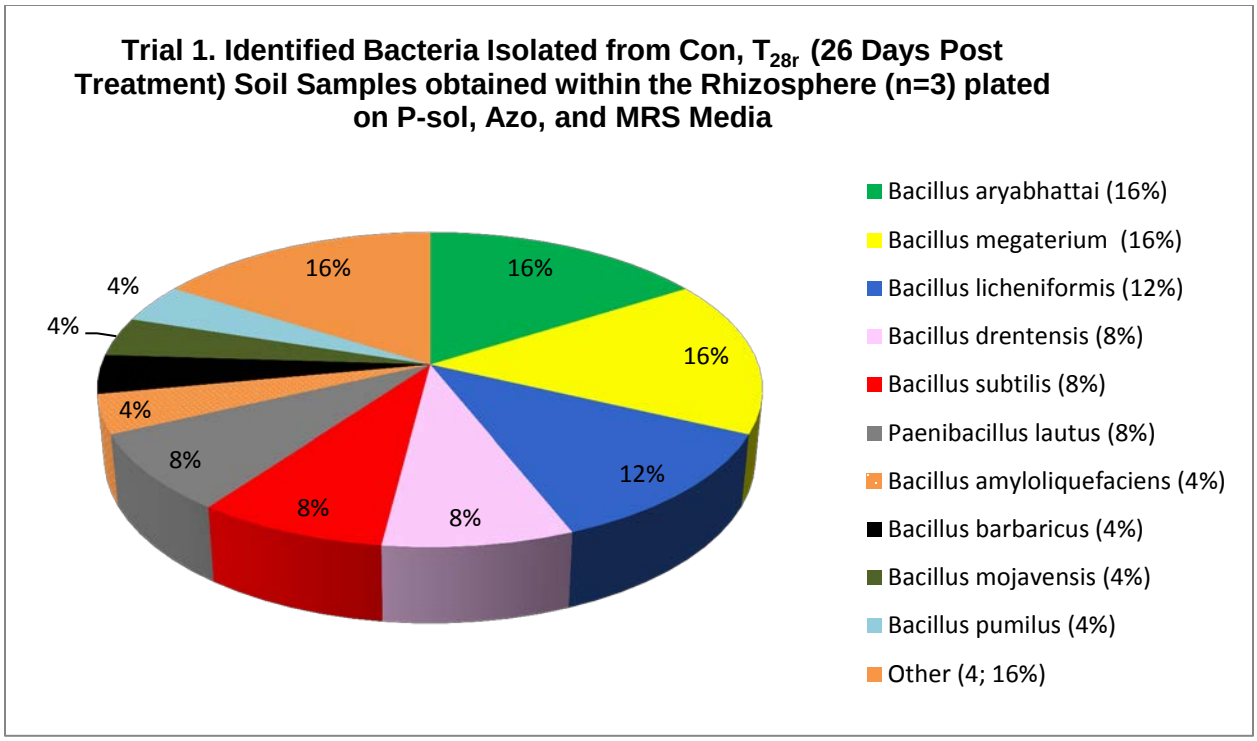



**Figure 9**. Pie chart summarizing the distribution of identified bacteria isolated from soil samples collected (n=3) from the Con plots 26 days post treatment; plated on P-Sol, Azo, and MRS Media. The soil contained an abundance of Bacillus *aryabhattai* (16%), Bacillus *megaterium* (16%), and Bacillus *licheniformis* (12%). Other identified bacteria present in the soil that make up 16% of the isolates identified include Bacillus *sonorensis*, Bacillus *tequilensis,* Brevibacillus *laterosporus,* and Terribacillus *saccharophilus*.

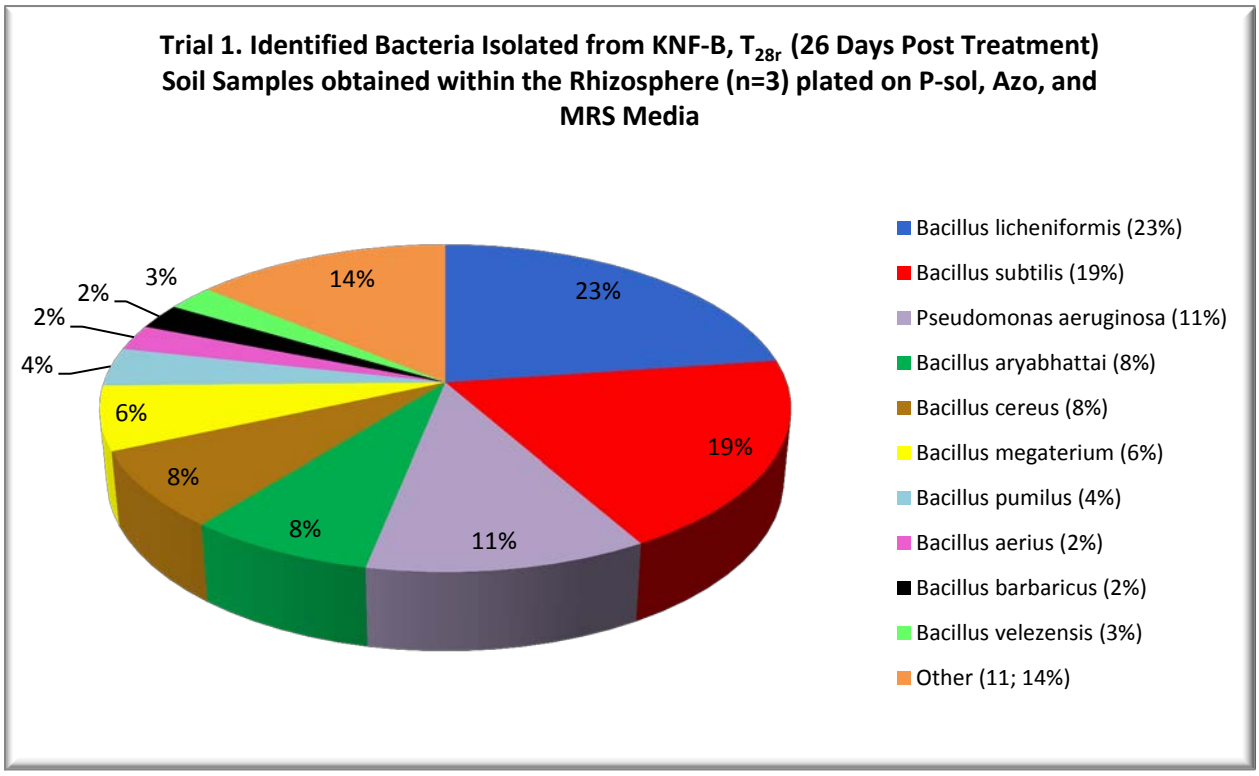



**Figure 10.** Pie chart summarizing the distribution of identified bacteria isolated from soil samples collected (n=3) from the KNF-B plots; plated on P-Sol, Azo, and MRS Media. This soil sample contained a wide variety of bacteria. Many of the isolates identified were that of Bacillus *licheniformis* (23%) and Bacillus *subtilis* (19%). Other identified bacteria present in the soil that make up 14% of the isolates identified include Lysinibacillus *fusiformis*, Bacillus *amyloliquefaciens*, Bacillus *drentensis*, Bacillus *oleronius*, Bacillus *sonorensis*, Bacillus *subterraneus*, Bacillus *tequilensis*, Bacillus *thuringiensis*, Cellulosimicrobium *funkei*, Nocardioides sp., and Brevibacillus *laterosporus.*

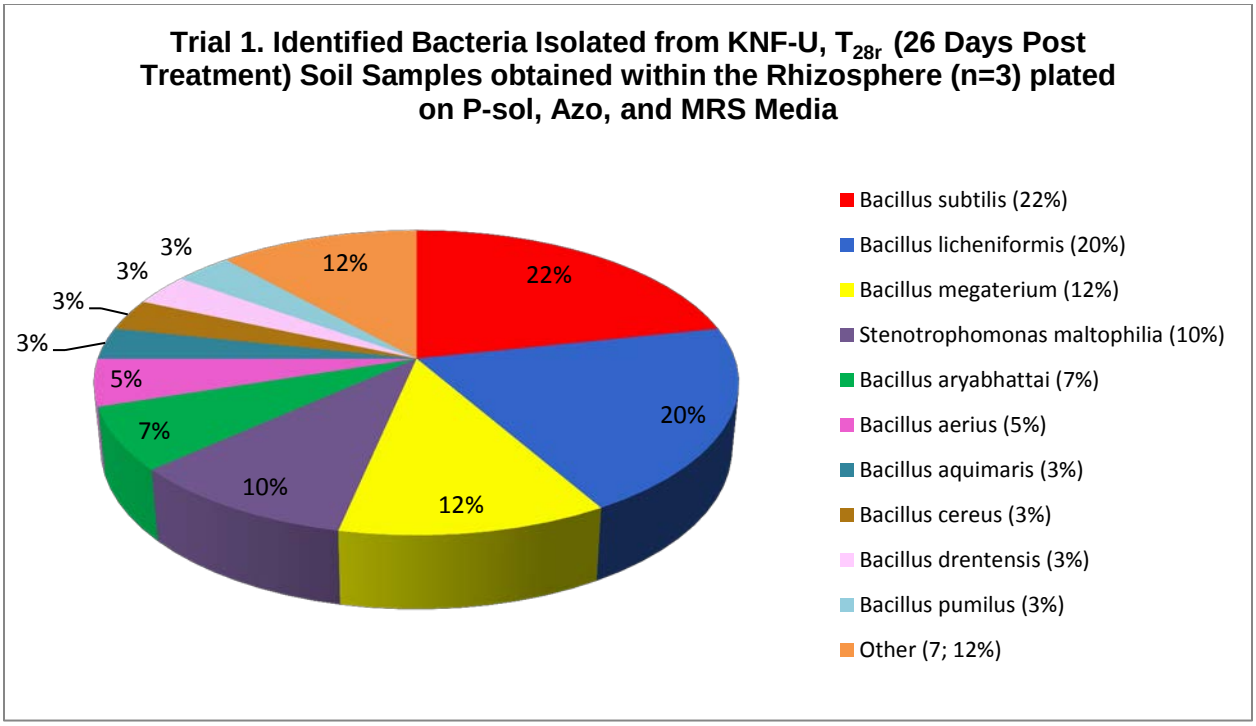



**Figure 11.** Pie chart summarizing the distribution of identified bacteria isolated from soil samples collected (n=3) from the KNF-U plots; plated on P-Sol, Azo, and MRS Media. Bacillus *subtilis* (22%) and Bacillus *licheniformis* (20%) make up close to half of the bacteria identified. Other identified bacteria present in the soil that make up 12% of the isolates identified include Bacillus *amyloliquefaciens*, Bacillus *safensis*, Bacillus *sonorensis*, Streptomyces *cinerochromogenes*, Streptomyces *thermocarboxydus*, Brevibacillus *laterosporus*, and Bacillus *velezensi*.

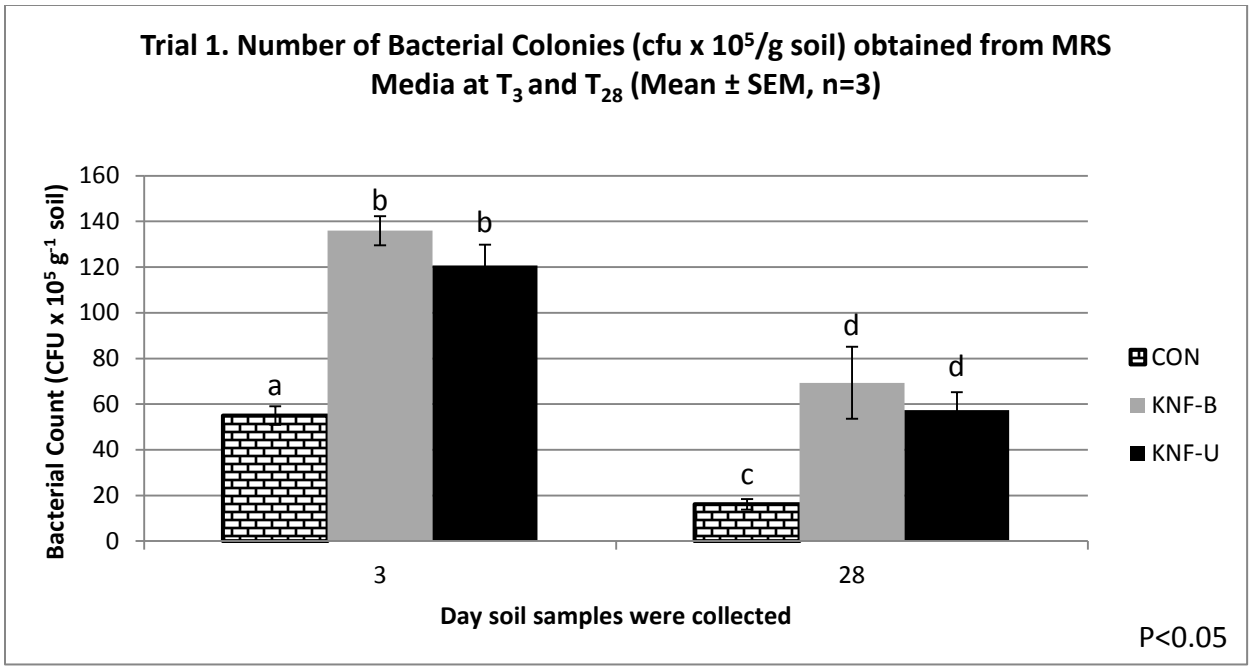



**Figure 12**. Trial 1. Bacterial population (cfu/g) on day 3 and day 28. Data represents the bacterial colonies cultured on MRS media. Lowercase alphabet indicates a significant difference at P<0.05 with each time of collection. KNF-B and KNF-U samples collected on day 3 ($T_3$; 136 x $10^5$ ± 6.4 CFU/g and 120.6 x $10^5$ ± 9.1 CFU/g, respectively) and 28 ($T_{28}$; 69.3 x $10^5$ ± 15.8 CFU/g and 57.3 x $10^5$ ± 7.9 CFU/g, respectively) contained a significantly higher amount of bacterial colonies compared to CON ($T_3$ = 55 x $10^5$ ± 4.1; $T_{28}$ = 16.1 x $10^5$ ± 22.7 CFU/g; P<0.05).

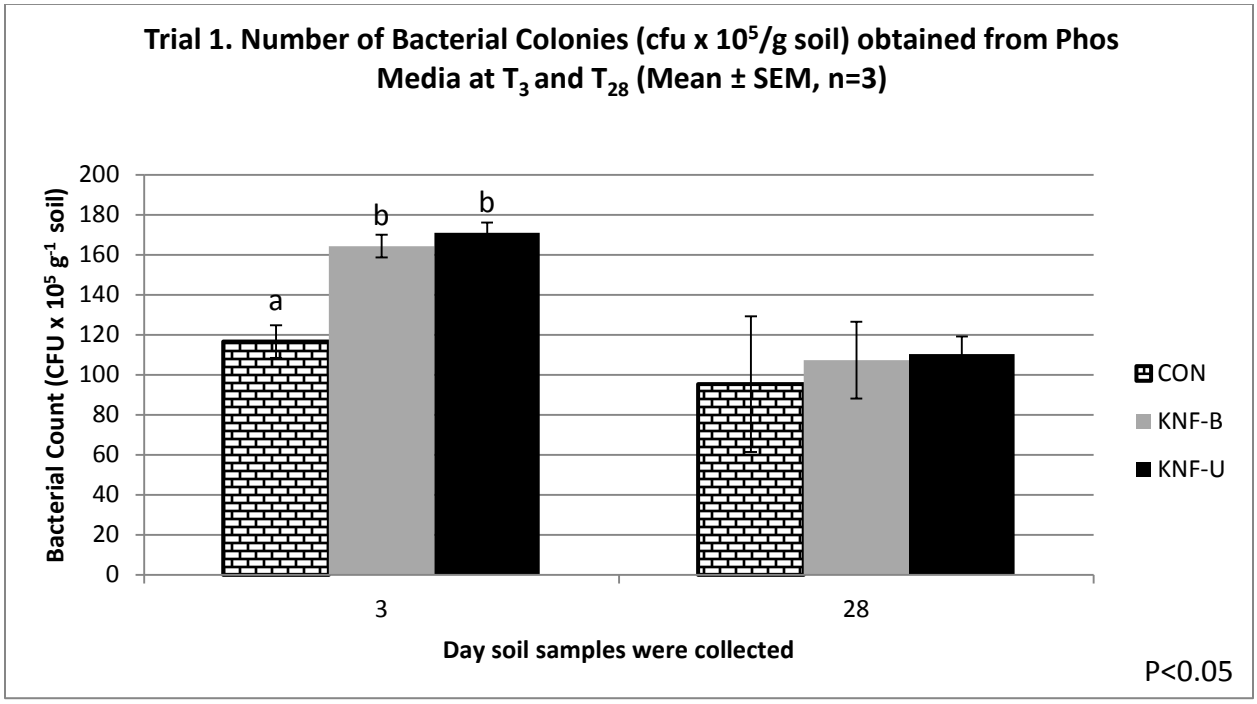



**Figure 13.** Trial 1. Bacterial population (cfu/g) on day 3 and day 28. Data represents the bacterial colonies cultured on phosphorus-solubilizing media. Lowercase alphabet indicates a significant difference at P<0.05 with each time of collection. KNF-B and KNF-U samples collected on day 3 ($T_3$; 164.3 x $10^5$ ± 5.7 CFU/g and 171 x $10^5$ ± 5.2 CFU/g, respectively) contained a significantly higher amount of bacterial colonies compared to CON (116.6 x $10^5$ ± 8.1 CFU/g; P<0.05). There was no significant difference between the soils collected at $T_{28}$.

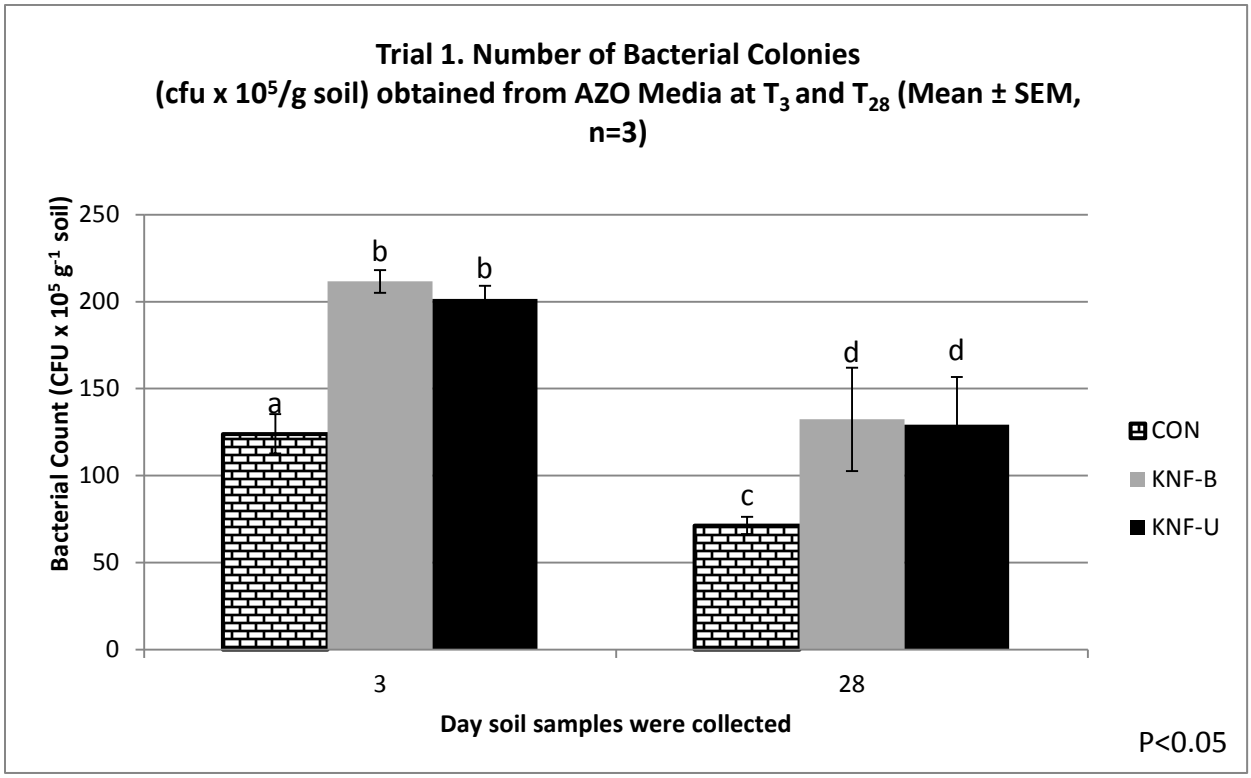



**Figure 14.** Trial 1. Bacterial population (cfu/g) on day 3 and day 28. Data represents the bacterial colonies cultured on AZO media. Lowercase alphabet indicates a significant difference at P<0.05 with each time of collection. KNF-B and KNF-U samples collected on day 3 ($T_3$; 211.6 x $10^5$ ± 6.5 CFU/g and 201.7 x $10^5$ ± 7.5 CFU/g, respectively) and 28 ($T_{28}$; 132.3 x $10^5$ ± 29.7 CFU/g and 129.3 x $10^5$ ± 27.4 CFU/g, respectively) contained a significantly higher amount of bacterial colonies compared to CON ($T_3$ = 124 x $10^5$ ± 11.3; $T_{28}$ = 71.3 x $10^5$ ± 4.95 CFU/g; P<0.05).

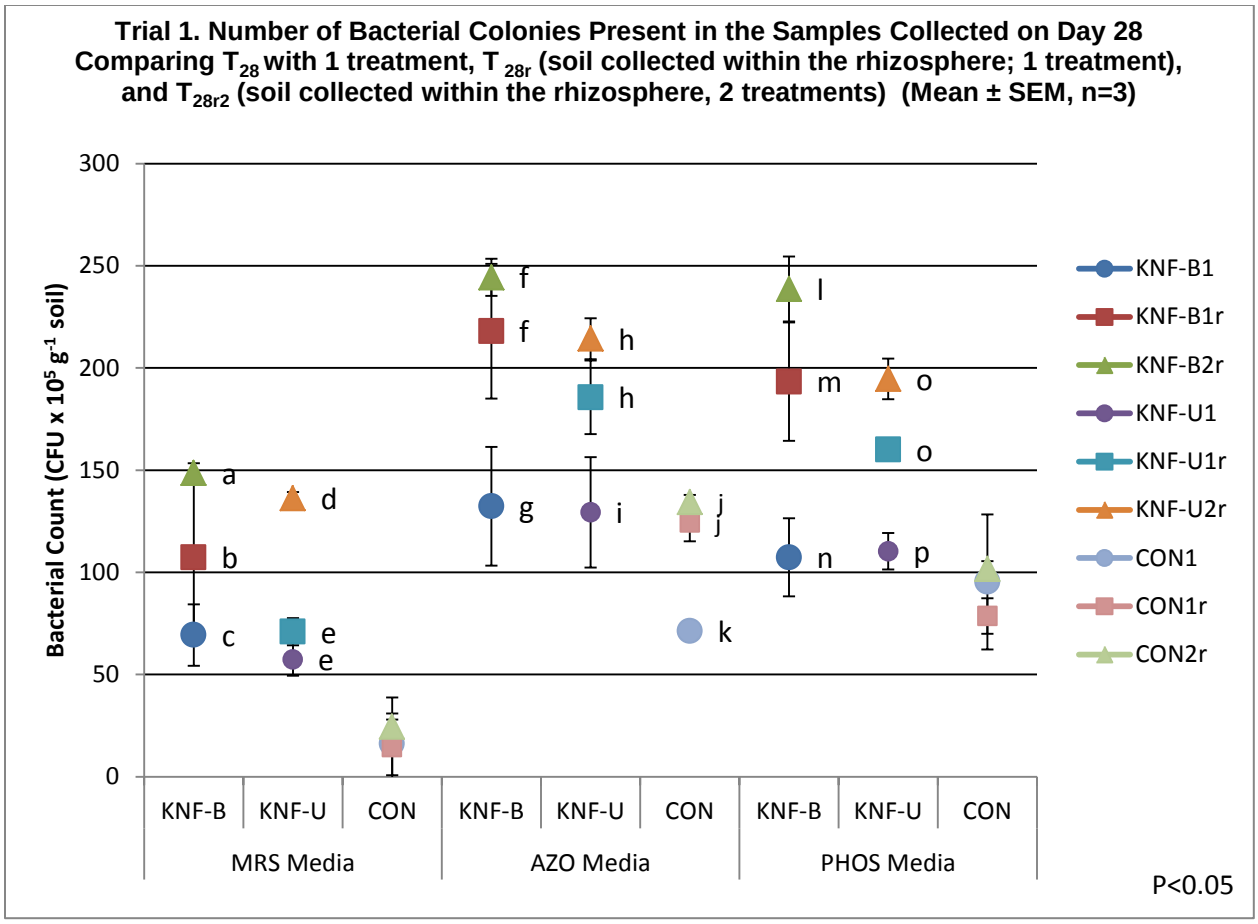



**Figure 15**. Trial 1. A statistical analysis was performed on the CFU count obtained from the soil samples collected on day 28. Three sets of soil samples were compared within each farming system; $T_{28}$ (soil collected from in-between the plants and received one application on day 1; $CON_1$, $KNF\text{-}B_1$, and $KNF\text{-}U_1$), $T_{28r}$ (soil collected within the rhizosphere, post-harvest, and received one application on day 1; $KNF\text{-}B_{1r}$, $KNF\text{-}U_{1r}$, and $CON_{1r}$), and $T_{28(2r)}$ (soil collected within the rhizosphere, post-harvest and from the plots that received 2 applications, the first on day 1 (plantlets planted on day 2) and the second on day 14; $CON_{2r}$, $KNF\text{-}B_{2r}$, and $KNF\text{-}U_{2r}$). Three different culturing media was used to obtain the CFU/g of soil; MRS media, Phosphorus-solubilizing media (PHOS), and Azospirillum media (AZO). In regards to the CON samples plated on the MRS and PHOS media, there was no significant difference between $CON_1$, $CON_{1r}$, and $CON_{2r}$. However, $KNF\text{-}B_{2r}$ contained significantly higher bacterial compared to the soil collected from in-between the plants that received just one application. Based on the data, reapplying IMO for a 2nd time in the KNF-B and KNF-U plots increased bacterial count overall. CFU is highest within the rhizosphere.

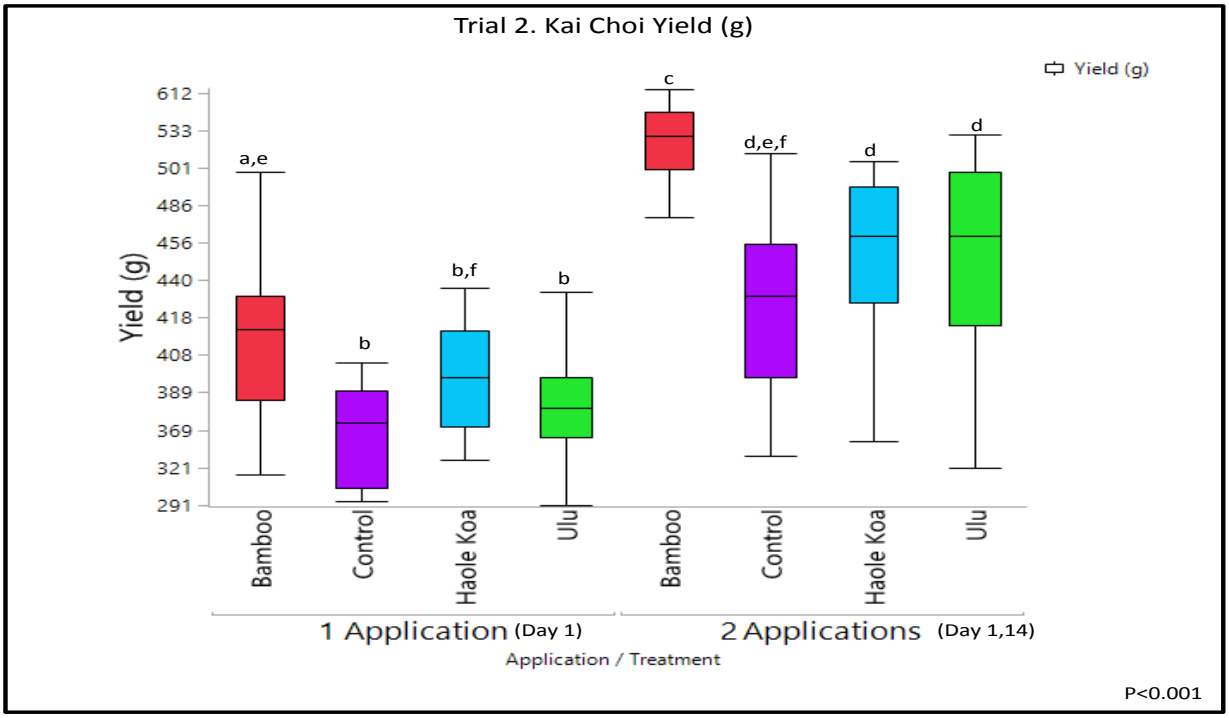



**Figure 16.** Trial 2. Box-plot of Kai Choi plant yield. A multiple comparisons (Tukey HSD) test was performed on the data. The band inside the box is the median, the bottom and top of the box are the 25% and 75% quartiles, respectively, and the lower and upper whiskers represent the minimum and maximum values, respectively. In regards to plots that received 1 application, KNF-B (IMO cultivated with Bamboo) had a significantly higher yield (mean=411.33 SE=8.3; P<0.001) than CON (Conventional; mean=358g SE=12.8; P<0.001), KNF-U (IMO cultivated with Ulu; mean=377.4g SE=8.54; P<0.001), and KNF-H (IMO cultivated with Haole koa; mean=392g SE=9.7; P<0.001). In reference to two applications, KNF-B (mean=535.15g SE=9.47; P<0.001) was significantly higher than CON (mean=428.2g SE=14.9; P<0.001), KNF-U (mean=455.4g SE=9; P<0.001) and KNF-H (mean=455.4g SE=13.4; P<0.001).

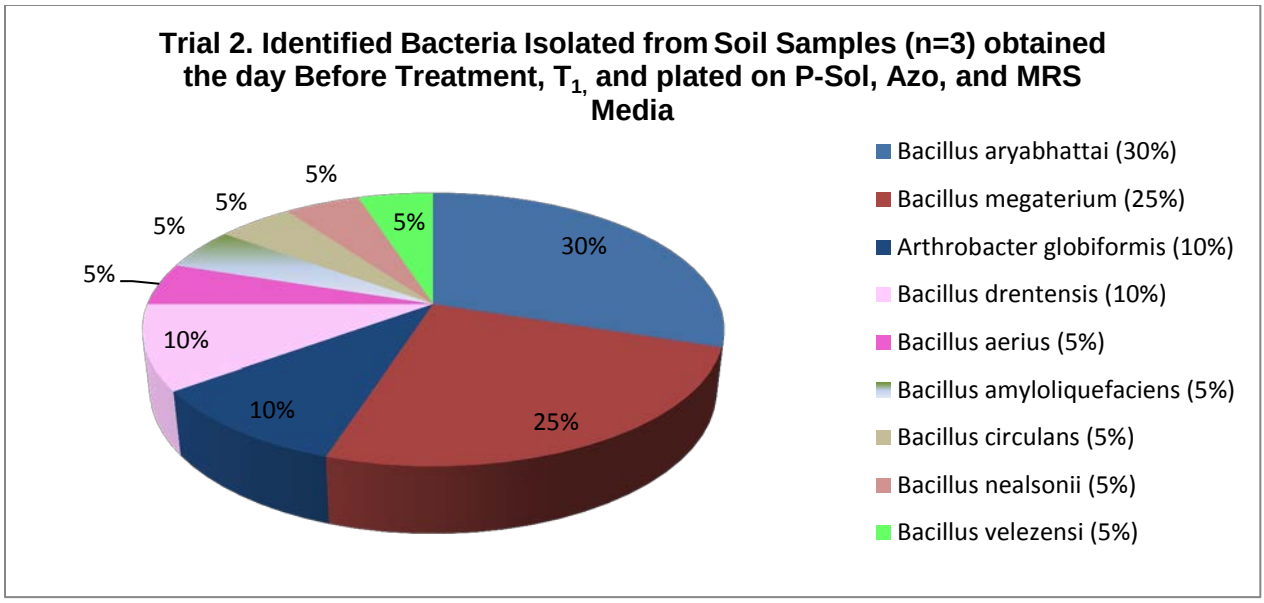



**Figure 17**. Pie chart summarizing the distribution of identified bacteria isolated from soil samples collected (n=3) prior to treatment; plated on P-Sol, Azo, and MRS Media. Many of the isolates identified were Bacillus *aryabhattai* (30%) and Bacillus *megaterium* (25%).

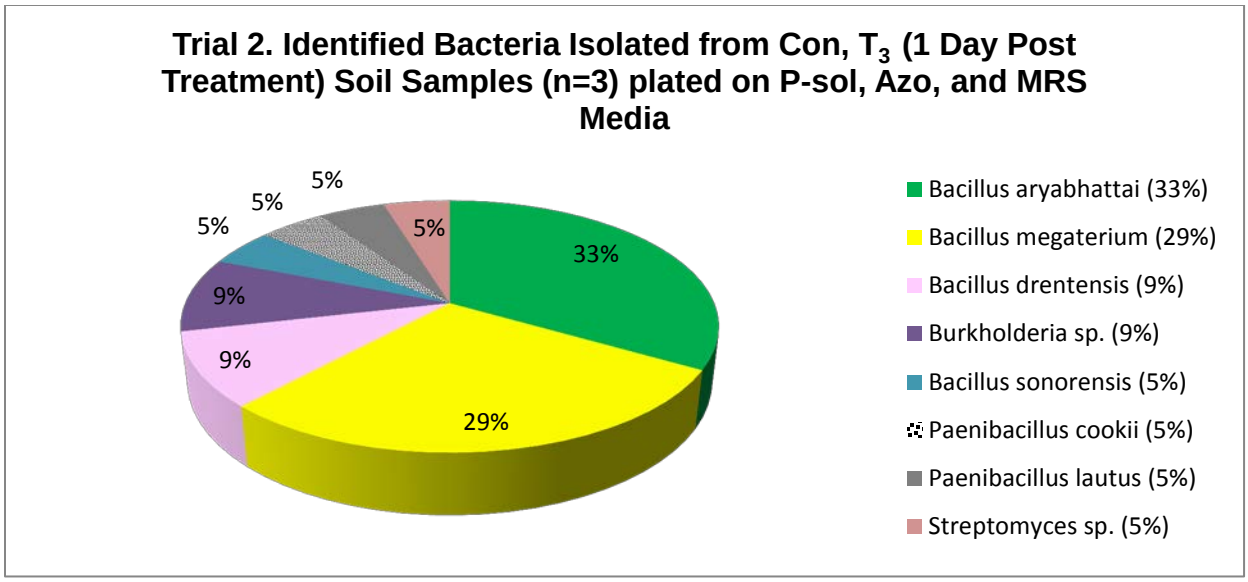



**Figure 18**. Pie chart summarizing the distribution of identified bacteria isolated from soil samples collected (n=3) from the Con plots one day after applying inorganic fertilizer to the soil; plated on P-Sol, Azo, and MRS Media. Many of the isolates identified were Bacillus *aryabhattai* (33%) and Bacillus *megaterium* (29%).

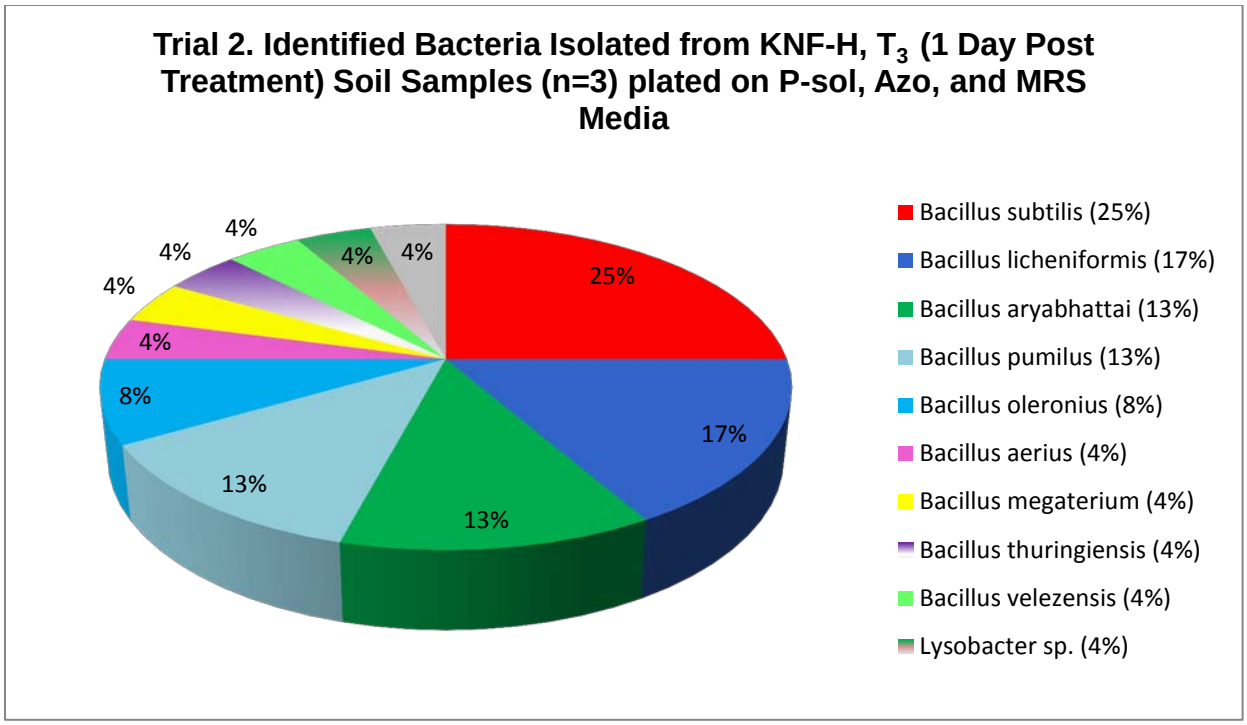



**Figure 19**. Pie chart summarizing the distribution of identified bacteria isolated from soil samples collected (n=3) from the KNF-H plots one day after the soil had been treated IMO #4 cultivated from haole koa (leucaena); plated on P-Sol, Azo, and MRS Media. Bacillus *subtilis* (25%) and Bacillus *licheniformis* (17%), Bacillus *aryabhattai* (13%) and Bacillus *megaterium* (13%) make up more than half of the bacteria identified.

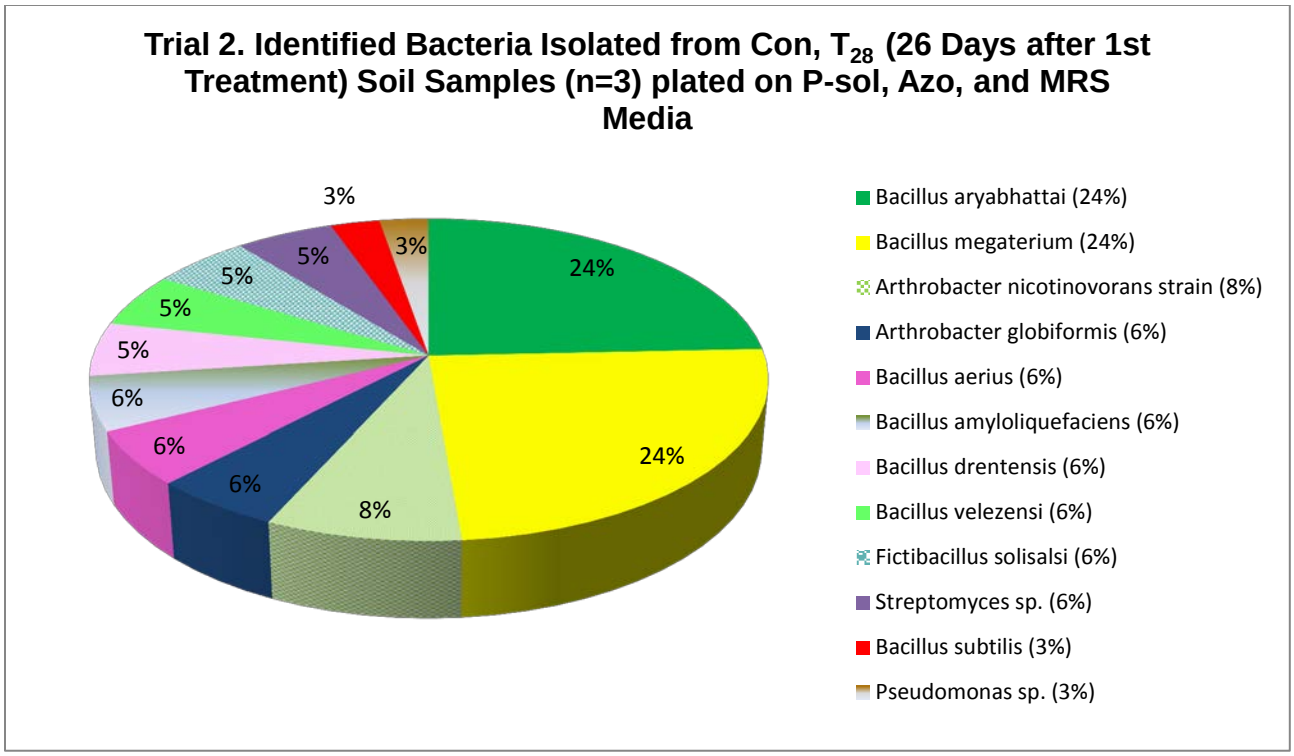



**Figure 20.** Pie chart summarizing the distribution of identified bacteria isolated from soil samples collected (n=3) from the Con plots 26 days post treatment; plated on P-Sol, Azo, and MRS Media. Bacillus *aryabhattai* (24%) and Bacillus *megaterium* (24%) were the most abundant.

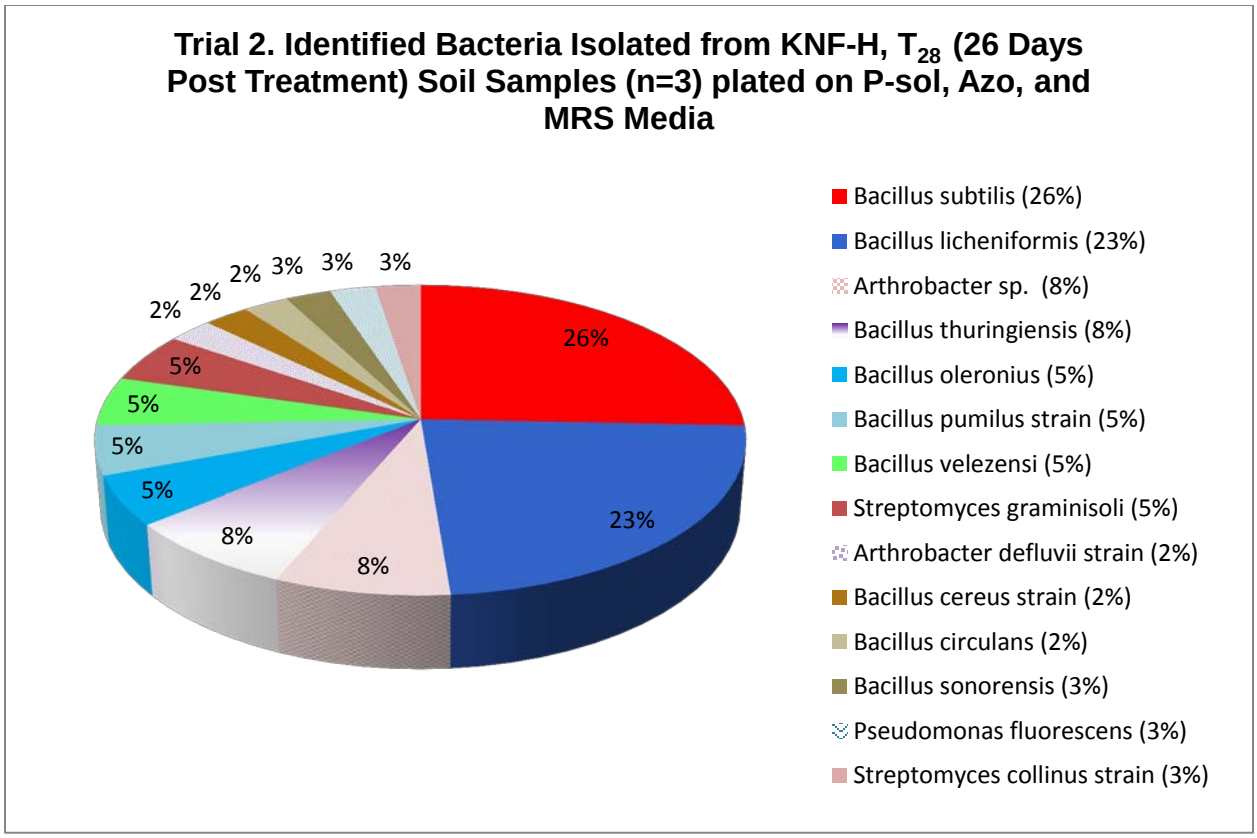



**Figure 21.** Pie chart summarizing the distribution of identified bacteria isolated from soil samples collected (n=3) from the KNF-H plots 26 days post treatment; plated on P-Sol, Azo, and MRS Media. Bacillus *subtilis* (26%) and Bacillus *licheniformis* (23%) make up nearly half of the bacteria identified.

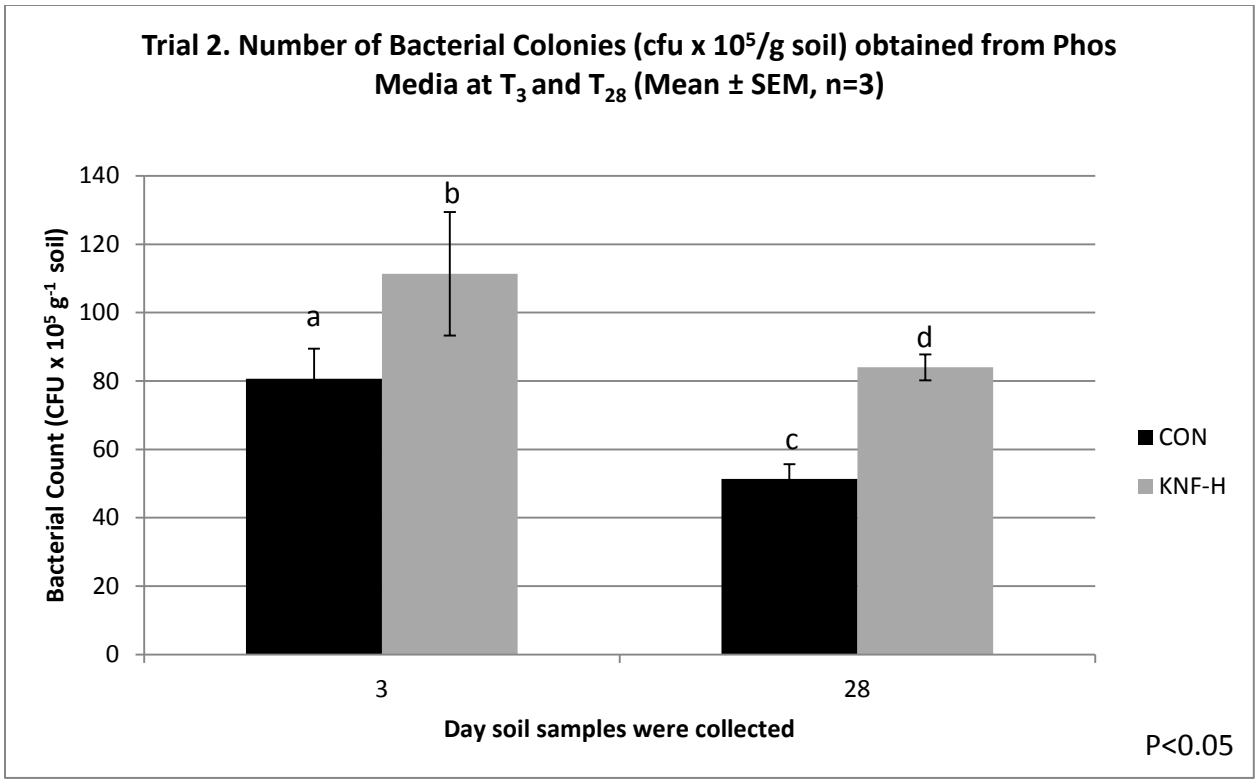



**Figure 22**. Trial 2. Bacterial population (cfu/g) on day 3 and day 28. Data represents the bacterial colonies cultured on phosphorus-solubilizing media. Lowercase alphabet indicates a significant difference at P<0.05 with each time of collection. KNF-H (IMO cultivated with Haole koa) soil samples collected on day 3 ($T_3$) and day 28 ($T_{28}$) contained significantly higher bacterial colonies (111.3 x $10^5$ ± 18.1 CFU/g and 84 x $10^5$ ± 3.8 CFU/g, respectively) compared to CON ($T_3$ = 80.7 x $10^5$ ± 8.8 CFU/g; $T_{28}$ = 51.3 x $10^5$ ± 4.4 CFU/g; P<0.05).

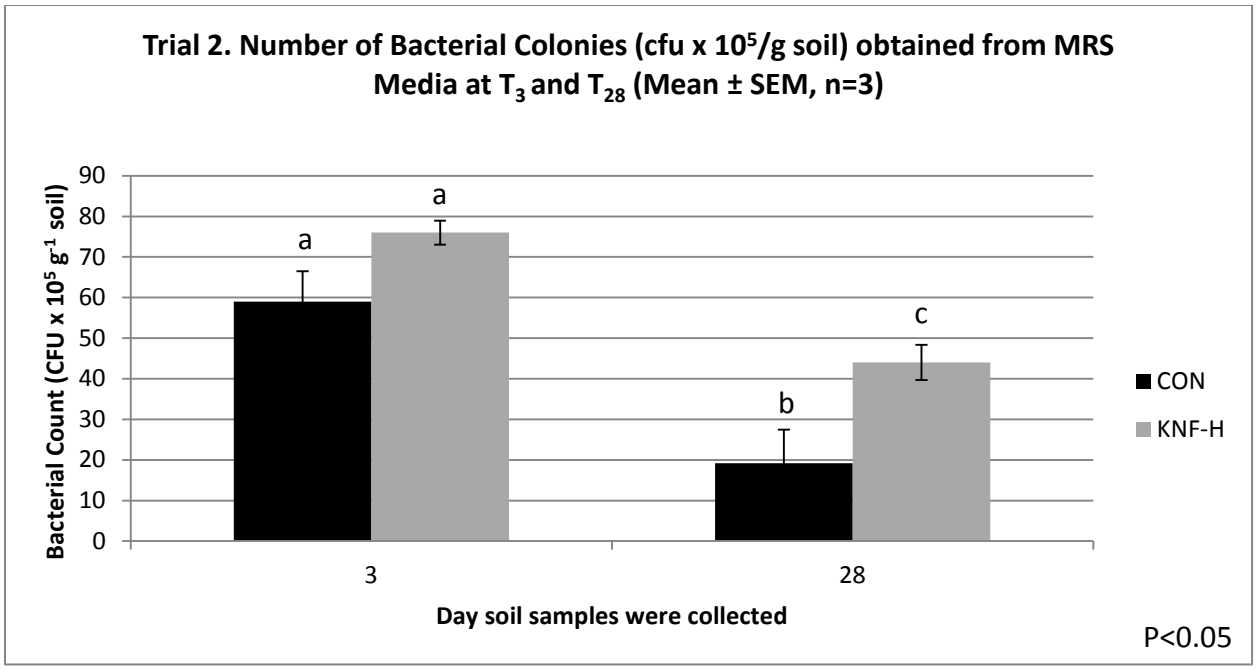



**Figure 23.** Trial 2. Bacterial population (cfu/g) on day 3 and day 28. Data represents the bacterial colonies cultured on MRS media. Lowercase alphabet indicates a significant difference at P<0.05 with each time of collection. Soil samples obtained from KNF-H (IMO cultivated with Haole koa) plots on day 28 ($T_{28}$) contained significantly higher bacterial colonies (44 x $10^5$ ± 4.3 CFU/g) compared to CON ($T_{28}$ = 19.2 x $10^5$ ± 8.3 CFU/g). There was no significant difference between the soils (KNF-U and CON) collected on day 3, $T_3$.

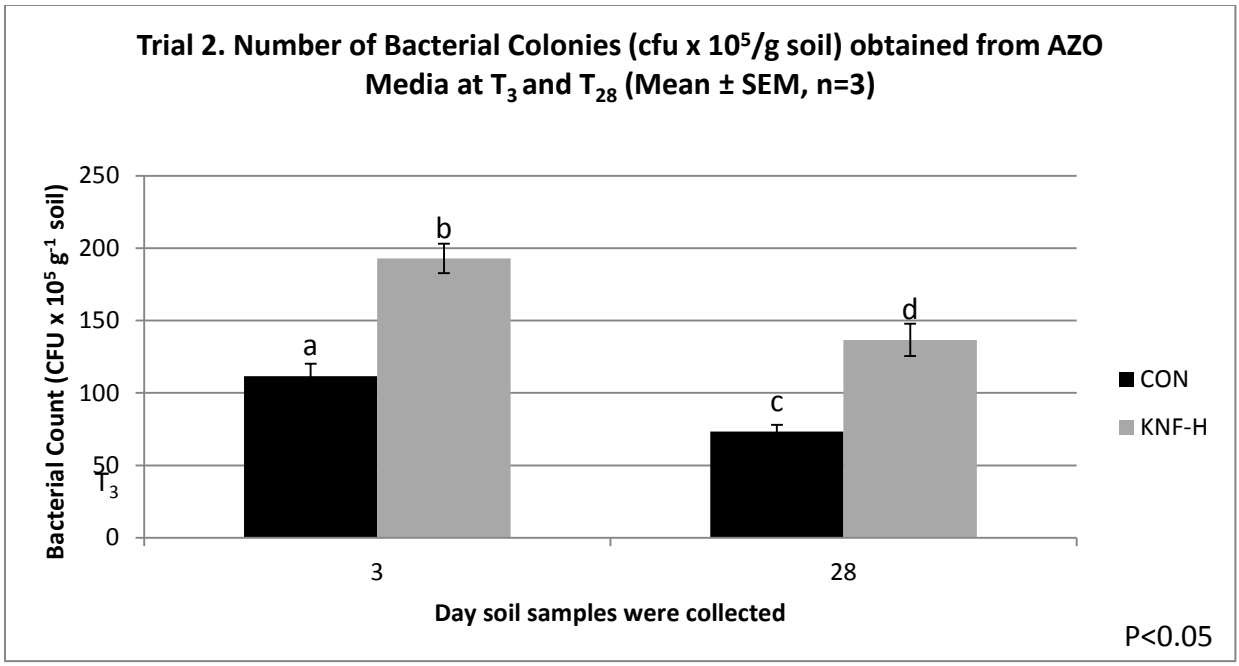



**Figure 24**. Bacterial population (cfu/g) on day 3 and day 28. Data represents the bacterial colonies cultured on AZO media. Lowercase alphabet indicates a significant difference at P<0.05 with each time of collection. KNF-H (IMO cultivated with Haole koa) soil samples collected on day 3 ($T_3$) and day 28 ($T_{28}$) contained significantly higher bacterial colonies (193 x $10^5$ ± 10.1 CFU/g and 136 x $10^5$ ± 11 CFU/g, respectively) compared to CON ($T_3$ = 111.7 x $10^5$ ± 8.6 CFU/g; $T_{28}$ = 73.3 x $10^5$ ± 4.6 CFU/g; P<0.05).

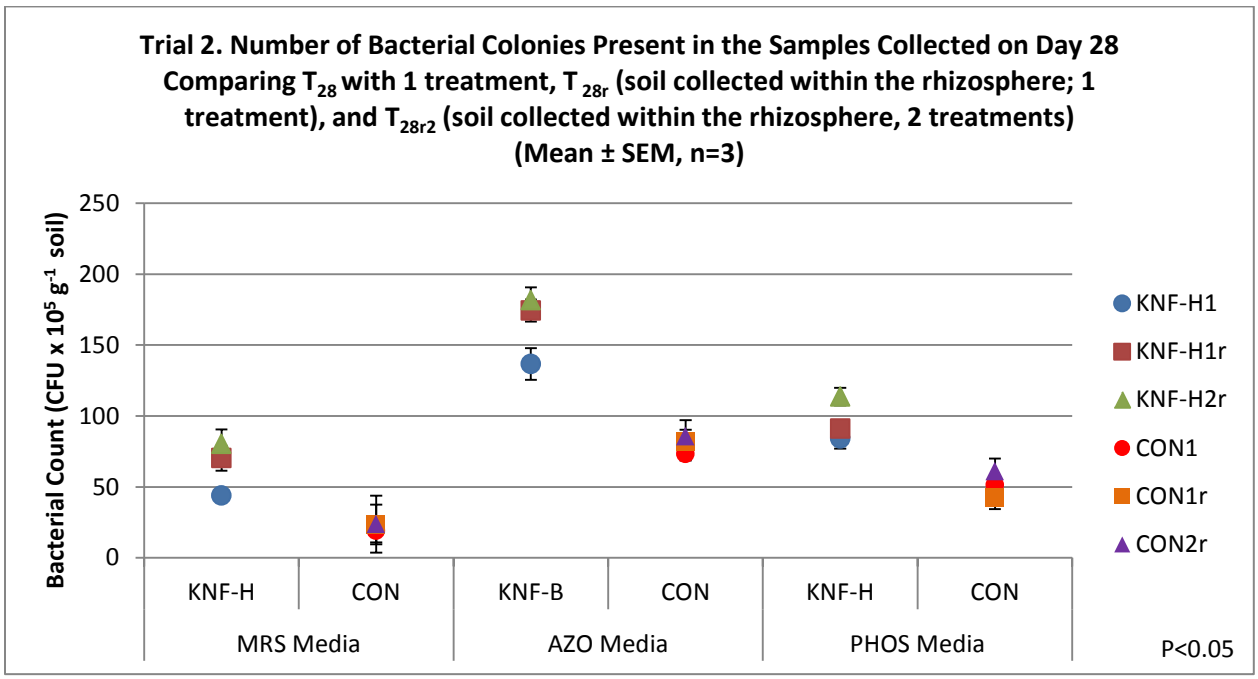



**Figure 25.** Trial 2. A statistical analysis was performed on the CFU count obtained from the soil samples collected on day 28. Three sets of soil samples were compared within each farming system; $T_{28}$ (soil collected from in-between the plants and received one application on day 1; $CON_1$ and KNF-$H_1$), $T_{28r}$ (soil collected within the rhizosphere, post-harvest, and received one application on day 1; KNF-$H_{1r}$ and $CON_{1r}$), and $T_{28(2r)}$ (soil collected within the rhizosphere, post-harvest and from the plots that received 2 applications, the first on day 1 (plantlets planted on day 2) and the second on day 14; $CON_{2r}$ and KNF-$H_{2r}$). Three different culturing media was used to obtain the CFU/g of soil; MRS media, Phosphorus-solubilizing media (PHOS), and Azospirillum media (AZO). There were no significant difference between any of the treatments/media.

# Chapter 5

## Enhanced growth of Genovese basil (*Ocimum basilicum*) in tissue culture systems by *Bacillus subtilis* and *Pseudomonas Aeruginosa*

## 5.1 Abstract

In recent years, there has been a growing interest in Korean Natural Farming (KNF). However, there has been minimal scientific documentation as to how this system works or what types of bacteria are prevalent within the system. Recently, an attempt been made to unravel the soil bacteria population associated with KNF. These studies have shown that KNF contained an abundance of *Bacillus subtilis*. It had also been determined that the collection site of IMO affects plant yield – IMO cultivated from Bambusoideae (KNF-B; bamboo) produced a higher crop yield (Brassica *juncea*) than IMO cultivated from Artocarpus *altilis* (breadfruit/ulu), Leucaena *leucocephala* (haole koa) and conventionally treated plants. KNF-B contained a ubiquitous amount of *B. subtilis* and *Pseudomonas aeruginosa* in the soil samples collected near the rhizosphere – the home of many plant growth-promoting rhizobacteria (PGPR). Hence, the sole purpose of this study was to investigate the effects of *B. subtilis* and *P. aeruginosa* on plant growth in the hopes of providing a better understanding as to the role it may play in KNF. *Ocimum basilicum* (genovese basil) seeds were surface sterilized and inoculated with the following treatments – 1) MRS broth, 2) *B. subtilis* ($10^9$ CFU/mL), 3) azospirillum broth, 4) *P. aeruginosa* ($10^9$ CFU/mL), 5) *B. subtilis* + *P. aeruginosa* ($10^9$ CFU/mL), and 6) deionized water. The inoculated seeds were then placed into a sterile vessel containing growth media. The vessels were then placed under light-emitting diode (LED) grow lights with a photoperiod of 16 h of light and 8 h of darkness (25 ± 3◦C). There were 9 seeds per vessel and 8 vessels per treatment (72 seeds per treatment). Germination rate was recorded on day 2, 3, 4, and 5. Root length, stem length, and emergence of lateral roots were recorded on day 7. On day 9, a second treatment was re-applied (1mL inoculated directly onto the surface of the growth media) to half of the culture vessels (4 vessels per treatment). On day 16, a third application was re-applied (1mL inoculated directly onto the surface of the growth media) to two vessels per treatment (the two vessels were randomly selected from the 4 vessels that received 2 applications). Hence, each treatment contained 4 vessels with one application (day 1), 2 vessels with two applications (days 1 and 9) and 2 vessels with three applications (days 1, 9, and 16). Dry weights of the root and stem/leaf were recorded on day 21 (half of the plants in each vessel were selected at random) and 28 (remaining plants were weighed). The results showed that the seeds treated with MRS broth, *B. subtilis*, azospirillum broth, *P. aeruginosa*, and *B. subtilis* + *P. aeruginosa* had a significantly higher germination rate than the seeds

treated with deionized water, for all days. In terms of leaf/stem dry weight, plants inoculated with *B. subtilis* (1 application = 0.177 ± 0.007g; 2 applications = 0.00.165 ± 0.0081g; and 3 applications = 0.181 ± 0.015g) and *P. aeruginosa* + *B. subtilis* (1 applications = 0.171 ± 0.00273g; 2 applications = 0.168 ± 0.0044g; and 3 applications = 0.169 ± 0.005g), was significantly higher than all other treatments/applications.

**5.2 Introduction**

Photosynthetic plants play a crucial role in regulating the life cycles of living organisms and nutrient cycling (Wright and Jones, 2006; Hartmann *et al*., 2009). They maintain the atmosphere by converting carbon dioxide into oxygen which is essential for cellular respiration for all aerobic microorganisms (Costa *et al.*, 2006). Roots play a significant role in the growth and development of plants as they provide structural support, absorb water and minerals, and store nutrients (Berg and Smalla, 2009). In addition, roots provide shelter and nutrients to microorganisms such as protozoa, fungi, and bacteria. In turn, these microorganisms, specifically bacteria, aid the root system in nutrient uptake, nitrogen fixation, and defense against pathogens. This type of symbiotic relationship that occurs between bacteria and plant roots takes place within the rhizosphere. The rhizosphere is the narrow zone of soil specifically influenced by the root system (Dobbelaere *et al.,* 2003). This zone is rich in nutrients due in part to the accumulation of varying plant exudates that include sugars, amino acids, polysaccharides, and ectoenzymes (Gray and Smith, 2005). The relationship between bacteria and plants can be positive, negative, or neutral (Dobbelaere *et al.,* 2003). Beneficial bacteria that colonize plant roots and promote plant growth are referred to as plant growth-promoting rhizobacteria (PGPR; Beneduzi *et al.,* 2012). Some PGPR possess the ability to inhibit the growth of harmful nematodes, control the biological function of plants, create a pathogen-induced system (make plant varieties more resistant to specific pathogens), decrease the fungal growths around various plants, and solubilize nutrients for easy uptake by plants (Akhtar *et al.,* 2012; Beneduzi *et al.,* 2012; Prathap, & Kumari, 2015; and Usha, 2015). PGPR's role in stimulating plant growth can be broadly categorized as either direct or indirect (Kloepper, 1993). With direct stimulation, PGPR facilitates plant growth by producing metabolites or compounds (i.e. modulating plant hormone levels or assisting in resource acquisition). Indirect growth promotion, on the other hand,

affects other factors in the rhizosphere (i.e. by decreasing the inhibitory effects of different pathogens on plant growth) which in turn results in plant growth stimulation.

These bacteria are commonly used as bio-fertilizers in organic farming and sustainable agriculture ecosystems. One farming practice whose methods are based on utilizing such beneficial bacteria is Korean natural farming (KNF). KNF is a self-sufficient system that involves collecting and cultivating indigenous microorganisms (IMO) – fungi, bacteria, and protozoa – and reintroducing them into nutrient depleted soil, further enhancing soil microorganism activity and fertility (Essoyan, 2011). Previous studies (Poamoho and Kula Trial) have shown that not only does KNF have a greater diversity of bacteria compared to organic and conventional methods but also higher bacterial colonies (refer to Chapter 2). It was also determined that KNF contained an abundance of *Bacillus subtilis* and *Bacillus Licheniformis*. In the Makaha Farm trial (Chapter 4), we learned that the collection site of IMO affects plant yield – IMO cultivated from Bambusoideae (KNF-B; bamboo) produced a higher crop yield (Brassica *juncea*) than IMO cultivated from Artocarpus *altilis* (KNF-U; breadfruit/ulu), Leucaena *leucocephala* (KNF-H; haole koa) and conventionally treated plants (synthetic fertilizer, NPK 16-16-16). Additionally, this study showed that $\geq$ 40% of the bacteria isolated and identified from KNF-U, KNF-B, and KNF-H were *B. subtilis* and *B. licheniformis* and that a ubiquitous amount of *Pseudomonas aeruginosa* was present only in KNF-B. Provided this information, this begs the question as to what makes bamboo the optimal site for collecting and cultivating IMO in a KNF system. Part of the answer may lie in the presence of these specific bacteria.

*B. subtilis* is a PGPR that has been the sole focus of many studies and has shown much promise in promoting plant growth in many ways (Errington 2003; Yanez-Mendizabal et al. 2012). In fact, given the right conditions, B. *subtilis* has the ability to accomplish the primary functions of most PGPR (Qiao *et al*., 2017). Because of its growth-promoting capabilities and its ability to produce heat-resistant endospores, some seed companies will inoculate their seeds (i.e. cotton seeds, tomato seeds, cowpea seeds) with *B. subtilis* (in addition to other seed treatment insecticides and fungicides) in order to increase germination rate/viability and overall health of seedling (Minaxi *et al.* 2012). Inoculating seeds with *B. subtilis* has

shown to increase phosphorus solubilization and anti-fungal and ACC (1-Aminocyclopropane-1-Carboxylate) deaminase activity.

*P. aeruginosa,* on the other hand, is nonspore-forming and less resilient. While some studies prove *P. aeruginosa* to be beneficial to plants, in terms of growth promoting, others have shown this bacterium to be an opportunistic pathogen of both humans and plants (Weihui *et al.,* 2015). Whether it will have a positive or negative effect is highly dependent on the strain (Walker *et. al.,* 2004; Radhapriya *et al.,* 2015). In one study, inoculating wheat with *P. aeruginosa* improved nutrient uptake and plant biomass in wheat under Zn (zinc) stress (Islam *et al., 2014*). In another study, *P. aeruginosa* inhibited the growth of arabidopsis and genovese basil by infecting the root system which in turn decreased nutrient uptake of the plants (Walker *et. al.,* 2004).

Hence, in order to determine what role *B. subtilis* and *P. aeruginosa* plays on plant growth, *Ocimum basilicum* (genovese basil) seeds will be inoculated with each bacterium obtained from the previous study or in combination to determine the effects. Furthermore, the seeds will be surface sterilized prior to inoculation and then placed into a sterile jar containing basal growth media (in-vitro). This will allow for control of what's present in the medium and what's not. Had we chosen to plant the inoculated seeds in soil, which farmers commonly practice, it would be impossible to tell what other types of microorganisms are present (within the soil) and how they affect the treatments, whether direct or indirect. Performing the experiment in-vitro means that no other microorganisms, besides the inoculum/treatment, will be present. *B. subtilis* was selected for this study for the fact that it was the bacteria most prevalent in KNF. *P. aeruginosa* was chosen because of its dominance in KNF-B. The sole purpose of this study was to investigate the effects of these bacteria on plant growth in the hopes of providing a better understanding as to the role it may play in KNF.

### 5.3 Materials and Methods

**Isolation of Bacteria from Soil.** The two bacterial strains used in this experiment, *Bacillus subtilis* and *Pseudomonas aeruginosa*, were obtained from a previous study conducted at Hoa Aina O Makaha, Hawai'i. Please refer to chapter 4 for a detailed guide of the isolation process.

**Inoculum Preparation.** *Bacillus subtilis* was grown (37◦C overnight) and maintained in MRS broth (De Man, Rogosa, and Sharpe; $l^{-1}$: Difco Lactobacilli MRS Broth, 55 g (BD™, Franklin Lakes, New Jersey)). *Pseudomonas aeruginosa* was grown (37◦C overnight) and maintained in azospirillum broth ($l^{-1}$: $K_2HPO_4$, 5 g; $MgSO_4 \cdot 7H20$, 0.975 g; NaCl, 1 g; yeast extract, and 0.5 g; the pH was adjusted with 1M HCl to 6.8 prior to autoclaving; Hurst *et al.*, 2000). The bacterial growth was measured by optical density at 600 nm (OD600) and serial dilution with plate counts, as described by Zhang *et al.* (2008). At the appropriate intervals (approximately every 4±8 h during lag and stationary phases and about every 2 h during log phase), the absorbance was measured in triplicate at 660 nm. To maintain accuracy, each culture was vortexed prior to absorbance readings. Absorbance was transformed into cell concentration using calibration curves. The target inoculum concentration for both bacteria (used in this study) was $10^9$ colony forming units $mL^{-1}$ (Kacena *et al.*, 1999; LaBauve *et al.*, 2012). The tubes containing the correct concentration ($10^9$ CFU/mL) of both samples were then placed into a centrifuge for 10 minutes at 8000rpm. Following centrifugation, the supernatant was removed with a 10 mL syringe and then filtered through a 0.2 µm syringe-driven membrane filter into an empty sterile tube (Fisher Scientific, Waltham, MA, USA). The samples, which were now ready to use, were stored 2–8°C prior to use.

**Plant Growth Media Preparation.** The growth media contained the following ingredients $l^{-1}$: deionized water, 963.5 mL; Murashige and Skoog (MS) medium w/vitamins, 4.43 g; sucrose, 30 g; gelrite, 2.25g, 15 g; the pH was adjusted to 5.6 prior to autoclaving (Murashige and Skoog,1962). Approximately 75 mL of growth media was added to 48 sterilized culture vessels (75mm x 75mm x 98mm; PTL-100™, Phytotech, Lenexa, Kansas).

**Seed Sterilization Treatment.** The seeds used in this experiment, genovese basil (*Ocimum basilicum*), were obtained from Burpee (Warminster, PA). Approximately 360 seeds were placed into a 50mL centrifuge tube followed by 35mL of 70% (v/v) isopropyl alcohol. The tube was immediately capped and shaken by hand for 1 minute. The seeds were then placed into a strainer and rinsed continuously (over a non-sterile sink) with deionized water for 1 minute. The next steps were performed in a laminator flow hood (aseptic environment). The rinsed seeds were placed into a beaker containing the following ingredients $l^{-1}$: 5 % (v/v) sodium hypochlorite; 0.05% Tween 20; bring up the volume to 1L with sterile

deionized water. The seeds remained in the solution, with occasional stirring, for ten minutes followed by three washes with sterilized deionized water. After the final rinsing, the seeds were placed into a sterile 6 well plates (~ 60 seeds per well), ready to be inoculated.

**Inoculation of Seeds.** The well plates (6 in total) were inoculated (day 1) with 1 mL of the following: well 1 – sterile deionized water (control); well 2 – azospirillum broth (control); well 3 – MRS broth (control); well 4 – *B. subtilis* suspended in MRS broth; well 5 – *P. aeruginosa* suspended in azospirillum broth; and well 6 – *B. subtilis* (suspended in MRS broth) and *P. aeruginosa (*suspended in azospirillum broth). The seeds remained in the well plate, exposed to these inoculums for 24 h (22 ± 2◦C in darkness). Following this period, the seeds were then placed into the culture vessel containing the growth media. There were 9 seeds per vessel and 8 vessels per treatment (72 seeds per treatment). The vessels were then placed under led grow lights with a photoperiod of 16 h of light and 8 h of darkness (25 ± 3◦C; Saha *et al*., 2016). Germination rate was recorded on days 2, 3, 4, and 5. Root length, stem length, and the emergence of lateral roots were recorded on day 7. On day 9, a second treatment was re-applied (1mL inoculated directly onto the surface of the growth media) to half of the culture vessels (4 vessels per treatment). On day 16, a third application was re-applied (1mL inoculated directly onto the surface of the growth media) to two vessels per treatment (the two vessels were randomly selected from the 4 vessels that received 2 applications). Hence, each treatment contained 4 vessels with one application (day 1), 2 vessels with two applications (days 1 and 9) and 2 vessels that received three applications (days 1, 9, and 16). Dry weights of the root and shoot were recorded on day 21 (half of the plants in each vessel were selected at random) and 28 (remaining plants were weighed). To obtain the dry weights, the shoots and stems were first air-dried overnight and then placed into an oven set at 70◦C for 24 hours.

The two bacteria used in this study, *B. subtilis* and *P. aeruginosa,* were suspended/cultured in MRS and azospirillum broth, respectively. As a result, MRS and azospirillum broth were two of the six treatments used in this experiment, in addition to the seeds inoculated with sterile deionized water. These three treatments were used as controls. Differences between MRS broth and *B. subtilis* suspended in MRS broth as well as azospirillum broth and *P. aeruginosa* suspended in azospirillum broth may be determined.

**5.4 Results**

Figure 1 represents the cumulative total of newly germinated seeds observed on days 2, 3, 4, and 5 of the experiment (initial inoculation took place on day 1). The data shows that the seeds treated with MRS (inoculated with MRS broth), BS/MRS (inoculated with *B. subtilis* suspended in MRS broth), AZO (inoculated with azospirillum broth), PA/AZO (inoculated with *P. aeruginosa* suspended in azospirillum broth), and BS/PA (inoculated with both *B. subtilis* and *P. aeruginosa*) had a significantly higher germination rate than the CON (inoculated with deionized water) treated seeds, for all days. In terms of lateral roots, MRS (43.8%), BS/MRS (63.3%), and BS/PA (59.6%) had a significantly higher emergence of lateral roots by day 7 than CON (control; 26.6%), AZO (16.3%), and PA/AZO (28.3%; P>0.001; Figure 2). The stem/shoot height (mm) was measured and recorded on day 7 (n=72; Figure 3). There were no significant difference between the plants treated with MRS broth (MRS; mean=7.4mm SE=0.253; P<0.001), *B. subtilis* (BS/MRS; mean=7.94mm SE=0.185; P<0.001), and *P. aeruginosa* + *B. subtilis* (BS/PA; mean=8.12mm SE=0.206; P<0.001). However, all three had significantly greater stem lengths than the plants treated with deionized water (CON; mean=6.14mm SE=0.253; P<0.001), azospirillum broth (AZO; mean=6.06mm SE=0.258; P<0.001) and *P. aeruginosa* (PA/AZO; mean=6.91mm SE=0.376; P<0.001). Root length (mm) was measured and recorded on day 7 as well (n=72; Figure 4). There were no significant difference between the plants treated with deionized water (CON; mean=29.07mm SE=1.076; P<0.001), azospirillum broth (AZO; mean=31.27mm SE=1.152; P<0.001), and *P. aeruginosa* (PA/AZO; mean=31.46 SE=1.075; P<0.001). Plants treated with *B. subtilis* (BS; mean=47.9mm SE=0.149; P<0.001) and *P. aeruginosa* + *B. subtilis* (BS/PA; mean=48.56mm SE=0.821; P<0.001) were significantly higher than all other treatments. Figure 5 represents the root weight (DM) obtained from 21 day old plants. Lowercase alphabet indicates a significant difference at P<0.0001 within each application. Plants inoculated with *B. subtilis* (1app = 0.0378 ± 0.00127g) and *P. aeruginosa* + *B. subtilis* (1app = 0.0374 ± 0.00138g) had a significantly higher DM root weight than plants treated with deionized water (1app = 0.029 ± 0.0018g). There was no significant difference between any of the treatments that received 2 or 3 applications/inoculations. Figures 6 and 7 represents the dry weights of stem/leaf collected on day 21 and roots collected on day 28, respectively. The results showed that there was no significant difference between any of the treatments or application rate.

Figure 8 represents dry weights of stem/leaf collected on day 28. In regards to the plants that received one inoculation, those treated with *B. subtilis* (1app = 0.177 ± 0.007g) had a significantly higher leaf/stem weight than plants treated with deionized water (No trt; 1app = 0.0.145 ± 0.00591g), MRS (1app = 0.15 ± 0.004g), AZO (1app = 0.148 ± 0.007g), and *P. aeruginosa* (1app = 0.148 ± 0.008g). There was no significant difference between the treatments that received two inoculations. As for the plants that received three applications, those treated with *B. subtilis* (3app = 0.18 ± 0.015g) had a significantly higher dry stem/leaf weight than plants treated with AZO (3app = 0.0.14 ± 0.007g).

**5.5 Discussion**

The sole focus of this study was to investigate the effect *B. subtilis* and *P. aeruginosa* have on plant growth in the hopes of providing a better understanding as to the role they may play in KNF. The data showed that seeds treated with MRS broth, azospirillum broth, *B. subtilis*, *P. aeruginosa*, or *B. subtilis* + *P. aeruginosa* had a significantly higher germination rate than the control (seeds treated with deionized water; Figure 1). According to Pérez-Fernández *et al*. (2006), pH greatly affects seed germination. For most plants, the optimal pH range is 5.0 - 7.0 (Kumar *et al.*, 2014). Unfortunately, the pH of each inoculum was not measured and recorded. Additionally, to my knowledge, no study has been conducted to determine the optimum pH for germinating genovese basil. Therefore, whether or not pH played a role cannot be determined.

Seeds inoculated with MRS broth, *B. subtilis*, and *B. subtilis* + *P. aeruginosa*, all of which contain MRS, had significantly higher lateral root formation and longer stems (on day 7) compared to seeds inoculated with *P. aeruginosa*, deionized water, and azospirillum broth (Figures 2 and 3). The data seems to suggest that MRS promotes plant growth to some capacity. MRS consists of proteose peptone, beef extract, yeast extract, dextrose, polysorbate 80, ammonium citrate, sodium acetate, magnesium sulfate, manganese sulfate, and dipotassium phosphate (Fisher Scientific, Pittsburgh, PA). The plant growth media used in this experiment contained the following nutrients: sucrose, ammonium nitrate, boric acid, calcium chloride, cobalt chloride, cupric sulfate, $Na_2EDTA$, ferrous sulfate, magnesium sulfate, manganese sulfate, molybdic acid, potassium iodide, potassium nitrate, potassium phosphate, zinc sulfate, glycine, myo-inositol, nicotinic acid, pyridoxine, and thiamine (Murashige and Skoog, 1962).

There are similarities between the MRS broth and growth media. Many of the ingredients present in MRS broth have proven to promote plant growth. When applied to the soil, ammonium citrate increased plant biomass of wheat (Sanaullah, 1986). When yeast extract was applied to potato plants (*Solanum tuberosum L.),* the results showed that foliar application of yeast extract increased vegetative growth in terms of plant and stem length, number of leaves, and biomass (Ahmed *et al.,* 2011). Manganese and magnesium (sulfate) are both essential nutrients plants require for optimal growth. A deficiency in either of these nutrients may result in interveinal chlorosis, yellowing of leaves, and stunted growth (Brenchley, 1936). In addition to the vitamins and minerals present in the plant growth media, the basil seedlings may have also been utilizing the nutrients from the MRS broth as well to aid in growth. This could explain why the seedlings containing MRS broth (including *B. subtilis* and *P. aeruginosa* + *B. subtilis*) outperformed all other treatments.

In terms of dry weight (obtained on day 21 and 28), the plants treated with *B. subtilis* was significantly higher than plants treated with deionized water and azospirillum broth. There was no significant difference in the number of applications (1 application vs. 2 applications vs. 3 applications) between each treatment (Figures 5 and 6). In this case, *B. subtilis* seemed to promote plant growth over the course of 3-4 weeks whereas *P. aeruginosa* did not. Many studies have shown *B. subtilis* to be a beneficial PGPR and that seems to hold true in this study. As for *P. aeruginosa*, inoculating basil with this bacterium proved to have little to no benefit in terms of growth.

*B. subtilis* is a well-known antagonistic microorganism that has the ability to suppress the growth of phyto-pathogens such as *Fusarium graminearum*, *Macrophomina phaseolina, Fusarium oxysporum*, and *Rhizoctonia solani* (Zhao *et al.*, 2014; Torres *et al.*, 2016; Beneduzi *et al.*, 2012). This is accomplished by colonizing the roots via biofilm formation. Biofilms are created when a group of microorganisms (i.e. *B. subtilis*) within a matrix of extracellular polymeric substances adhere to surfaces such as roots (Allard-Massicotte *et al.*, 2016). It is through the root system where most communication between plants and bacteria occur (Choudhary and Johri, 2009). In addition to suppressing soil-borne pathogens, *B. subtilis* also possesses the ability to synthesize plant hormones such as auxins, cytokinins, gibberellins, abscisic acid (ABA), and ethylene (Arkhipova *et al*., 2005; Wang *et al.,* 2011). These

hormones regulate the following stages of plant development - cell division/elongation (cytokinin, gibberellin), apical dominance (auxin), germination (gibberellins), secondary plant growth (auxin), tissue differentiation, lateral root formation (auxin), and ripening of fruit (ethylene; Costacurta and Vanderleyden, 1995). In one study, Arkhipova *et al*. (2005) inoculated growth media (calcinated sand) containing lettuce plants (*Lactuca sativa* L.,cv Lolla Rossa) with *B. subtilis.* The authors concluded that *B. subtilis* increased the amount cytokinin present in the roots which in turn increased the plant biomass (root and shoot) by ~ 30% (compared to the control). Another study focused on the production of auxin and how it correlates to plant growth. In that study, spinach plants (*Spinacia oleracea*) were inoculated with *B. subtilis* (mixed into the soil). The data showed that there is a direct correlation between plant growth (root, stem, and leaves) and the amount of auxin present within the plant (Lim and Kim, 2009). Spinach plants inoculated with this bacterium were not only bigger in biomass (by 20%) compared to the control, but also contained higher amounts of auxin. In another study conducted by Adesemoye *et al.* (2008), both *B. subtilis* and *P. aeruginosa* were used to inoculate the seeds of *Solanum lycopersicum L.* (tomato), *Abelmoschus esculentus* (okra), and *Amaranthus sp.* (African spinach). There were three treatments in total (per plant species); 1) *B. subtilis*, 2) *P. aeruginosa*, and 3) distilled water (control). Germination rate and dry biomass (harvested 60 days post inoculation) was significantly higher (>30%) in seeds treated with either bacteria compared to the seeds treated with distilled water. It's worth mentioning that a preliminary trial was conducted by the same authors using a different strain of *P. aeruginosa*. The results for that trial showed that the seeds treated with *P. aeruginosa* did not promote plant growth; they were no different from the control. A second and final trial was conducted using a strain that ultimately proved beneficial to plant growth. This study demonstrated that different bacterial strains used in a study make a significant difference in terms of plant growth.

In the present study at hand, it's warranted to state that the *P. aeruginosa* strain used did not enhance the growth of genovese basil (in-vitro). However, that doesn't mean this particular strain would not promote plant growth in a field setting. Other trials would need to be conducted in order to determine whether or not this strain of *P. aeruginosa* promotes plant growth when inoculated into the soil (outdoor setting). Alternatively, it may be plausible that *P. aeruginosa* promotes plant growth only when surrounded by other microorganisms. On the contrary, B. subtilis proved beneficial to genovese basil

even by itself. Based on previous studies, an influx of auxin and/or cytokinin production could be a factor that's promoting plant growth in genovese basil inoculated with *B. subtilis*.

In conclusion, *B. subtilis* promotes plant growth in terms of germination rate, lateral root formation, root length, stem elongation, and overall biomass under sterile conditions (in-vitro). Applying *P. aeruginosa,* on the other hand, proved to have little to no effect on plant growth at these early stages. The abundance of *B. subtilis* in KNF could very well be one of many reasons why KNF is beneficial for plants. More studies need to be conducted on bacteria isolated and identified in KNF IMO4 stage in order to determine the role they play in plant growth.

**5.6 References**

Adesemoye, A. O., Obini, M., & Ugoji, E. O. 2008. Comparison of plant growth-promotion with Pseudomonas aeruginosa and Bacillus subtilis in three vegetables. Brazilian Journal of Microbiology, 39(3), 423–426. http://doi.org/10.1590/S1517-83822008000300003

Ahmed, M.M.H., El-Baky, M.F., Zaki, M.F., Zaki, F.S., and El-Aal, A. 2011. Effect of foliar application of active yeast extract and zinc on growth, yield and quality of potato plant (Solanum tuberosum L.). Journal of Applied Sciences Research 7(12):2479-2488

Akhtar, A., Hisamuddin, A., Robab, M., Sharf, A., & Sharf, R. 2012. Plant growth promoting Rhizobacteria: An overview. Scholars Research Library. Retrieved from http://www.scholarsresearchlibrary.com/

Allard-Massicotte, R., Tessier, L., Lécuyer, F., Lakshmanan, V., Lucier, J.-F., Garneau, D., … Beauregard, P. B. (2016). Bacillus subtilis Early Colonization of Arabidopsis thaliana Roots Involves Multiple Chemotaxis Receptors. mBio, 7(6), e01664–16. http://doi.org/10.1128/mBio.01664-16

Arkhipova, T.N., Veselov, S.U., Melentiev, A., Martynenko, E.V., and Kudoyarova, G.R. 2005. Ability of bacterium *Bacillis subtilis* to produce cytokinins and to influence the growth and endogenous hormone content of lettuce plants, Plant and Soil, vol. 272 (pg. 201-209)

Beneduzi, A., Ambrosini, A., & Passaglia, L. 2012. Plant growth-promoting rhizobacteria (PGPR): Their potential as antagonists and biocontrol agents. Genetics and Molecular Biology, 35(4), 1044-1051. Retrieved from http://www.sbg.org.br/

Berg G., Smalla K. 2009. Plant species and soil type cooperatively shape the structure and function of microbial communities in the rhizosphere. FEMS Microbiology. Ecol. 68 1–13 10.1111/j.15746941.2009.00654.x

Brenchley, W., 1936. The essential nature of certain minor elements for plant nutrition, The Botanical Review, 2, 4, (173).

Cho, H.-K., and Cho, J.-Y. 2010. Natural Farming. Han-Kyu Cho. CGNF. Seoul, S. Korea

Choudhary, D. and Johri, B. 2009. Interactions of Bacillus spp. and plants – With special reference to induced systemic resistance (ISR),Microbiological Research. Volume 164, Issue 5.Pages 493-513, ISSN 0944-5013, https://doi.org/10.1016/j.micres.2008.08.007.

Costacurta, A. and Vanderleyden, J. 1995. Synthesis of phytohormones by plant-associated bacteria. Critical Review Microbiology 21: 1-18

Costa R., Götz M., Mrotzek. N., Lottmann. J., Berg. G., Smalla. K. 2006. Effects of site and plant species on rhizosphere community structure as revealed by molecular analysis of microbial guilds. FEMS Microbiology. Ecol. 56 236–249 10.1111/j.1574-6941.2005.00026.x

Dobbelaere S., Vanderleyden J., Okon Y. 2003. Plant growth-promoting effects of diazotrophs in the rhizosphere. Critical Review Plant Science 22 107–149 10.1080/713610853

Errington, J. 2003. Regulation of endospore formation in *Bacillus subtilis*. Nature Rev. Microbiol. 1: 117-125

Essoyan, S. 2011. Natural selection: A self-sufficient system of farming is increasing yields across Hawai'i. Star Advertiser. Retrieved October 1, 2013, from http://www.staradvertiser.com/news/20110118_Natural_selection.html?id=114074644

Gray, E.J. and Smith, D.L. 2005. Intracellular and extracellular PGPR: Commonalities and distinctions in the plant-bacterium signaling processes. Soil Biology Biochemistry; 37:395–412.

Hartmann, A., Schmid, M., Tuinen, D., and Berg G. 2009. Plant-driven selection of microbes. Plant Soil 321 235–257 10.1007/s11104-008-9814-y

Hurst C J, Knudsen G R, Mclnerny M J and Stetzenbatch L D. 2000. “Role of Azospiriillum in Nitrogen Fixation”, Journal of Manual Envronmental Microbiology, Vol. 6, No. 3, pp. 104-105.

Islam, F., Yasmeen, T., Ali, Q., Ali, S., Arif, M.S., Hussain, S., and Rizvi, H. 2014. Influence of *Pseudomonas aeruginosa* as PGPR on oxidative stress tolerance in wheat under Zn stress, Ecotoxicology and Environmental Safety,Volume 104,Pages 285-293, ISSN 0147-6513, https://doi.org/10.1016/j.ecoenv.2014.03.008.

Kacena, M., Merrell, G., and Manfredi, B. 1999. Applied Microbiology Biotechnology. 51: 229. https://doi.org/10.1007/s002530051386

Kloepper, J.W. 1993. Plant growth-promoting rhizobacteria as biological control agents. In: Metting FB, ed. Soil microbial ecology—applications in agricultural and environmental management. New York: Marcel Dekker, 255–274.

Kołodziejek, J., & Patykowski, J. 2015. Effect of Environmental Factors on Germination and Emergence of Invasive Rumex confertus in Central Europe. The Scientific World Journal, 2015, 170176. http://doi.org/10.1155/2015/170176

Kumar, B., Gupta, E., Yadav, R., Singh S.C., and Lal, R.K. 2014. Temperature Effects on Seed Germination Potential of Holy Basil (*Ocimum tenuiflorum*) Seed Technology. Vol. 36, No. 1, pp. 75-79 of Plant Nutrition, 9:3-7, 975-979, DOI: 10.1080/01904168609363497

LaBauve, A. E., & Wargo, M. J. 2012. Growth and Laboratory Maintenance of Pseudomonas aeruginosa. Current Protocols in Microbiology, 0 6, Unit–6E.1. http://doi.org/10.1002/9780471729259.mc06e01s25

Lim, J. H. and Kim, S. D. 2009. Synergistic Plant Growth Promotion by the Indigenous Auxins-producing PGPR *Bacillus subtilis* AH18 and *Bacillus licheniforims* K11 J. Korean Soc. Applied Biological Chemistry, 52 (5): 531-538.

Lopez, D., Vlamakis, H., & Kolter, R. 2009. Generation of multiple cell types in *Bacillus subtilis*. FEMS Microbiology Reviews.

Murashige, T, Skoog, F.1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Plant Physiology;15:473–97.

Pérez-Fernández, M.A., Calvo-Magro, E., Montanero-Fernández, J., and Oyola-Velasco, J.A. 2006. Seed germination in response to chemicals: effect of nitrogen and pH in the media. Journal of Environmental Biology 13-20. PubMed PMID: 16850869.

Prathap, M. & Kumari, R. 2015. A critical review on plant growth promoting rhizobacteria. Plant Pathology & Microbiology, 6(4). doi: http://dx.doi.org/10.4172/2157-7471.1000266

Qiao, J., Yu, X., Liang, X., Liu, Y., Borriss, R., Liu, Y. 2017. Addition of plant-growth-promoting *Bacillus subtilis* PTS-394 on tomato rhizosphere has no durable impact on composition of root microbiome. BMC Microbiology, 17(131). doi: 10.1186/s12866-017-1039-x

Radhapriya, P., Ramachandran, A., Anandham, R., & Mahalingam, S. 2015. *Pseudomonas aeruginosa* RRALC3 Enhances the Biomass, Nutrient and Carbon Contents of Pongamia pinnata Seedlings in Degraded Forest Soil. PLoS ONE, 10(10), e0139881. http://doi.org/10.1371/journal.pone.0139881

Saha, S., Monroe, A., & Day, M. R. 2016. Growth, yield, plant quality and nutrition of basil (Ocimum basilicum L.) under soilless agricultural systems. Annals of Agricultural Sciences, 61(2), 181-186., https://doi.org/10.1016/j.aoas.2016.10.001

Sanaullah, S. 1986. Effect of ammonium iron (III) citrate as liquid fertilizer on wheat, Journal of Plant Nutrition, 9:3-7, 975-979, DOI: 10.1080/01904168609363497

Torres, M.J., Pérez Brandan, C., Petroselli, G., Erra-Balsells R., Audisio, M.C. 2016. Antagonistic effects of Bacillus subtilis subsp. subtilis and B. amyloliquefaciens against Macrophomina phaseolina: SEM study of fungal changes and UV-MALDI-TOF MS analysis of their bioactive compounds, Microbiological Research. Volume 182, Pages 31-39, ISSN 0944-5013, https://doi.org/10.1016/j.micres.2015.09.005.

Usha, B. 2015. PGPR Interaction: An Ecofriendly Approach Promoting the Sustainable Agriculture System. Advances in Botanical Research, Academic Press, Volume 75, Pages 81-113. ISBN 9780124201163, https://doi.org/10.1016/bs.abr.2015.09.006.

Walker, T. S., Bais, H. P., Déziel, E., Schweizer, H. P., Rahme, L. G., Fall, R., & Vivanco, J. M. 2004. Pseudomonas aeruginosa-Plant Root Interactions. Pathogenicity, Biofilm Formation, and Root Exudation. Plant Physiology, 134(1), 320–331. http://doi.org/10.1104/pp.103.027888

Wang, Y. H., & Irving, H. R. 2011. Developing a model of plant hormone interactions. Plant Signaling & Behavior, 6(4), 494–500. http://doi.org/10.4161/psb.6.4.14558

Wright J. P. and Jones, C. G. 2006. The concept of organisms as ecosystem engineers ten years on: progress, limitations, and challenges. BioScience 56 203–209 10.1641/0006-3568(2006)056[0203:TCOOAE]2.0.CO;2

Weihui, W., Yongxin, J., Fang, B., and Shouguang, J. 2015. Chapter 41 - *Pseudomonas aeruginosa*. Molecular Medical Microbiology (Second Edition),Academic Press, Pages 753-767,ISBN 9780123971692,https://doi.org/10.1016/B978-0-12-397169-2.00041

Yanez-Mendizabal, V., Vinas, I., Usali, J., Canamas, T., and Teixido, N. 2012. Endospore production allows use of spray-drying as a possible formulation system of the biocontrol agent *Bacillus subtilis* CPA-8. Biotechnology Lett. 34:729–735. doi: 10.1007/s10529-011-0834-y.

Zhang, H., Xie, X., Kim, M.S., Kornyeyev, D.A., Holaday, S., and Pare, P.W. 2008. Soil bacteria augment Arabidopsis photosynthesis by decreasing glucose sensing and abscisic acid levels in plant. The Plant Journal 56, 264–273.doi:10.1111/j.1365-313X.2008.03593.x

Zhao, Y., Selvaraj, J. N., Xing, F., Zhou, L., Wang, Y., Song, H., and Liu, Y. 2014. Antagonistic Action of *Bacillus subtilis* Strain SG6 *on Fusarium graminearum* . PLoS ONE, 9(3), e92486. http://doi.org/10.1371/journal.pone.0092486

**5.7 Appendix**

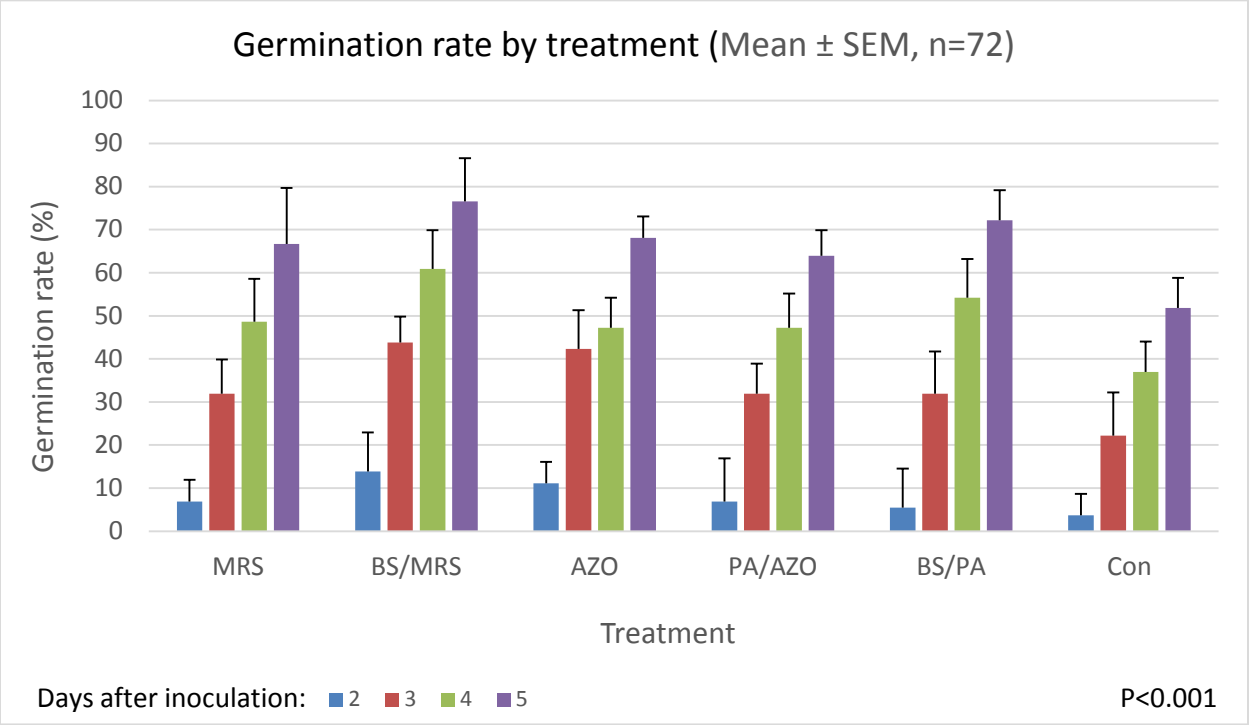



**Figure 1** Germination data for Genovese basil seeds inoculated with various treatments. This data represents the cumulative total of newly germinated seeds observed on days 2, 3, 4, and 5 of the experiment. MRS, BS/MRS, BS/PA, AZO, and PA/AZO are significantly higher (P<0.001) than CON (for all days).
Legend: MRS – broth used to grow *B. subtilis*; BS – *B. subtilis*; AZO – broth used to culture *P. aeruginosa*; PA – *P. aeruginosa*; Con – contains no bacteria or broth media

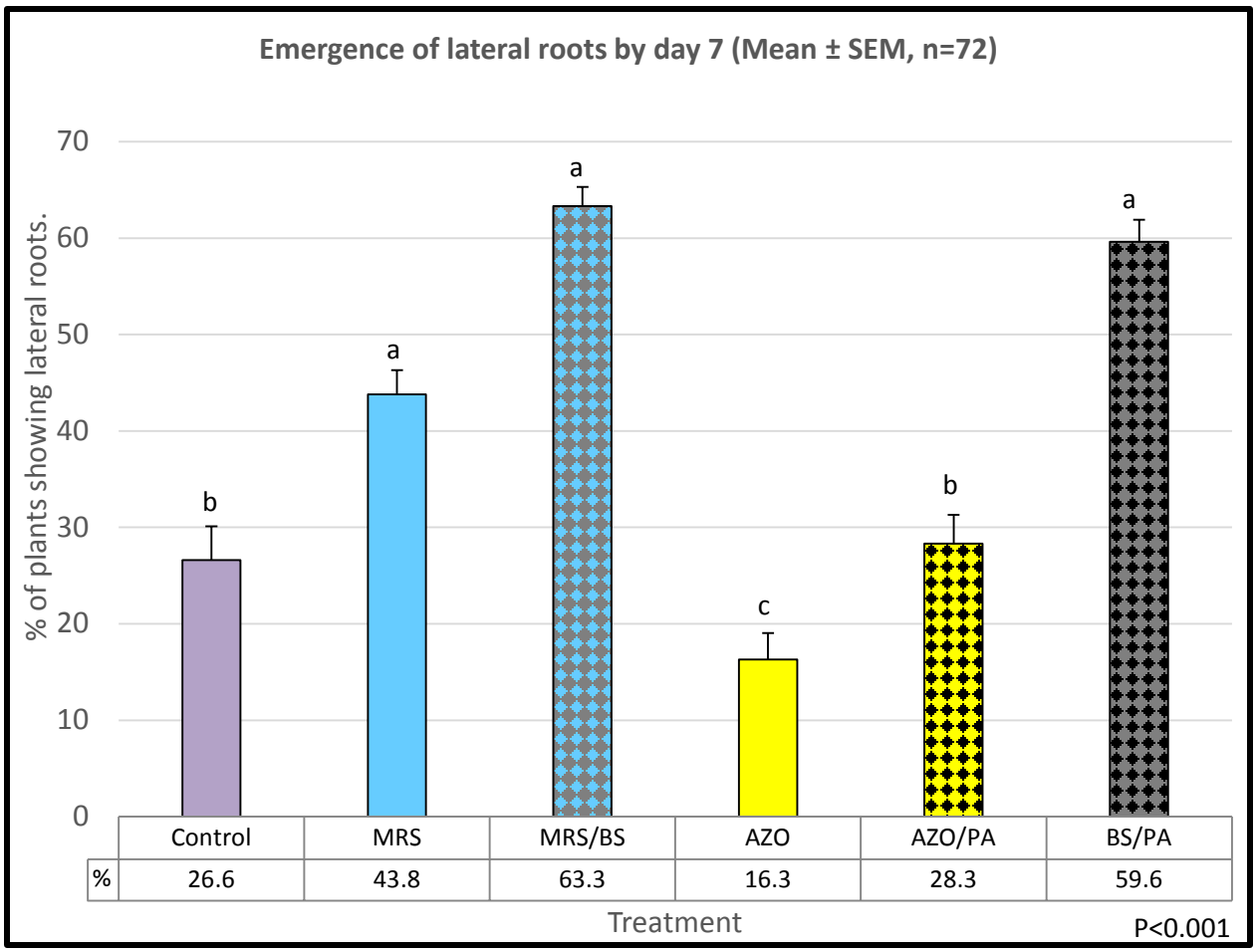



**Figure 4.** The data represents the percentage of plants showing lateral roots by day 7. MRS (43.8%), BS/MRS (63.3%), and BS/PA (59.6%) had a significantly higher emergence of lateral roots than CON (control; 26.6%), AZO (16.3%), and PA/AZO (28.3%; P>0.001). A one-way ANOVA was performed on the data using JMP statistical software.

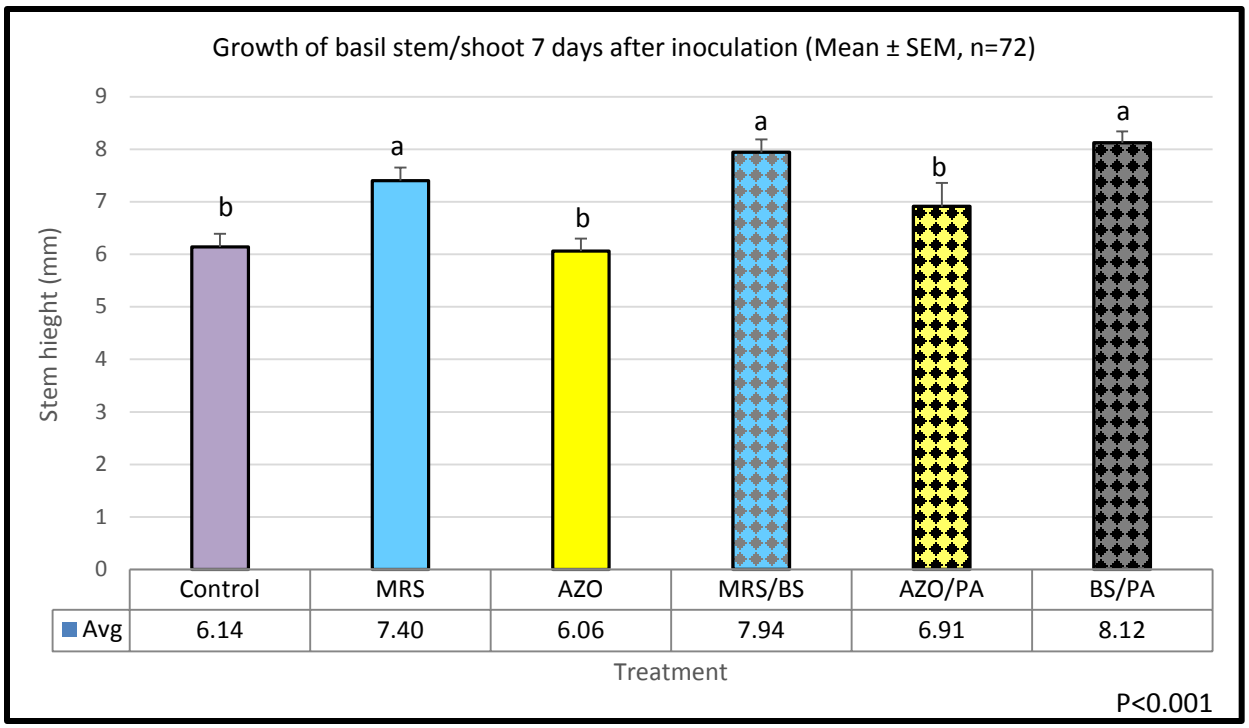



**Figure 3** The stem/shoot height (mm) was measured and recorded on day 7 (n=72). There were no significant difference between the plants treated with MRS broth (MRS; mean=7.4mm SE=0.253; P<0.001), *B. subtilis* (BS/MRS; mean=7.94mm SE=0.185; P<0.001), and *P. aeruginosa* + *B. subtilis* (BS/PA; mean=8.12mm SE=0.206; P<0.001). However, all three had significantly greater stem lengths than the plants treated with deionized water (CON; mean=6.14mm SE=0.253; P<0.001), azospirillum broth (AZO; mean=6.06mm SE=0.258; P<0.001) and *P. aeruginosa* (PA/AZO; mean=6.91mm SE=0.376; P<0.001). ). A one-way ANOVA was performed on the data using JMP statistical software.

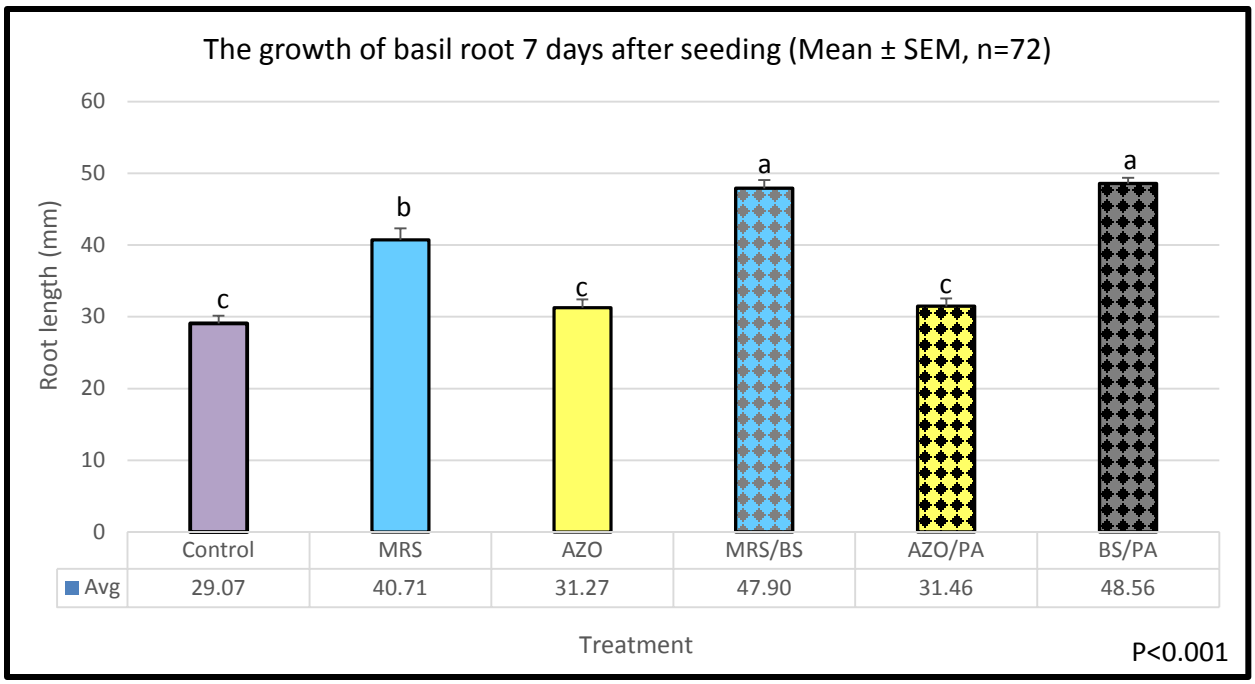



**Figure 4** The root length (mm) was measured and recorded on day 7 (n=72). There were no significant difference between the plants treated with deionized water (CON; mean=29.07mm SE=1.076; P<0.001), azospirillum broth (AZO; mean=31.27mm SE=1.152; P<0.001), and *P. aeruginosa* (PA/AZO; mean=31.46 SE=1.075; P<0.001). Plants treated with *B. subtilis* (BS; mean=47.9mm SE=0.1.149; P<0.001) and *P. aeruginosa* + *B. subtilis* (BS/PA; mean=48.56mm SE=0.821; P<0.001) were significantly higher than all other treatments. ). A one-way ANOVA was performed on the data using JMP statistical software.

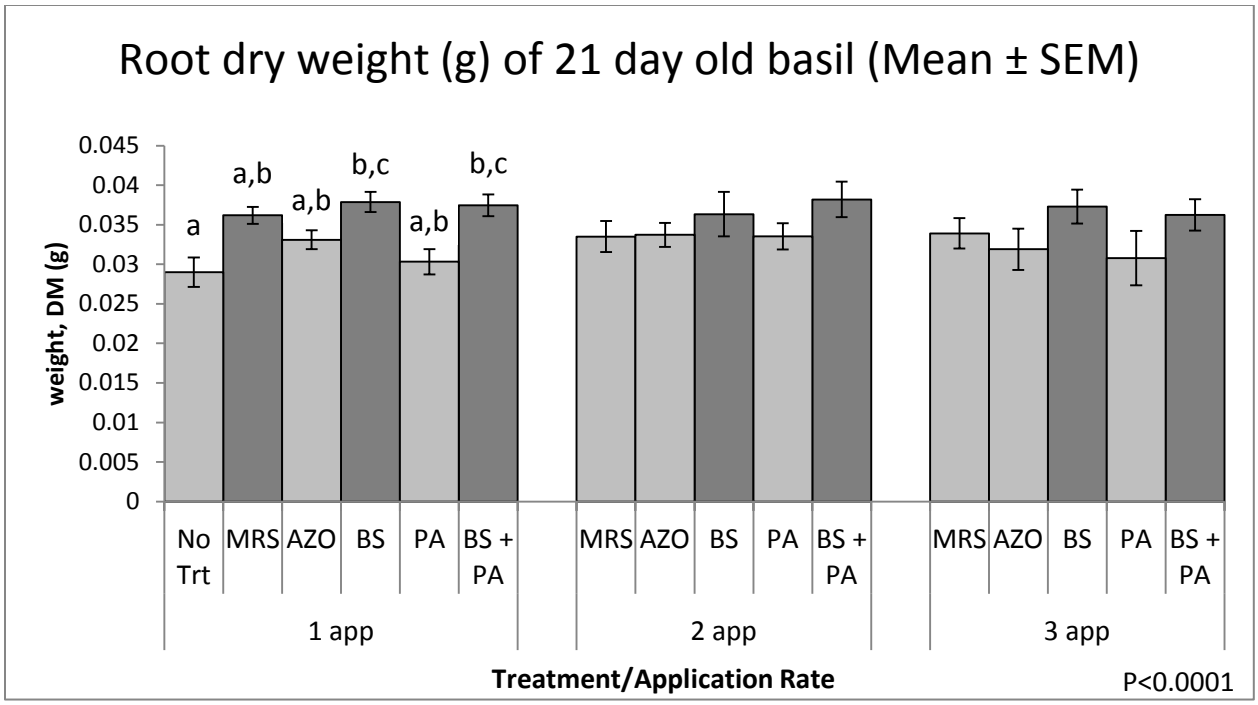



**Figure 5**. Each treatment contained 8 vessels total - 4 vessels with one application (day 1), 2 vessels with two applications (days 1 and 9) and 2 vessels that received three applications (days 1, 9, and 16). Dry weights of the root were recorded on day 21. Lowercase alphabet indicates a significant difference at P<0.0001 within each application. Plants inoculated with *B. subtilis* (1app = 0.0378 ± 0.00127g) and *P. aeruginosa* + *B. subtilis* (1app = 0.0374 ± 0.00138g) had a significantly higher DM root weight than plants treated with deionized water (1app = 0.029 ± 0.0018g). There was no significant difference between any of the treatments that received 2 or 3 applications/inoculations.

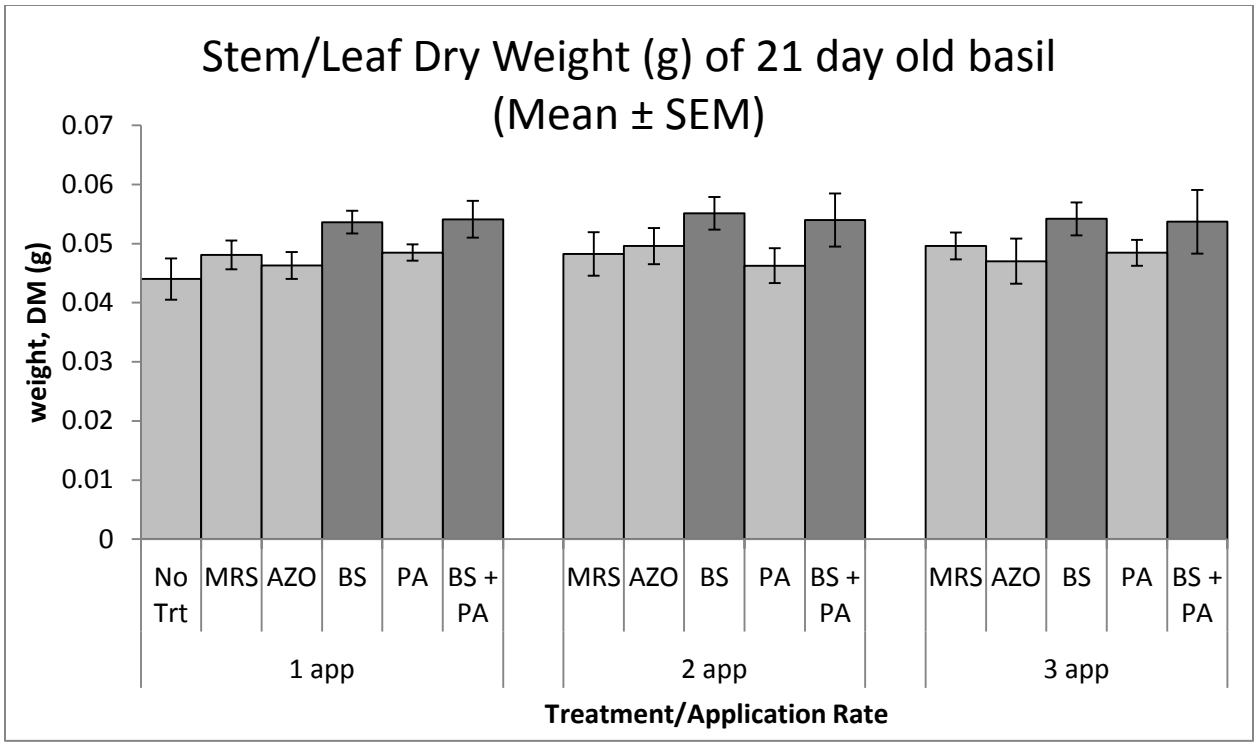



**Figure 6** The data represents dry weights of roots collected on day 21. A multiple comparisons (Tukey HSD) test was performed on the data. The results showed that there was no significant difference between any of the treatments or application rate.

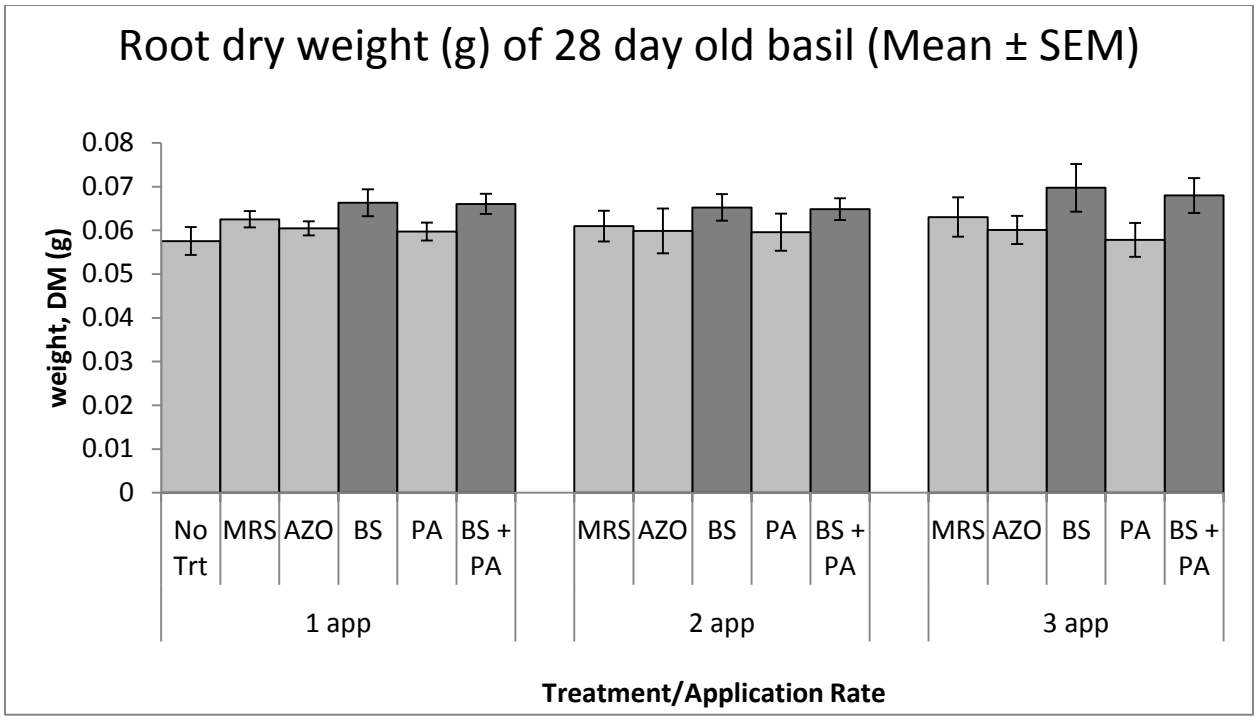



**Figure 7**. The data represents dry weights of roots collected on day 28. A multiple comparisons (Tukey HSD) test was performed on the data. The results showed that there was no significant difference between any of the treatments or application rate.

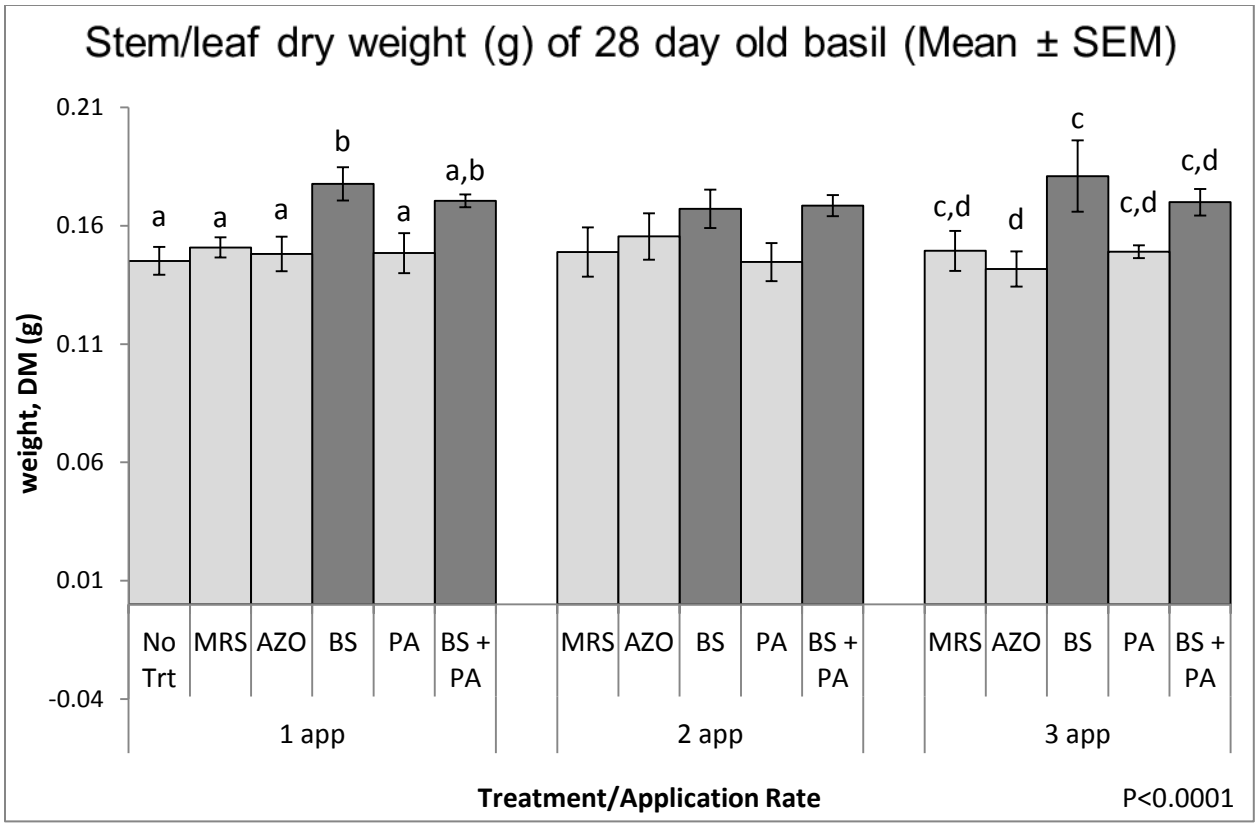



**Figure 8.** The data represents dry weights of stem/leaf collected on day 28. In regards to the plants that received one inoculation, those treated with *B. subtilis* (1app = 0.177 ± 0.007g) had a significantly higher leaf/stem weight than plants treated with deionized water (No trt; 1app = 0.0.145 ± 0.00591g), MRS (1app = 0.15 ± 0.004g), AZO (1app = 0.148 ± 0.007g), and *P. aeruginosa* (1app = 0.148 ± 0.008g). There was no significant difference between the treatments that received two inoculations. As for the plants that received three applications, those treated with *B. subtilis* (3app = 0.18 ± 0.015g) had a significantly higher dry stem/leaf weight than plants treated with AZO (3app = 0.0.14 ± 0.007g).

# Chapter 6

# Conclusion

## 6.1 Conclusion

The overall goal of this research was to provide a better understanding of KNF and how it works, from a scientific perspective. We know that farmers who practice this farming technique have seen positive results in terms of plant growth, based on visual assessment. The program promotes the idea that indigenous microorganisms are the driving force behind KNF. However, it remained unknown what are the types of microorganisms present in the soil treated under KNF conditions and whether or not the collection site (of IMO) plays an integral role in soil fertility. Wang determined that KNF significantly suppressed weed growth and that it also enriched the soil with beneficial earthworms, enchytraeids, and nematodes (i.e. bacterivores, fungivores, and omnivores). The authors suggest that these soil dwellers improved soil tilth and nutrient cycling which in turn promoted plant growth. KNF system pride itself as a self-sufficient farming system that involves the culturing of indigenous microorganisms (IMO) – bacteria, fungi, nematodes, and protozoa. Thanks to previous studies, we have an idea as to how nematodes contribute to the benefits of KNF practitioners. Though the presence of fungi and protozoa are of vital importance for plant growth and soil fertility, the primary focus of this study was to identify the types of bacteria prevalent in KNF.

There were four main objectives: 1) identify the bacteria present in KNF, specifically phosphorus-solubilizing and nitrogen-fixing bacteria, 2) determine whether or not the collection site plays an integral part in plant growth, 3) how often to re-apply IMO 4 to the soil, and 4) inoculate seeds with bacteria isolated from KNF in the hopes of providing a better understanding as to the role it may play.

In the studies conducted in Kula, Poamoho, and Makaha, *Bacillus megaterium* and *Bacillus aryabhattai* were present in all soil samples collected (organic, KNF, and conventional). Additionally, KNF had a greater diversity of bacteria and CFU count ($10^4$ CFU/g). Some of the bacteria identified in KNF include *Bacillus simplex, Arthrobacter defluvii, Bacillus oleronius, Bacillus pseudomycoides, Bacillus thuringiensis, Paenibacillus glucanolyticus, Streptomyces djakartensis, Streptomyces galilaeus,* and *Bacillus cereus.* *Bacillus subtilis* and *Bacillus licheniformis* were dominant only in the KNF system.

In the Makaha trail, the bacterial population was greatest in the soil within the rhizosphere, especially in the case of KNF-B and KNF-U. Some of the identified bacteria present in KNF-U, KNF-H,

and KNF-B include *Bacillus drentesis, Baccilus oleronius, Bacillus thuringiensis, Bacillus circulans, Flavobacterium johnsoniae, Arthrobactor globiformis and Herbaspirillum massiliense.* KNF-$B_2$ (IMO cultivated from bamboo, with 2 applications) had the highest plant yield (535.15g ± 9.47; P<0.001) compared to KNF-U (IMO cultivated from breadfruit), KNF-H (IMO cultivated from haole koa), and conventionally (16-16-16 NPK) treated plots that received either one (applied on day 1) or two (applied on day 14) applications. More than 50% of the bacteria isolated from KNF-B were that of *B. subtilis* (23%), *B. licheniformis* (19%), and *Pseudomonas aeruginosa* (11%).

In order to determine what makes bamboo the optimal site for collecting and cultivating IMO in a KNF system, genovese basil seeds (and growth media) were inoculated with *B. subtilis* (most abundant in KNF soil) and *P. aeruginosa* (only found in KNF-B). The experiment was performed in-vitro. The results showed that *B. subtilis* promoted plant growth in terms of germination rate, lateral root formation, root length, stem elongation, and overall biomass. Applying *P. aeruginosa* on the other hand proved to have little to no effect on plant growth. It may be due to the stage of growth as other studies showed that *P. aeruginosa* helped in making Phosphorus available for the kreb-cycle in photosynthesis.

This research has provided much insight into KNF. We were able to identify the various types of bacteria present in KNF. Many of these bacteria (as described in Chapter 1) are proven to have PGPR We have been stored them for future experiments. We have also determined that the collection site of microbes yields different types of bacteria and they effects plant growth. The IMO cultivated with bamboo worked best. Additionally, we learned that applying two applications (2 weeks apart) as opposed to one application significantly increased plant growth (kai choi). And lastly, *B. subtilis* proved to have a beneficial effect on plant growth.

Knowing where to collect/cultivate IMO and how often to apply it to the soil will be of great use to farmers who currently practice KNF. This study also provided statistical data that shows KNF to be more effective than conventional farming methods when sufficient bacteria are applied to the soil in a regular schedule. Natural farming is the key to a sustainable future.