LOCAL EFFECTIVE MICROORGANISMS: EFFECTS ON SOIL AND PLANT HEALTH PARAMETERS IN THE VENETIAN PLAIN

by

BRENDAN FATZINGER

(Under the Direction of Dorcas Franklin)

ABSTRACT

Application of locally-sourced microbiological supplements (local effective microorganisms- LEM) at the time of fertilization with organic fertilizers has the potential to increase nitrogen retention and availability of certain nutrients such as carbon and phosphorus in the soil and bolster microbial communities in the soil system, ultimately contributing to increased soil, plant and environmental health. LEM/False-LEM consists of many different species of naturally occurring microorganisms including bacteria, yeasts and fungi. The goal of this research was to identify the effects of LEM application in conjunction with mineral and organic fertilizers in intensively managed soil from Italy's Venetian Po River Valley. We conducted a factorial experiment of three fertilizer treatments (organic, inorganic, no fertilizer) combined with three inoculant treatments (LEM, False-LEM, and water) to evaluate effects on soil and wheat or bean crops. Soil microbial biomass, soil soluble organic solids, plant biomass, and leaf chlorophyll content were significantly elevated by the microbial inoculum treatments in the first month, whereas nitrogen leaching was significantly lowered.

INDEX WORDS: soil health; effective microorganisms; local effective microorganisms; agronomic indices; plant health; nutrient cycling efficiency

LOCAL EFFECTIVE MICROORGANISMS: EFFECTS ON SOIL AND PLANT HEALTH PARAMETERS IN THE VENETIAN PLAIN

by

BRENDAN FATZINGER

B.S., University of Georgia, 2017

M.S., University of Padova, 2019

A Thesis Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

MASTER OF SCIENCE

ATHENS, GEORGIA

2019

LOCAL EFFECTIVE MICROORGANISMS: EFFECTS ON SOIL AND PLANT HEALTH PARAMETERS IN THE VENETIAN PLAIN

by

BRENDAN FATZINGER

Major Professor:	Dorcas Franklin
Committee:	Antonio Berti
	Miguel Cabrera

Electronic Version Approved:

Ron Walcott
Interim Dean of the Graduate School
The University of Georgia
December 2019

ACKNOWLEDGEMENTS

I would like to convey my most sincere gratitude to my professors, Dory Franklin and Antonio Berti, for the opportunity to work for them on this project. Their guidance was paramount in developing this research and I am beyond grateful for every minute of counsel they extended to me as I pursued it. Many thanks also to Riccardo Polese and his staff at the Lucio Toniolo Experimental Farm in Legnaro for their help in constructing various implements and general technical assistance with the project. Francesco Morari and Ilaria Piccoli deserve a massive amount of thanks for helping me with numerous little (and most likely very annoying) things, like navigate administration details, lending advice and transporting samples to Venice- thank you so much for making my life easier. Thank you to every Italian friend who made me feel welcome and at home for my stay in your beautiful country.

I am very grateful also for my UGA people- Kishan Mahmud, Laura Ney, Patrick Gebrayel, Logan Moore, Aaron Bruce, Carson Dann and Pietro Sica. All of you were pivotal in my grad school experience in significant ways.

Thank you to my family, for being so supportive in every way and reaching out regularly to see how I was doing, and deepest gratitude to my pen-pal turned girlfriend, Gina Borg, who was a source of such comfort and joy for me before I even arrived at UNIPD.

Finally, thank you to my Father. I shudder to think what I would be if not for your continuous intervention in my life.

TABLE OF CONTENTS

Page

LIST OF FORMULAS AND FIGURES

Page

CHAPTER 1

Introduction and Literature Review

1.1 Local Effective Microorganisms

Advancements in technology, increased population growth, and heightened consumer expectations have resulted in the adoption of many high-throughput agricultural methodologies such as monoculture, synthetic fertilizers, pesticides and other practices, al of which combined have ultimately lead to a marked increase in food and fiber production (Giller, et al. 1997). Indeed, this is certainly a positive but there is evidence to suggest that this has led to a decline in overall soil microbial health and nutritional value in the foods being produced as well as wasted phosphorus, a limited resource (Cóndor Golec, et al. 2007; Davis, 2009). In recent decades, studies have identified that some agricultural chemicals have had a negative impact on the environment by adversely affecting microbial populations as a whole; even seemingly unrelated chemicals and organisms can have interactions, such as the fungicide mancozeb on aerobic nitrogen fixing bacteria (Doneche et al., 1983; Baxter and Cummings, 2008). In addition to their poor environmental legacy, these products and technologies are prohibitively expensive to subsistence farmers and there is distinct difficulty in meeting the demand for them at an attainable price (Kumar and Gopal, 2015). Furthermore, the use of certain agricultural chemicals poses a significant risk to farm workers, potentially diminishing their quality of life (Megali, Schlau, and Rasmann, 2015). As such, more sustainable practices are continually being explored as potential alternatives. Though there are many possible implications related to the use of biotechnology in agriculture, one facet that has not been fully explored is that of microbiological diversity, particularly in the soil, and its effects in relation to sustainable, subsistence, and closed-loop agricultural systems, particularly those that rely heavily on organic fertilizers such as manure and

litters from animals and plant-comprised “green manures” (Javaid, Bajwa, and Anjum, 2008). Use of organic fertilizers can be problematic to growers because they must undergo mineralization before plant uptake (Compant et al., 2010) This mineralization is reliant on biology in the soil, and if the soil is lacking sufficient populations of key functional groups then timing of nutrient availability with crop needs can be mismatched resulting in poor yields. Much research has been done to maximize food productivity in fields such as plant breeding and fertility management, but relatively little has been focused towards using native soil microorganisms to help boost nutrient cycling and retention (Hu and Qi, 2012). Currently, there exist many elucidated and cultured microorganisms that contribute to modern human life, yet in agriculture there are still gaps in understanding regarding effective microorganisms (EM) and their potential relation to plant health (Kumar and Gopal, 2015). Arguably, benefits provided by diverse soil dwelling microbial populations are numerous, yet research is regularly discovering potential advantages these organisms may lend to various cropping systems (Wright and Jones, 2006). Particularly for sustainable and closed-loop oriented growers, the main services leveraged are to provide better soil structure, pathogen suppression, increased nutrient availability, minimization of nutrient loss, weed suppression, and bio-remediation of soil contaminates (Giller, et al., 1997; Singh and Singh 2016; Müller-Stöver et al., 2016; Smith and Goodman, 1999). Mitigating the untapped potential that EM’s may have to offer will be a key factor in trying to combat world hunger in the future, and further investigation of these beneficial microorganisms with their ability to reduce the amount of inputs used in modern agroecosystems could prove to be advantageous to many branches of agriculture.

1.2 Mechanisms of Improvement

A proposed key to combatting the undesirable effects of agricultural intensification on soil health is to bolster overall biodiversity within the system. In nature, plant root systems are in close proximity and physical contact with a wide range of soil microbial populations (Berg and Smalla, 2009). The microbial community associated with plant roots can be referred to as the rhizo-microbiome (Chaparro et al., 2013). As such, it is necessary to allow for the proliferation of these populations in order to ensure that agroecosystems reach their full potential. A methodology to leverage the benefits of the interactions between plants and microorganisms is to apply effective microorganism cultures to the soil. Local effective microorganism cultures are conglomerations of distinct species of effective microorganisms harvested local to the agricultural system in which they are intended to be applied (Kumar and Gopal, 2015; Higa and Parr, 1994). The organisms are a part of various taxonomic groups and may be present simultaneously in any combination in a given soil in the wild (Kyselková et al., 2009; Almario et al., 2013a). Based on studies by Higa (1998), LEM contains about 80 species of microorganisms divided into photosynthesizing bacteria, lactic acid bacteria, yeasts, actinomycetes, and fermenting fungi which all have significant roles in the soil system. It is also reported that the main organisms represented in the cultures are *Rhodopseudomonas palustrus* and *Rhodobacter spaeroides* (photosynthetic bacteria), *Lactobacillus plantarum, L. casei,* and *Streptoccus lactis* (lactic acid bacteria), *Saccharomyces cerevisiae* and *Candida utilis* (yeasts), *Streptomyces albus* and *S. griseus* (actinomycetes), and *Aspergillus oryzae*, *Penicillium* sp. and *Mucor hiemalis* (fermenting fungi) (Ahn, et al., 2014; Diver, 2001). Use of this biological technology has the added benefits of not being genetically-engineered and nor chemically synthesized, and is therefore acceptable for implementation in even the strictest legal frameworks (Zakaria, Gairola, and Shariff, 2010). Additionally, in making LEM

cultures, cost effective and locally available ingredients are used, which makes it a much more viable technique to be used in even marginal, low income farms. Within the rhizosphere, the expression of effective microorganism's plant-beneficial properties can be affected by both abiotic factors (such as pH, oxygen, clay mineralogy, heavy metals, etc.) and biotic factors (for example, compounds exuded by plants or the rhizosphere-dwelling microbes) that can lead to distinct expressions in spatial and temporal patterns, possibly with different effects observed on the host plant (Piccoli and Bottini, 1994; Pothier et al., 2008; Prigent-Combaret et al., 2008; Dutta and Podile, 2010; Almario et al., 2013b; Drogue et al., 2013).

Plants have evolved many different types of biotic relationships with soil microbial populations that range from commensalism to mutualism. This interaction between plants and their rhizo-microbiome is complex and varies with the plant genotypes and microbial soil inhabitants (Vacheron et al., 2013; Hartmann, 2009). Organic exudates and nutrients in the form of organic acids, flavonoids, phytosiderophores, sugars, vitamins, amino acids, nucleosides, and mucilage comprise the backbone of these rhizo-microbiological relationships and by acting as signals to attract and proliferate microbial populations (Zak, et al. 2003; Bais et al., 2006; Pothier et al., 2007; Badri et al., 2009; Shukla et al., 2011; Drogue et al., 2013). As a result of this important communication between plants and microorganisms, healthy soils tend to have a rich microbiological community, with the rhizosphere containing up to 10^{10} bacteria per gram of soil (Gans et al., 2005; Roesch et al., 2007) and encompassing a wide diversity of taxa (Kyselková et al., 2009; Gomes et al., 2010). Many of these bacteria are able to colonize the surface and inner tissue of root systems and can stimulate the growth and health of plants (Barea et al., 2005). With greater diversity in plant species, there are many more resources available to organisms that improve soil and plant health (Hu and Qi, 2013). Thus, crops grown within a system affect soil

health not only directly, but also indirectly. Within the rhizo-microbiome, it has been shown that microorganisms can provide better plant health and promote plant growth through several indirect or direct mechanisms (Couillerot et al., 2009; Richardson et al., 2009; Desbrosses et al., 2011). These beneficial symbiotic plant-microbe interactions are such that costs and benefits are shared by the plants and the microorganisms (Odum and Barrett, 2005; Bulgarelli et al., 2013).

Effective microorganisms can improve plant germination, root development, and growth through the production of enzymatic activities or phytohormones, as well as promote the establishment of rhizobial or mycorrhizal symbioses (Cassán et al., 2009; Mantelin and Touraine, 2004). They can enhance plant nutrition via phosphate solubilization, associative nitrogen fixation, or phytosiderophore production (Richardson et al., 2009). Others can protect plants by inhibiting phytoparasites based on antagonism or competition mechanisms, and/or possibly by eliciting plant defenses (Couillerot et al., 2009; Lugtenberg and Kamilova, 2009). Some plant growth promoting rhizobacteria (PGPR) such as *Phragmites australis*, *Pseudomonas asplenii*, *Streptomyces spp.*, and *Bacillus licheniformis* can also help plants withstand abiotic stresses including heat and cold stress, drought, and contamination by heavy metals or other pollutants (Bhattacharyya and Jha, 2012; Jing et al., 2007; Lim and Kim, 2013; Saharan and Nehra, 2011; Tak et al., 2013; Yang et al., 2009). Effective microorganisms may stimulate root hair elongation in vitro (Dobbelaere et al., 1999; Contesto et al., 2008) and increase the number and/or length of lateral roots (Combes-Meynet et al., 2011; Chamam et al., 2013). Consequently, mineral and water uptake, and therefore whole plant growth, can be increased (El Zemrany et al., 2006; Minorsky, 2008; Veresoglou and Menexes, 2010; Walker et al., 2012). These physiological plant modifications are thought to be a result of direct manipulation of phytohormone pathways involved in root development, particularly cytokinin, auxin, and ethylene, but also gibberellin and abscisic acid (Moubayidin et

al., 2009; Stepanova and Alonso, 2009; Dodd et al., 2010; Overvoorde et al., 2011). Plant-microbe interactions have also been shown to modify chemical composition and structural properties of plant cell walls (El Zemrany et al., 2007; Zhang et al., 2007). Additionally, studies have observed changes in metabolism triggered by effective microorganism inoculation (Lavania et al., 2006; Shaw et al., 2006).

1.3 Impacts on Crops

Benefits seen in experimental trials with EM include increased yield in onion by 29%, increase in pea yields by 31%, and increase in sweetcorn cob weights by 23%. Additionally, treated soil respired an additional 46% more carbon than the control soil in a New Zealand silt-loam soil (Daly and Stewart, 1999). Used in conjunction with green manure amendments, applying EM enhanced rice yield by 46% (Javaid, 2011). In corn trials conducted by Megali, Schlau, and Rasmann (2015), there was a 16% increase in overall biomass observed across nine different species. These studies indicate that the presence of LEM in a cropping system, at the very least, allows for greater incorporation of raw cell building materials into plant tissue leading to increased plant health.

In healthy soils, active fungal hyphae growth is also present and serves to increase soil health. This is important because the hyphae's thin, hair-like reticulation form a cohesive framework to create better structure in the soil. In dense clayey soils, this structure can increase porosity which aids in water infiltration, aeration, and plant root growth. In sandy soils, the hyphae can help create aggregate stability by holding together the large, loose mineral particles associated with sandy soils, which can help increase water holding capacity and prevent erosion (Kumar and Gopal, 2015).

Increased nutrient cycling and availability to plants has been correlated with more active and diverse soil dwelling microbial populations (Burns et al.,2012; Mantelin et al., 2006). Rhizobacteria can not only directly increase nutrient supply in the rhizosphere, but they can also stimulate ion transport systems in roots (Zhang et al., 2008). Phosphate solubilization is another key facet of effective microorganisms on plant nutrition. Soils generally contain adequate amounts of phosphorus for plant growth, which accumulates as a result of regular fertilizer applications, but only a small proportion is available for plant uptake (Richardson and Simpson, 2011).

Phosphorous may be made less limited to plants by accessing the reserves strongly held in the soil (Abdu, 2006; Chen, 2002). More labile phosphorus (P) in soil is correlated with the presence of mycorrhizal fungi. Labile P is more easily utilized from the soil CEC and is therefore more available for plant uptake. Ca-, Fe-, and Al- P compounds can be recalcitrant and those soil cation exchange sites considered to be non-labile forms. Recalcitrant P compounds are not readily taken up by plants even though adequate P is present in the soil (Wang, et al., 2016; Richardson et al., 2009). Some mycorrhizal fungi can access phosphorous even in its non-labile form and provide it to plants in exchange for carbohydrates (El Mrabet, et al., 2013; Parniske, 2008). Plants inoculated with mycorrhizal fungi have been shown to take in 3-5 times more P than controls (Indriani, et al., 2016). It is important to note that plants are not entirely limited in phosphorus uptake and are able to absorb mono and dibasic phosphate on their own, but organic or recalcitrant forms of phosphate-compounds need to be mineralized or solubilized by microorganisms. *Pseudomonas, Bacillus, Rhizobium* are able to dissolve recalcitrant forms of phosphate and make them plant available (Richardson et al., 2009; Ramaekers et al., 2010). This is significant not just in the case of phosphorus but also in the case of other agriculturally important nutrients which are in the form of biological complex molecules and are only plant available either

in very low levels, or not available entirely due to their inability to assimilate into the soil solution. For plant material and animal wastes to be used by plants, they must first be decomposed to a mineral form, and active microbiological populations in the soil are critical to this process (Mahanta, et al., 2014). Mycorrhizal fungi and rhizobacteria can improve plant uptake of nitrogen, potassium, magnesium, zinc, copper, boron, and molybdenum (Giri and Mukerji, 2004; Kim, et al., 2010: Leyval & Berthelin, 1989; Ramasamy, et al., 2011). They also have been shown to mitigate ammonium volatilization when organic fertilizers are applied to fields, consequently resulting in less nitrogen lost to the atmosphere, which is then therefore more available to the plants (van Vliet, Bloem, and de Goede, 2005; Richardson et al., 2009). Iron transporters can also be upregulated by microorganisms which leads to an increase in iron assimilation (Zhang et al., 2009).

Another well documented benefit of soil dwelling microorganisms is nitrogen fixation, either by free-living microorganisms or symbiotic production between genus Rhizobium and legumes (Roesch et al., 2007). By leveraging this mechanism for use in agricultural systems, biological N_2 fixation technology can decrease the need for N fertilizer application and reduce environmental risks associated with excessive N fertilization (Raimam et al., 2007). This process has been shown to contribute as much as 75 kg N ha^{-1} per crop cycle with means of 8 to 30 kg N ha^{-1} (Irissarri and Reinhold-Hurek, 2001). Soybeans and peanuts are often inoculated with Rhizobium at planting to supplement soils with lower bacteria populations (Higa and Parr, 1994).

Pathogen suppression is another important function of healthy and diverse soil microorganism populations (García-Gutiérrez et al., 2013). Mitigating the presence of plant pathogenic organisms within a cropping system is important for growers because pathogens are capable of significantly reducing production or leading to the complete demise of important cash crops (Khaliq, Abbasi, and Hussain, 2005). There are two distinct mechanisms by which this

suppression can occur, and the first is general suppression. General suppression is non-targeted suppression of pathogens through multi-species competition for limited resources. This means that single populations are only allowed to grow to a certain extent, the size of which is usually small enough to keep potential plant pathogens from becoming a nuisance. The second mechanism is specific suppression, or targeted suppression. This describes a predatory, parasitic, or defensive capability of a non-plant pathogenic organism that allows for it to directly antagonize a specific plant pathogenic organism (Javaid, 2010; Mazzola and Gu, 2002). An example of this can be seen in a recent study with *Bacillus amyloliquefaciens*, which resulted in fewer cases of Fusarium wilt in cucumbers caused by *Fusarium oxysporum f. sp. cucumerinum* (Huang, et al, 2017). Free living nematodes also provide a certain degree of direct suppression against various plant pathogenic nematodes (McSorley, 2011).

Another argument for cultivating strong and active microorganism communities in an agroecosystem is that over time they have the ability to suppress the weed seed bank in the soil (Müller-Stöver et al., 2016). In any agricultural system, weeds compete with cash crops for water, nutrients, and light, ultimately diminishing the overall health of the crop. Certain fungi and bacteria can either directly attack seeds in the soil or they can exude compounds that inhibit the germination of the seeds (allelopathy). Data collected over the course of three years by Maramble and Sangakkara (1998) from tomato plots treated with EM seems to agree with this theory. During the first year, the initial increase in nutrient availability from the EM made the weed biomass increase above the that of the plots treated with mineral fertilizer (179 g/m^2 and 175 g/m^2, respectively) but in the subsequent years there was a significant decrease in weed biomass. Year two EM and mineral fertilizer plots contained 156 g/m^2 and 172 g/m^2 (respectively) while year three saw an increased separation of 114 g/m^2 and 190 g/m^2, respectively. This could be of immense importance

to sustainable agriculture, because a significant portion of most sustainable farm incomes are diverted to weed control, either chemical or mechanical. Chemical weed control, although generally used to a lesser extent in sustainable agriculture, relies on patented compounds that in many cases are strictly regulated, and therefore can be quite costly to subsistence or low-income farmers. Mechanical weed control generally relies on hired labor to be effective and can significantly reduce a farmer's net income.

Abiotic stressors can also be mitigated with the presence of effective microorganisms. Rhizobacteria can help plants withstand saline stress by stimulating sodium transporters (Bharti et al., 2013). Cold stress resistance and drought stress resistance has also been shown to be enhance by the presence of certain microorganisms in the rhizosphere through their modulation of phytohormones and solute concentrations within plant tissue (Ait Barka et al., 2006; Arkhipova et al., 2007; Jaleel et al., 2007).

In addition to effecting root health, effective microorganisms can modify metabolite composition of the whole plant, potentially increasing levels of compounds beneficial to humans within crops. Plant metabolites are important in crops that are intended for animal and human consumption because they are the driving force behind key factors of economic importance such as flavor, scent and plant defense as well as biologically interactive compounds like carotenoids (a beta-carotene precursor), flavonoids (a group of important antioxidizing compounds) and cannabinoids (bioactive components in cannabis). Increased levels of these nutritionally and pharmaceutically important compounds were shown in medicinal plants following inoculation by certain rhizosphere-dwelling microorganisms (Manero et al., 2003; Jaleel et al., 2007; Bharti et al., 2013). An increase in secondary metabolites in wheat and barley roots was demonstrated when a rhizosphere bacterium was applied with arbuscular mycorrhizal fungus

(Fester et al., 1999). Leaf secondary metabolites, such as total phenols and ortho dihydroxy phenols, as well as leaf mineral content (phosphorus, copper, potassium, zinc, and iron) were maximal when *Begonia malabarica* or *Solanum viarum* were inoculated with effective microorganism mixtures containing two fungi and a *Bacillus coagulans* strain (Selvaraj et al., 2008; Hemashenpagam and Selvaraj, 2011).

1.4 Historical Use of Local Effective Microorganisms

Though the exact mechanics behind the effectiveness of microbial soil communities in agricultural soil are still being elucidated, their use has been leveraged in Central and South American subsistence farms for generations to increase the vitality of their marginal and generally infertile soils. Multiple modes of implementation are used, but all rely on leaf litter collected from forests local to the cropping systems. The litter is transported to the farms and then either directly incorporated into the top layer of the soil or spread beneath stable animals for a period of about two weeks. The resulting mixture of broken-down leaves and animal manure is then worked into the fields. The rates of litter applied vary as greatly as the number of farmers who use it. Though the exact effects have not been quantified in peer-reviewed studies, the results are thought to be significant enough to the farmers to warrant the extra steps of applying the forest leaf litter to their agroecosystems **(**Altieri, 1995**).**

Effective microorganisms fit into a unique niche within the agricultural additives sector. Though modern farms have benefited greatly from the relatively new technologies introduced during the Green Revolution, it can be argued that these technologies have done little to modify ways of life for subsistence farmers **(**Pearse, 1980**)**. Many areas of the developing world are characterized by small traditional farms that primarily provide food, animal feed, and fiber to

the families that work them. These farms are often located in remote and geographically isolated regions with little modern infrastructure. The many agricultural advancements that have come about in recent years are scale dependent and based on modern scientific knowledge, which tends to neglect both traditional knowledge and local scale participation. Additionally, access to necessities such as technical support, information, and credit has been historically difficult for poor farmers to obtain. The lack of these things, when purchasing highly specialized inputs, imposes more risk and specialized problems for marginalized farmers. Even something as simple and common place to first world farmers as mineral nitrogen fertilizer can pose issues to less secure subsistence farmers. The fertilizer itself costs money that these farmers do not have, and meaningful quantities of it are very heavy and difficult or purely impossible to transport over poorly developed infrastructure like a mountain pass or marsh area. The challenge for international research in agriculture now lies in reorienting its efforts and focusing on marginalized farmers and the agroecosystems they manage. In the case of this present research, a process already adopted by the marginalized farmers was taken as a starting point and then modified to better understand the effects and mechanisms at work. By doing this, there is the possibility to redistribute this research to the farmers with greater understanding, higher efficiency and more significant impact. Much research in sustainable agriculture leads to frustration because low-input practices are unable to outperform conventional practices when compared in juxtaposition. The low-input methods are typically disregarded when they do not display direct agronomic gains, despite their systemic benefits **(**Vandermeer, 1997**).**

Several products and approaches have been attempted to modulate the soil microbiome. In the early 1970s, research at the University of the Ryukyus, Okinawa, Japan resulted in the development of effective microorganisms (EM), which was a heterogeneous culture

of beneficial and naturally occurring microorganisms, including lactobacilli, yeasts, and actinomycetes (Higa, 2000). Though EM was produced and distributed by EM Research Organization, Inc. (Uruma City, Okinawa, Japan) and its licensees, locally sourced microbial inoculants have continued to be cultured and applied in many regions. For example, farmers in Central and South America utilize a microbial product called microorganismos de la montaña (mountain microorganisms) that is cultured on substrates enriched with carbohydrates and inoculated with locally sourced microbes found in partially decomposed leaf litter. These local, effective microorganisms (LEM) have gained popularity because of a demonstrated ability to reduce foul odors and flies when applied to composts, pig pens, dairy corrals, and chicken coops. Agricultural extension organizations throughout Central and South America, such as the Advisory Foundation for the Ciudad de Dios Rural Sector (as La Fundación de Asesorías para el Sector Rural Ciudad de Dios; FUNDASES) in Colombia, The Ministry of Agriculture and Livestock (el Ministerio de Agricultura y Ganadería; MAG) in Costa Rica (Tencio, 2016), and The National Association for the Promotion of Agroecology (la Asociación Nacional para el Fomento de la Agricultura Ecológica; ANAFAE) in Honduras (ANAFAE, 2016), have actively promoted the use of LEM (Ney et al., 2019).

Even still, despite its widespread use in these regions, there is little published research that explores the potential challenges or benefits associated with modulating soil microbiomes when local inoculum sources are applied with varying nutrient sources.

While there has been some research conducted concerning the ability of commercial EM inoculant to improve soil function and crop productivity, reports of its effectiveness vary. Though several researchers found positive effects of EM additions to fertilizer regimes on crop yield and/or soil fertility (Daly and Stewart, 1999; Daur and Abusuwar, 2015;

Javaid and Bajwa, 2010), others found little or no beneficial effects from incorporating EM into production (Mayer et al., 2010; Zu Schwiensberg-Mickan and Müller, 2009; Vliet et al., 2006), there has been little effort put into investigating the effectiveness or consequences of the locally-produced LEM inoculant on soil function and crop productivity. The research that has been published suggests that it has the potential to improve agricultural production. For example, Kamla et al. (2008) investigated the effects of a locally-produced microbial inoculant similar to LEM on cowpea (*Vigna unguiculata* (L.) Walp.) yield and found that when used in combination with an organic amendment, the fermented bio-extract (LEM) resulted in higher yields than those achieved with the addition of the organic amendment alone. It is possible that the use of locally derived microbes to make LEM is not only more affordable and accessible to farmers, but it may also be more effective than the commercial equivalent. Campo-Martinez et al. (2014) investigated the effects of both EM and locally produced, LEM/ mountain microbe bacterial inoculants on the growth of chard and found that the LEM performed significantly better than the commercial EM product.

As denoted in previous research, LEM shows promise as biological stimulant but may be affected by many factors including climate, soil type, organic material, crop type, and others. Indirect effects of microbial community fluctuations such as nitrogen retention, plant tissue nutrient levels and plant biomass are only a few ways to quantify changes that may be occurring within soil microbial populations in an agroecosystem.

1.5 Decline of Food Nutrient Levels

As primary producers, plants serve as distinct intermediaries in the food chain for every succeeding trophic level. In this position, they translate nutrients from the soil to whatever

may be consuming them. In the case of humans, food is the main source through which it is possible to receive the mineral elements necessary for basic life functions. Wheat remains the most important agricultural crop in the world in terms of number of people it sustains and high quantities are always needed. Since the early 1900's, there has a been a drastic increase in the productivity and yield of crop plants per hectare, with modern farming practices and genetic improvement interacting to increase wheat yields by more three times the record quantity of 860 kg ha^{-1} back near the turn of the century in order to cope with demand for food (Garvin, Welch, and Finely, 2006). However, research suggests that along with this increase in yield, there has been a corresponding decrease in overall nutrition despite adequate nutrient levels in the soil being made available to the plants (Fan, et al., 2008). In light of this, there is need to explore alternative methods for restoring historical nutrient levels in crop plants.

1.6 Implementation in Cropping Systems

There are three main methodologies that can be used to incorporate EM into cropping systems. The first is bioaugmentation, in which selected and known strains of microorganisms are introduced to the system. The advantage for using specific EM inoculum is that there is a higher probability of getting highly efficient strains, but that efficiency comes with the prohibitive cost of not only purchasing the inoculum, but also having to incorporate it into the cropping system. The second method is biostimulation, or to modify management practices in such a way that encourages the growth and proliferation of the indigenous populations of microorganisms already within the system. Although indigenous EM may be relatively less efficient than the lab proven strains available for purchase, they can be advantageous because they are already adapted to the local environment and its associated conditions (Roy-Bolduc and Hijri,

2011). The promotion of indigenous EM is primarily driven by the amount and quality of nutrients and other metabolic building blocks present in the soil which are, in turn, mainly driven by the species of plants grown within the system. As mentioned previously, plants communicate with microorganisms and promote their activity by modifying the rhizosphere. This modification of the rhizosphere is accomplished by exuding carbohydrates and other nutrients through the roots (du Jardin, 2015). Additionally, as plant material dies the organic nutrients held within the biomolecules get incorporated into the soil system, further contributing to the soil nutrient pool that can potentially feed microorganisms. Many members of EM communities are also sensitive to harsh chemicals and can only be promoted within a cropping system by limiting the use of such compounds, particularly broad-spectrum biocides like fumigants and fungicides. Furthermore, the success of EM within a cropping system is largely impacted by the degree to which their populations are allowed to colonize the soil. Management is a distinct factor that affects microbial communities living in the soil (Orr et al., 2011). Heavy tilling has been shown to limit the presence of mycorrhizal associations, a distinctly important benefit of EM, therefore it is important to employ conservation or no-tilling practices if the full benefits of flourishing EM populations are to be gleaned (Giller, et al., 1997). The third method used to increase soil microbiological diversity combines the elements of both bioaugmentation and biostimulation by utilizing active cultures of organisms harvested from the decomposing layer in forests local to the agroecosystem that is being augmented. The LEM cultures are then applied to the local cropping system and their growth is promoted through the use of appropriate management techniques like those outlined above (Pushpa, et al., 2016). Ultimately, effective on-farm nutrient recycling will be a key facet in developing more efficient farms to keep pace with increasing demand for food and fiber. Local

effective microorganism cultures are a cheap and potentially effective way to accommodate this by boosting nutrient cycling efficiency while still adhering to sustainable agricultural practices.

1.7 Objectives

The intent of this study is to take what current literature has found, as outlined in this section, and validate it by utilizing a methodical experimental design that also incorporates comparisons between LEM treatments on mineral fertilizer and organic fertilizer. To that end, the objectives of the present study are to investigate and analyze:

1. The influence of LEM on important agronomic indices related to green bean and winter wheat grown in pots containing basic alluvial soil from the Po River Valley.
    a. Plant biomass
    b. Crop yield
    c. Crop tissue nutrient content
    d. Leaf chlorophyll content
2. The influence of LEM nitrogen retention in the growth system
    a. Leachate emissions from the pot systems
3. The influence of LEM on activity, size and composition of soil microbiological communities
    a. Soluble organic solids present in soil
    b. Microbial biomass
    c. DNA extraction and sequencing analysis of inoculum at the 16s RNA level

CHAPTER 2

AGRONOMIC RESPONSES TO LOCALLY SOURCED MICROBIOLOGICAL SUPPLEMENTATION (LEM)[1]

[1] Fatzinger, Brendan, D.H. Franklin, Antonio Berti, Andrea Squartini, and Miguel Cabrera. To be submitted to Soil Ecology Letters.

ABSTRACT

The application of locally-sourced microbiological supplements (local effective microorganisms- LEM) at the time of fertilization with organic fertilizers may increase the mineralization and availability of nutrients and bolster microbial communities in the soil system. LEM/False-LEM consists of many different species of naturally occurring organisms including, but not limited to, lactobacilli (*Lactobacillus plantarum*, *L. casei*, and *Streptococcus lactis*), yeasts (*Saccharomyces spp.*), Actinomycetes (*Streptomyces spp.*), and fungi. We conducted a factorial experiment of three fertilizer treatments (organic, inorganic, no fertilizer) combined with three inoculant treatments (LEM, False-LEM, and water) to evaluate effects on soil and wheat or bean crops. Variables measured were polymerase chain reaction (PCR), soil microbial biomass, soil soluble organic solids, plant tissue nutrient content, crop yield, plant biomass, leachate N content and leaf chlorophyll content. Of these, soil microbial biomass, soil soluble organic solids, plant biomass, N retention within the system, and leaf chlorophyll content were significantly elevated in systems receiving microbial inoculum treatments. These results show that LEM has potential to increase plant, soil and environmental health within the first month of application.

INDEX WORDS: soil health; effective microorganisms; local effective microorganisms; agronomic indices; plant health; nutrient cycling efficiency

1 Introduction

As agricultural intensification has been increasing over the past several decades due to advancement in technology, population growth, and heightened consumer expectations there has been a correlated increase in the use of monocultures, synthetic fertilizers, pesticides, and rigorous management practices, ultimately resulting in a marked increase in food production (Giller, et al. 1997). Indeed, this is certainly a positive but there is evidence to suggest that this has led to a decline in overall soil microbial health and nutritional value in the foods being produced as well as wasted phosphorus, a limited resource (Cóndor Golec, et al. 2007; Davis, 2009).

Studies observing the impacts of some agricultural chemicals on the environment have revealed that they can significantly affect microbial populations as a whole; even seemingly unrelated chemicals and organisms can have interactions, such as the fungicide mancozeb on aerobic nitrogen fixing bacteria (Doneche et al., 1983; Baxter and Cummings, 2008). In addition to their poor environmental legacy, these products and technologies are prohibitively expensive to subsistence farmers and there is distinct difficulty in meeting the demand for them at an attainable price (Kumar and Gopal, 2015). Furthermore, the use of certain agricultural chemicals poses a significant risk to farm workers, potentially diminishing their quality of life (Megali, Schlau, and Rasmann, 2015). As such, more sustainable practices are continually being explored as potential alternatives. Though there are many possible implications related to the use of biotechnology in agriculture, one facet that has not been fully explored is that of microbiological diversity, particularly in the soil, and its effects in relation to sustainable, subsistence, and closed-loop agricultural systems, particularly those that rely heavily on organic fertilizers such as manure and litters from animals and plant-comprised “green manures” (Javaid, Bajwa, and

Anjum, 2008). Use of organic fertilizers can be problematic to growers because they must undergo mineralization before plant uptake (Compant et al., 2010) This mineralization is reliant on biology in the soil, and if the soil is lacking sufficient populations of key functional groups then timing of nutrient availability with crop needs can be mismatched resulting in poor yields. Much research has been done to maximize food productivity in fields such as plant breeding and fertility management, but relatively little has been focused towards using native soil microorganisms to help boost nutrient cycling and retention (Hu and Qi, 2012). Currently, there exist many elucidated and cultured microorganisms that contribute to modern human life, yet in agriculture there are still gaps in understanding regarding effective microorganisms (EM) and their potential relation to plant health (Kumar and Gopal, 2015). Arguably, benefits provided by diverse soil dwelling microbial populations are numerous, yet research is regularly discovering potential advantages these organisms may lend to various cropping systems (Wright and Jones, 2006). Particularly for sustainable and closed-loop oriented growers, the main services leveraged are to provide better soil structure, pathogen suppression, increased nutrient availability, minimization of nutrient loss, weed suppression, and bio-remediation of soil contaminates (Giller, et al., 1997; Singh and Singh 2016; Müller-Stöver et al., 2016; Smith and Goodman, 1999).

A methodology to leverage the benefits of the interactions between plants and microorganisms is to apply effective microorganism (EM) cultures to the soil. Local effective microorganism cultures are conglomerations of distinct species of effective microorganisms harvested local to the agricultural system in which they are intended to be applied (Kumar and Gopal, 2015; Higa and Parr, 1994). The organisms are a part of various taxonomic groups and may be present simultaneously in any combination in a given soil in the wild (Kyselková et al.,

2009; Almario et al., 2013a). Based on studies by Higa (1998), EM contains about 80 species of microorganisms divided into photosynthesizing bacteria, lactic acid bacteria, yeasts, actinomycetes, and fermenting fungi which all have significant roles in the soil system. It is also reported that the main organisms represented in the cultures are *Rhodopseudomonas palustrus* and *Rhodobacter spaeroides* (photosynthetic bacteria), *Lactobacillus plantarum*, *L. casei*, and *Streptoccus lactis* (lactic acid bacteria), *Saccharomyces cerevisiae* and *Candida utilis* (yeasts), *Streptomyces albus* and *S. griseus* (actinomycetes), and *Aspergillus oryzae*, *Penicillium* sp. and *Mucor hiemalis* (fermenting fungi) (Ahn, et al., 2014; Diver, 2001). Local effective microorganism (LEM) cultures are a modified approach to EM and include the dimension of locality by culturing microorganisms suited for growth in the same environment as the agroecosystem in which they are applied. Use of this biological technology has the added benefits of not being genetically-engineered and nor chemically synthesized, and is therefore acceptable for implementation in even the strictest legal frameworks (Zakaria, Gairola, and Shariff, 2010). Additionally, in making LEM cultures, only cost effective and widely available ingredients are used, which makes it a much more viable technique to be used in even marginal, low income farms. Within the rhizosphere, the expression of effective microorganism's plant-beneficial properties can be affected by both abiotic factors (such as pH, oxygen, clay mineralogy, heavy metals, etc.) and biotic factors (for example, compounds exuded by plants or the rhizosphere-dwelling microbes) that can lead to distinct expressions in spatial and temporal patterns, possibly with different effects observed on the host plant (Piccoli and Bottini, 1994; Pothier et al., 2008; Prigent-Combaret et al., 2008; Dutta and Podile, 2010; Almario et al., 2013b; Drogue et al., 2013).

There is little existing data supporting or disproving the viability of LEM from an agronomic standpoint. This study aimed to provide more meaningful insight into the effects of locally sourced microbiological supplementation in the highly basic, carbonate rich Oxyaquic Eutrudept soils in Italy's Po River Valley by using a multifaceted approach including proximal and direct indicators at the plant and soil levels. As such, the objectives of this research were to 1) determine the influence of LEM on important agronomic indices (plant biomass, crop yield, crop tissue nutrient content, leaf chlorophyll content), 2) quantify the response of inorganic nitrogen retention in the soil system, and 3) analyze any changes in activity, size and composition of soil microbiological communities in response to LEM application.

2 Materials and Methods

2.1 Site and Experiment Description

Location was Legnaro, Padova, Italy (45° 21' 04" N, 11° 56' 51" E), 5 m a.s.l., Fluvi-Calcaric Cambisol (CMcf) according to the European Soil Bureau/ United Nations Food and Agriculture Organization UNESCO soil legend. The study area is in the low Venetian plain and is characterized by sedimentary loamy soils. The local climate is sub-humid, with annual rainfall of about 850 mm. Temperatures range from a average minimum of 1.5 °C in January to an average maximum of 27.2 °C in July. Pedo-climatic zone is Mediterranean North, Cambisol. The soil used in the experiment was under conventional management before being removed from the upper 15 cm and placed in containers. Soil at the site was an Oxyaquic Eutrudept, coarse-silty mixed, mesic according to the World Reference Base for Soil Resources (Morari, 2006), having a silt loam texture. Prior to treatment applications, total Kjeldahl nitrogen was 0.1%, pH was 7.5, available P was 17.8 mg kg^{-1} (Olsen), organic carbon content was 8.373 g kg^{-1} and electrical conductivity was 0.238 mS cm^{-1}. The experiment was set up in a randomized complete block design, with treatment position within each of the four blocks determined by random number generation in Microsoft Excel.

During the first season, green beans (*Phaseolus vulgaris* cv. Valentino) were planted in conventional black extruded polyethylene pots (38 liter capacity, top diameter: 37 cm, base diameter: 35.5 cm, height: 30 cm) in early July. In the second season, beginning in April the following year, winter wheat (*Triticum aestivum*, L.) was planted in custom pots fabricated out of 16 cm (inner diameter) PVC pipe, cut to a height of 50 cm, with a screen mesh partition installed at 15 cm from the bottom opening to allow for water to drain through to catch pans beneath the system. The fabricated pots had a capacity of 7.03 L. In both seasons, seeds were directly sown

into the pots. Fertilizer and inoculum treatments were applied immediately after sowing and incorporated into the top 5 cm of soil by hand. Bean trials were thinned to 4 plants per pot, while wheat trials were thinned to 10 plants per pot. Both trials were hand watered as needed with onsite, chlorine-free well water.

Fertilizers applied were Italpollina, a commercially available organic 4-4-4 (N-P_2O_5-K_2O) pelletized composted poultry manure, and mineral fertilizer. The mineral fertilizer used was a combination of ammonium sulfate, triple super phosphate and potassium chloride formulated specifically for this study. The mineral fertilizer was mixed to match the amounts of nitrogen, phosphorous and potassium determined from the analysis of the composted poultry manure used. Both fertilizers were applied at a rate of 60 kg N ha^{-1}, 60 kg P_2O_5 ha^{-1} and 60 kg K_2O ha^{-1}. No fertilization was added to control treatments.

LEM and False-LEM (FLEM) inoculum was prepared according to Ney, et al. (2019) and scaled down to facilitate a smaller 5 L batch. The LEM was made by collecting 15 kg of O horizon (loose and partly decayed organic matter) from healthy, well established upland, mixed pine and hardwood forests, which provided the microbial inoculant. The microbial inoculant was combined with 1.7 kg of organic semolina, 2 g of active dry baker's yeast and 0.77 kg of crushed hardwood charcoal. A liquid solution consisting of 0.31 L of feed-grade molasses, 0.31 L of unpasteurized, non-homogenized milk, and 0.31 L of non-chlorinated water was then blended into the solid mixture. Once the liquid and solid mixtures were well blended, the LEM was placed into a sheltered 14 L-container and sealed with silicone to ferment for 6 weeks at temperatures ranging from 17 - 21˚C. To make the liquid LEM solution, 0.5 kg of solid fermented LEM mixture was place in a porous sack and suspended in a 14 L-container with 4.7 L of water and 0.32 L of

molasses. The container was sealed with silicone and the liquid LEM solution was left to ferment for 2 additional weeks.

Because the growing media (molasses and semolina, yeast and raw milk) contains nutrients and microbes, the False-LEM (FLEM) treatment solution, which was intended to act as the false control, was formulated without the forest microbial inoculant and with only the nutrient media. The FLEM mixture is prepared under the same conditions as the LEM using the molasses, semolina, milk, yeast and charcoal and combined in the same ratio as the LEM.

The treatment design was a factorial of three fertilizer levels (organic composted poultry manure, mineral fertilizer, and blank control with no fertilization) and three inoculum treatments (LEM, FLEM, and pure water control) to generate 9 total treatments, which were arranged in a randomized complete block design with four replications. LEM and FLEM were applied at a rate of 18.337 L ha^{-1}, equivalent to 53 mL per pot. LEM/FLEM controls received 53 mL of pure well water. Immediately after treatment application, all pots were watered in with 50 mL of well water application to move applied materials into the soil.

2.2 Agronomic Indices

Plant biomass was determined by carefully extracting the plants from their pots and using water to wash away existing soil from the roots. The whole plants were then dried for 48 hours at 65°C. After drying, the samples were weighed. The plants were cut at the crown to separate the roots from the shots and each were weighed individually.

Bean yield was determined by harvesting the beans and counting the number of pods produced by each plant. The beans were then weighed to obtain fresh weight followed by

drying for 48 hours at 65°C to be weighed again for dry weight. Wheat yield was not quantified due to incomplete vernalization resulting in suboptimal seed set.

Nutrient content analysis of the crop tissue was performed by the University of Padova Centralized Chemical Laboratory (La-Chi) in Legnaro, Italy. Total nitrogen was determined via the Kjeldahl method (RB Bradstreet - Analytical Chemistry, 1954). Calcium, magnesium, zinc, and phosphorus were determined via inductively coupled plasma – atomic emission spectroscopy (ICP–AES) (Varian, Victoria, Australia).

Chlorophyll content was analyzed by using a SPAD 502 Plus Chlorophyll Meter (Spectrum Technologies, Inc., Aurora, IL). Three fully expanded leaves were selected from each pot at 1 month post germination with the SPAD meter placed in contact with the individual leaves until the reading was complete as indicated by the device. The three readings obtained from each leaf were then averaged together to give the final value for the respective plant.

2.3 Leachate Sampling and Nitrogen Retention

The amount of nitrogen retained within the soil/plant system in response to microbial inoculation was determined by analyzing the levels of nitrogen present in the leachate passing through the pots. This is an established method for quantifying inorganic N retention and is supported by previous studies (Dempster, et al., 2012; Hartz, et al., 2000; Mikkelsen, et al., 1994; Zheng, et al., 2013).

Leachate samples were obtained from the pots containing wheat by saturating the soil to capacity (determined by previous trials to be 1.9 L) and then adding an additional 50 mL of water and waiting two hours for full percolation. Approximately 50 mL leachate was collected in catch pans below the pots and was then transferred to resealable containers. The samples were

frozen at -5°C until they could be analyzed. To avoid consistent oversaturation of the soil from repeated sampling, weather data from ARPAV Centro Meteorologico di Teolo (Agenzia Regionale per la Prevenzione e Protezione Ambientale del Veneto, 2019) were used to track significant rain events that resulted in 40 mL of rain or more. Over the course of the study there were two such events, 4/23/2019 and 6/5/2019. The samples were collected immediately after those rain events to match the natural weather cycle as closely as possible without leaving the soil oversaturated. Leachate samples were analyzed by Veritas Laboratory (Veritas s.p.a., Venice, IT) for total nitrogen and NH_3, NO_2 and NO_3 specific fractions using ion chromatography.

2.4 Microbiological Indices and Community Structure

2.4.1 Soluble Organic Solids

Composite soil samples were taken from the top 5 cm of the profile at month 2 of the 4-month growing period, air dried and then sieved through a 1mm screen. Five g of the dried and sieved soil was then suspended in 10 mL of hexametaphosphate (HMP) buffer [$(NaPO_3)_6$ 35.7 g/L, Na_2CO_3 7.94 g/L] inside 15 mL plastic tubes with screw top closures. The samples were mixed with a vortex machine for 10 seconds and placed on a rotary shaker for 40 minutes at 140 rotations/minute. This was followed by 5 minutes at 5500 G's in a Sigma centrifuge (St. Louis, MO). A 0.5 mL aliquot of the supernatant was added to 1 mL NaCl (9.8 g/L) in Eppendorf polypropylene tubes. The contents of each tube were then transferred to a respective UV-spectrum cuvette and absorbance at the 260nm wavelength was read in a UV-visible light spectrophotometer (Pharmacia, Uppsala Sweden) using the 9.8 g/L NaCl solution as reference. For some samples with higher levels of organic matter, it was necessary to dilute the solution to a 1:10 ratio (soil solution : 9.8 g/L NaCl solution) before taking a reading.

2.4.2 Assay for Microbial Biomass

Composite soil samples were taken from the top 5 cm of the profile at month 2 of the 4-month growing period, air dried and then sieved through a 1--m screen. Next, 1 g was suspended in 5 mL 9.8g/L NaCl solution (pH adjusted to 7 with 0.1 N NaOH) inside a 15-mL plastic tube with screw top closures. The soil solution was then mixed with a vortex machine for 10 seconds followed by 40 minutes on a rotary shaker set to 140 rotations/minute. Two 1-mL aliquots of each sample were then deposited respectively into two Eppendorf polypropylene tubes. One tube received no further additives and served as the control while the other received 14 μL of fluorescein diacetate (FDA) (1 mg mL^{-1} FDA in acetone, stock solution stored at -20˚C). Tubes were then manually agitated to ensure full emulsion before being placed in an incubator at 30˚C for 2 hours. After incubation, the samples were centrifuged for 5 minutes at 14000 G's in an Eppendorf bench centrifuge. A supernatant volume of 1 mL was then transferred to a visible spectrum cuvette and the absorbance was read at the 490 nm in a UV-visible light spectrometer (Pharmacia, Uppsala Sweden). The 9.8g L^{-1} NaCl solution was used as a blank control. If absorbance readings were at or above 2 it was necessary to dilute the samples to a 1:10 ratio with 9.8 g L^{-1} NaCl solution. Absorption data were then translated to microbial biomass (mg kg^{-1}) via the following formulas derived from regression analysis of known microbial biomass values and corresponding standardized FDA hydrolysis:

$$Fluoresceine\ \mu g\ mL^{-1} = (4.9956 * 490nm\ Absorption) + 0.0019$$

$$Fluoresceine\ mg\ kg^{-1}\ soil = (Fluoresceine\ \mu g\ mL^{-1} * 5000\ kg)/1000 \mu g$$

$$Microbial\ Biomass\ mg\ kg^{-1}\ soil = 3.003 * Fluoresceine\ mg\ kg^{-1}\ soil$$

Formula 1. Translation of 490 nm light absorption readings to microbial biomass (mg kg^{-1} soil).

2.4.3 DNA Sequencing and Analysis

Characterization and elucidation of the functional bacterial groups present in the LEM and FLEM inoculum was achieved by using the Ion S5™ DNA sequencing system (Thermo Fisher Scientific, Life Technologies Corporation; Carlsbad, CA). One week after breaking the seal of the containers, the containers were gently agitated to homogenize the contents and 10 mL aliquots were taken from randomly varying points and depths in the fermenting containers housing the LEM and FLEM to obtain the highest possible heterogeneity in the samples. The samples were then prepared and run through the Ion S5 system as outlined by the Ion ReproSeq™ PGS Kits − Ion S5™/Ion GeneStudio™ S5 Systems quick reference guide (Thermo Fisher Scientific, Publication No. MAN0016713, Rev. C.0, 2018) and the resulting unique 16s RNA reads were defined and validated in the Ion AmpliSeq™ libraries (Torrent Suite™ Software 5.10.1, Life Technologies Corporation; Carlsbad, CA). The Ion AmpliSeq™ libraries then output the specific species present in the inoculum samples for further evaluation and visualization.

2.5 Statistical Analysis

Jmp Pro 14 software (SAS Institute Inc., 2019) was used for statistical analysis of all data in this present study. Normality of data was confirmed by the Shapiro-Wilk test and Bartlett's test and Levene's test for homogeneity of variances. Analysis of variance (ANOVA) was performed to ascertain significance, followed by Tukey’s test to identify differences between treatments. Treatment effects and the interactions among treatments were tested and, unless otherwise noted, all significance thresholds were $p = 0.05$.

3 Results and Discussion

3.1 Agronomic Indices

Wheat total biomass showed an increasing trend favoring LEM ($p = 0.0754$) (Table 1) (Fig. 1**A**) having 18% and 15% more overall biomass than control and FLEM treatments, respectively, whereas bean yield fresh and dry weight was not affected by the treatments ($p = 0.5768$ and $p = 0.5730$, respectively) (Fig. 1**B** and 1**C**). Plant biomass and general productivity are an important aspect to probe when considering any modification within an agroecosystem because most grower's income is related directly to those parameters. They are affected by many factors, however, including solar radiation, water availability, temperature, and nutrient availability (Simkin et al. 2015). In the case of this study, any effect of LEM on plant biomass and yield was most likely due to increased nutrient availability in the soil provided by the microorganisms because all other cultural factors were held constant for each of the treatments.

Chlorophyll content in wheat leaves displayed near significance favoring FLEM and LEM in terms of mean number of SPAD units ($p = 0.0653$) (Table 2) (Fig. 2**A**). Differences in green been leaf chlorophyll content showed no significance at the treatment level ($p = 0.7891$) (Fig. 2**B**). Chlorophyll leaf content serves as a strong indicator for overall plant health, vitality, and nitrogen mobility within the plant body. Elevated chlorophyll levels are correlated with higher quantities of nitrogen uptake in wheat (Bojović and Marković, 2009). This, in turn, is directly related to increased photosynthetic efficiency and CO_2 assimilation. Plant biomass and leaf area accumulation is largely driven by the efficiency of CO_2 assimilation (Evans, 1983), so while working within the framework the genetic constraints associated with the plants in question, it can be better understood how biomass was also positively affected by the microbial inoculum (Fig. 1**A)**.

Table 1. Wheat biomass and green bean yield ANOVA table.

Parameter	Effect	SS	DF	F	Prob F
Wheat Mass	Treatment	1518.7	2	2.88	**0.0754**
	Fertilizer	1150.4	2	2.18	0.1344
	Treatment x Fertilizer	1130.5	4	1.07	0.3915
Bean Fresh Yd.	Treatment	17.2	2	0.56	0.5768
	Fertilizer	21.1	2	0.69	0.5112
	Treatment x Fertilizer	20.4	4	0.33	0.8533
Bean Dry Yd.	Treatment	18.4	2	0.56	0.5730
	Fertilizer	4.3	2	0.13	0.8741
	Treatment x Fertilizer	11.0	4	0.17	0.9512

Note: Data is from a greenhouse study in which three fertilizer treatments (organic, inorganic, control) and three inoculant treatments (LEM, FLEM, water) were combined to generate a factorial of nine treatments that were applied to pots in which wheat or green bean was grown for 4 months. Significant probabilities indicated by bold type.

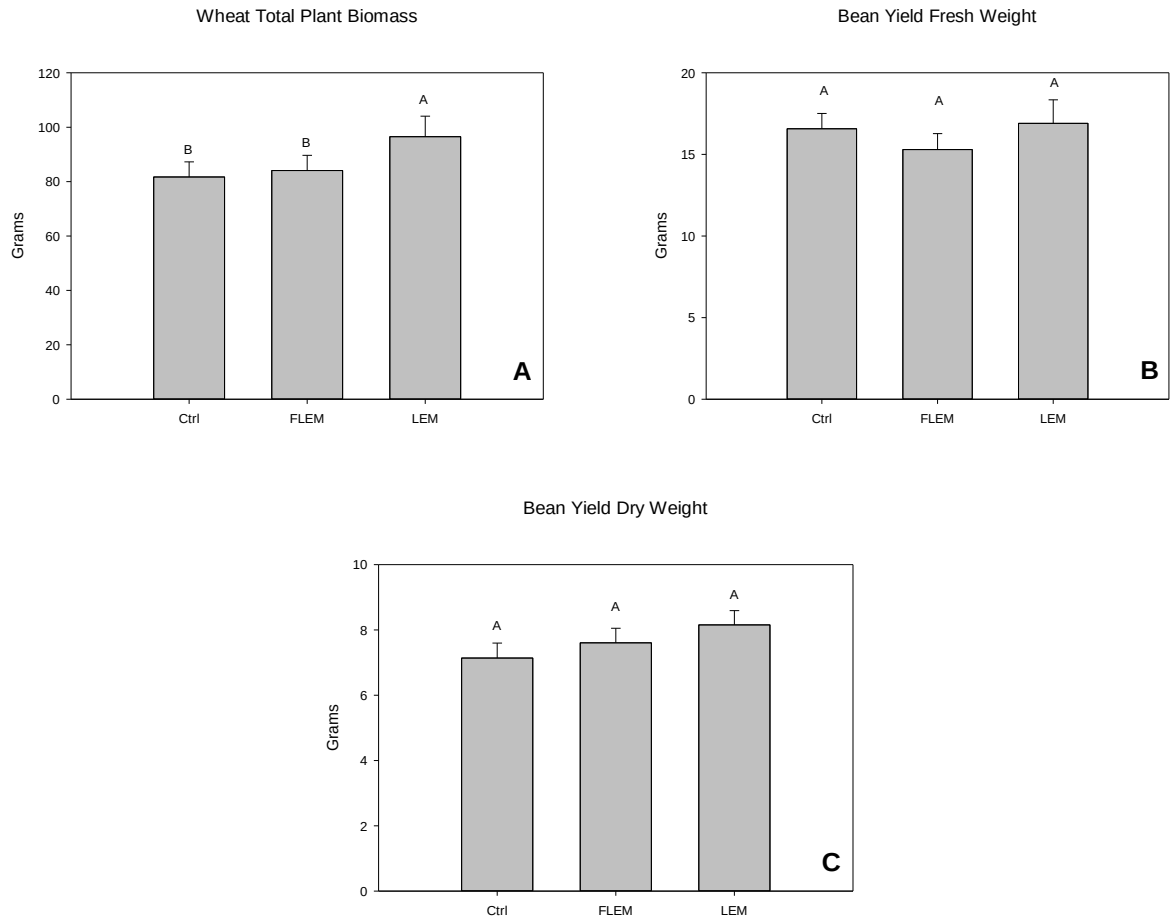

Figure 1. Biomass and yield responses in a greenhouse study in which three fertilizer treatments (organic, inorganic, control) and three inoculant treatments (LEM, FLEM, water) were combined to generate a factorial of nine treatments that were applied to pots in which wheat or bean was grown for 4 months. Graph **A** indicates wheat total plant biomass, graph **B** indicates green bean crop yield fresh weight and graph **C** indicates green bean crop yield dry weight. Different letters indicate significant differences between means according to pairwise comparisons using Tukey's test at p=0.05. Error bars represent standard error of the mean.

Table 2. Leaf chlorophyll content ANOVA table.

Chlorophyll Level	Effect	SS	DF	F	Prob F
Wheat	Treatment	69.0	2	3.06	**0.0653**
	Fertilizer	19.9	2	0.88	0.4259
	Treatment x Fertilizer	18.1	4	0.40	0.8040
Green Bean	Treatment	2.1	2	0.23	0.7891
	Fertilizer	15.6	2	1.79	0.1878
	Treatment x Fertilizer	2.6	4	0.15	0.9595

Note: Data is from a greenhouse study in which three fertilizer treatments (organic, inorganic, control) and three inoculant treatments (LEM, FLEM, water) were combined to generate a factorial of nine treatments that were applied to pots in which wheat or green bean was grown for 4 months. Significant probabilities indicated by bold type.

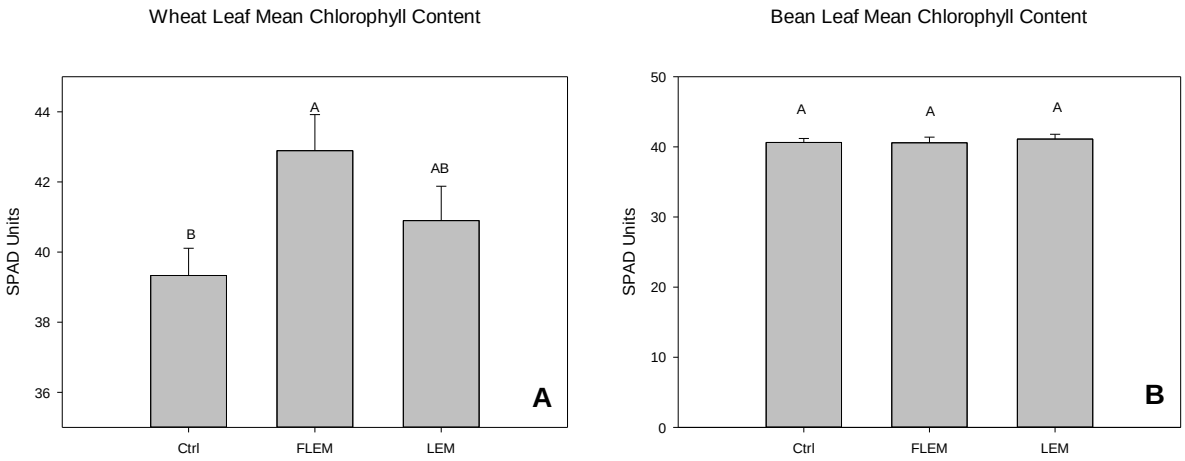

Figure 2. Results of SPAD readings for relative chlorophyll abundance in leaf tissue at one month post treatment in wheat (**A**) and green bean (**B**). Data is from a greenhouse study in which three fertilizer treatments (organic, inorganic, control) and three inoculant treatments (LEM, FLEM, water) were combined to generate a factorial of nine treatments that were applied to pots in which wheat or green bean was grown for 4 months. Different letters indicate significant differences between means according to pairwise comparisons using Tukey's test at p=0.05. Error bars indicate standard error of the mean.

Bean crop Ca, Mg, P, N and Zn concentrations from plants that received application of LEM and FLEM displayed no significant treatment differences compared to the controls at the $p < 0.05$ level (Table 3) (Fig. 3**A**-**E**). The beans did show a significant response to the fertilizer treatment only, with the organic fertilizer resulting in increased phosphorus accumulated in the

harvested tissue (Table 3). However, the general lack of response observed in green beans to the microbial inoculum treatments is thought to be primarily the result of two main contributing factors. First, the bean trial underwent higher levels drought and heat stress compared to the wheat trial because it was carried out in the summer months relatively unprotected from outside elements while the wheat trial was carried out in the late winter and spring months within the protection and stability of a green house. Second, green beans are legumes, which have evolved over time to form strong and highly favored symbiotic associations with members of *Rhizobiaceae*. The strength of this relationship between leguminous crops and *Rhizobiaceae* is such that members of *Rhizobiaceae* may be able to outcompete and limit the effectiveness of other microorganisms, thereby masking the effects of the microbial inoculum treatments.

Table 3. Bean crop tissue nutrient content ANOVA table.

Nutrient	Effect	SS	DF	F	Prob F
Ca	Treatment	466760.3	2	0.41	0.6663
	Fertilizer	1459295	2	1.29	0.2934
	Treatment x Fertilizer	2046788	4	0.90	0.4765
Mg	Treatment	38709.9	2	0.47	0.6293
	Fertilizer	198915.5	2	2.42	0.1097
	Treatment x Fertilizer	28304.4	4	0.17	0.9502
P	Treatment	670496.6	2	2.22	0.1303
	Fertilizer	1444502	2	4.78	**0.0178**
	Treatment x Fertilizer	539926.1	4	0.89	0.4827
TKN	Treatment	0.9	2	2.53	0.1002
	Fertilizer	0.2	2	0.59	0.5621
	Treatment x Fertilizer	0.3	4	0.45	0.7697
Zn	Treatment	110.7	2	1.63	0.2163
	Fertilizer	131.1	2	1.93	0.1665
	Treatment x Fertilizer	338.0	4	2.49	0.1399

Note: Data is from a greenhouse study in which three fertilizer treatments (organic, inorganic, control) and three inoculant treatments (LEM, FLEM, water) were combined to generate a factorial of nine treatments that were applied to pots in which wheat or green bean was grown for 4 months. Significant probabilities indicated by bold type.

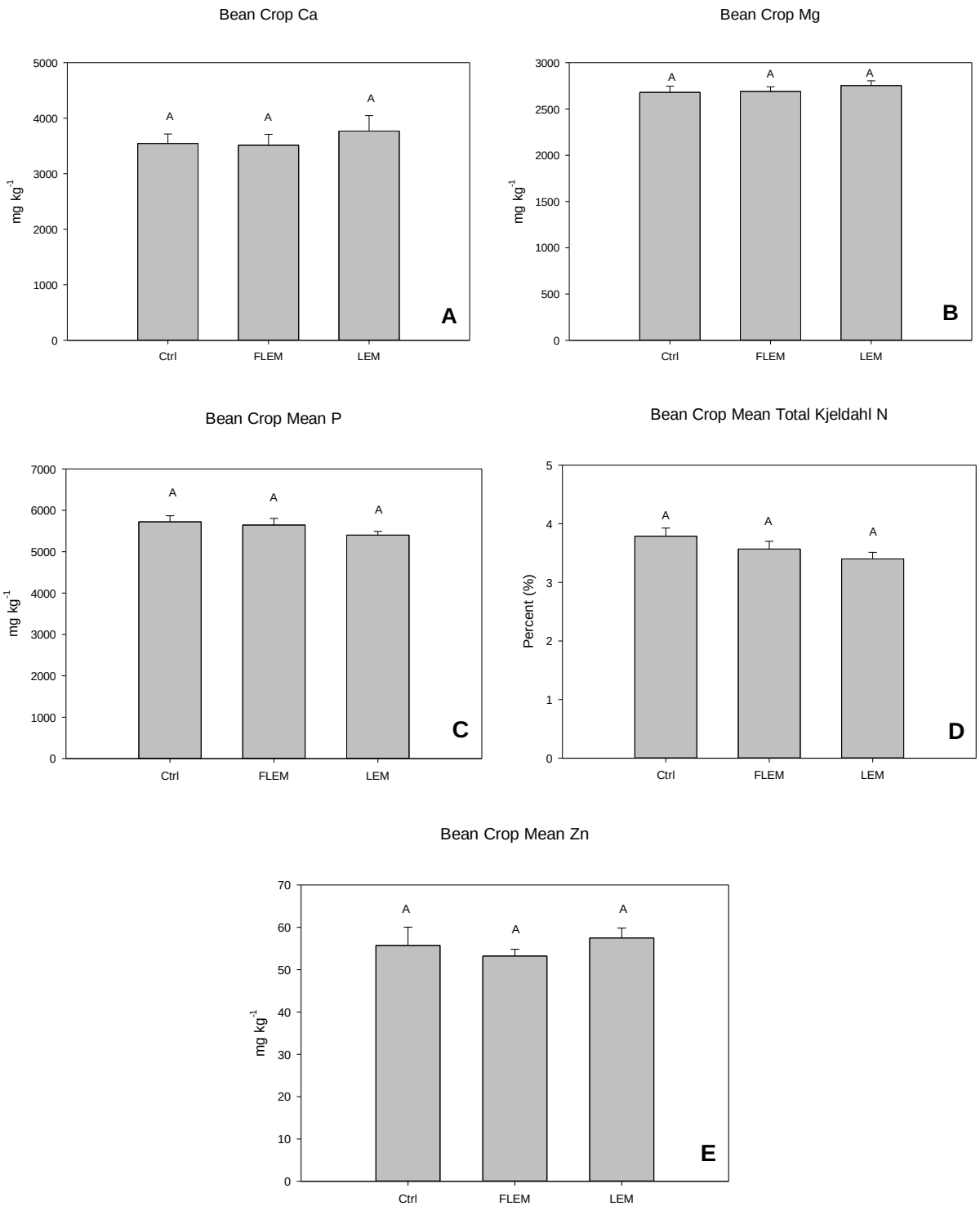

Figure 3. Bean tissue nutrient responses to differing treatments of microbial inoculum and fertilizer. Data is from a greenhouse study in which three fertilizer treatments (organic, inorganic, control) and three inoculant treatments (LEM, FLEM, water) were combined to generate a factorial of nine treatments that were applied to pots in which green bean was grown for 4 months. No differences were observed between inoculum treatments. Same letters indicate

no significant differences between means according to pairwise comparisons using Tukey's test at p=0.05. Error bars represent standard error of the mean.

3.2 Leachate Sampling and Nitrogen Retention

At both major rain events that occurred during the study, analysis of the leachate from the pots containing wheat confirmed previous research that organic fertilizer contributes to less short-term total nitrogen loss from the rhizosphere than mineral fertilizer (event 1 $p < 0.0001$ and event 2 $p < 0.0001$) (Dempster, et al., 2012; Hartz, et al., 2000; Mikkelsen, et al., 1994; Zheng, et al., 2013). Rain event 1 (Table 1) (Fig. 4) showed that there was a significant decrease in NO_2^- ($p = 0.0351$) (Fig. 4**B**) and NO_3^- ($p = 0.0523$) (Fig. 4**C**) leaching from soil columns treated with LEM and composted poultry manure compared to uninoculated control soil. No significant differences were observed in NH_4^+ leaching between LEM, FLEM, and control treatments (Fig. 4**A**).

Table 4. Rain event 1 ANOVA table.

Nutrient	Effect	SS	DF	F	Prob F
NH_4^+	Treatment	0.001	2	0.1	0.9048
	Fertilizer	0.05	2	4.27	**0.0257**
	Treatment x Fertilizer	0.03	4	1.09	0.3826
NO_3^-	Treatment	3893.284	2	1.92	0.1671
	Fertilizer	107067.3	2	53.04	**$1.56e^{-09}$**
	Treatment x Fertilizer	11047.83	4	2.73	**0.0523**
NO_2^-	Treatment	0.0001	2	1.33	0.2824
	Fertilizer	.00004	2	0.37	0.6923
	Treatment x Fertilizer	0.0007	4	3.17	**0.0315**
Total N	Treatment	4638.1	2	1.97	0.1604
	Fertilizer	115103.9	2	49.05	**$3.32e^{-09}$**
	Treatment x Fertilizer	12106.8	4	2.57	**0.0629**

Note: Data is from a greenhouse study in which three fertilizer treatments (organic, inorganic, control) and three inoculant treatments (LEM, FLEM, water) were combined to generate a factorial of nine treatments that were applied to pots in which wheat was grown for 4 months. Rain event 1 occurred approximately one month after microbial inoculum application. Fifty mL of leachate was collected from soil columns and assayed for nitrogen content. Significant probabilities indicated by bold type.

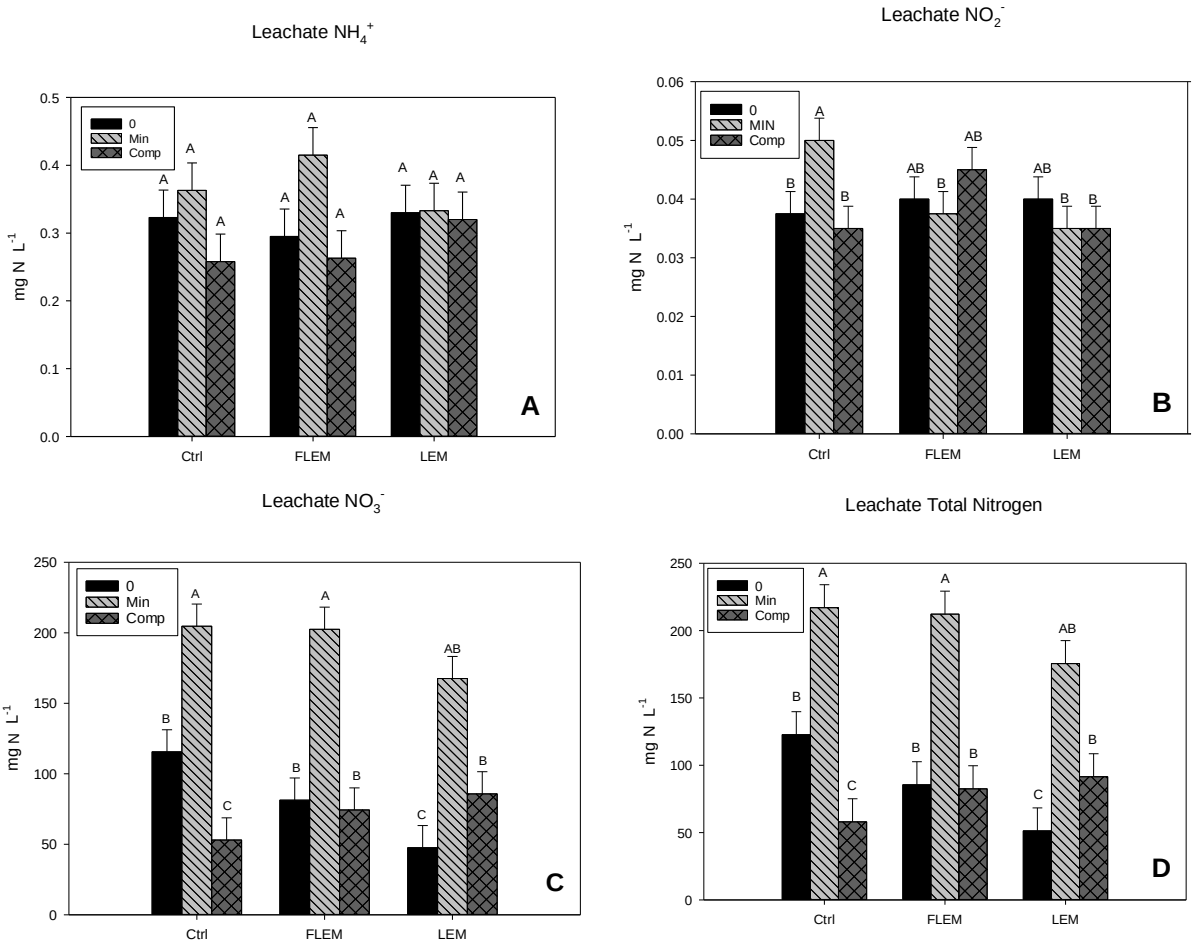

Figure 4. Rain event 1 leached nitrogen levels in 50 mL of leachate collected from soil columns receiving different inoculum and fertilizer treatments. Data is from a greenhouse study in which three fertilizer treatments (organic (Comp), inorganic (Min), control (0)) and three inoculant treatments (LEM, FLEM, water) were combined to generate a factorial of nine treatments that were applied to pots in which wheat was grown for 4 months. Rain event 1 occurred approximately one month after microbial inoculum application. Graph **A** indicates NH_4^+ fraction, graph **B** indicates NO_2^- fraction, graph **C** indicates NO_3^- fraction and graph **D** indicates total nitrogen. Different letters indicate significant differences between means according to pairwise comparisons using Tukey's test at p=0.05. Error bars indicate standard error of the mean.

Rain event 2 (Table 5) (Fig.5) resulted in reduced significance in the same forms of nitrogen loss at the LEM by composted poultry manure treatment level, though the quantities showed a similar trend as those in rain event 1. In a reversal, soil columns treated with LEM showed significantly increased levels of NH_4^+ ($p = 0.0388$) (Fig. 5**A**) in the leachate samples after rain event 2.

Table 5. Rain event 2 ANOVA table. Significance indicated by bold type.

Nutrient	Effect	SS	DF	F	Prob F
NH_4^+	Treatment	0.1	2	3.72	**0.0389**
	Fertilizer	0.01	2	0.86	0.4332
	Treatment x Fertilizer	0.003	4	0.09	0.9845
NO_3^-	Treatment	382.1	2	1.46	0.2499
	Fertilizer	5222.8	2	20.08	**$7.48e^{-06}$**
	Treatment x Fertilizer	725.6	4	1.39	0.2654
NO_2^-	Treatment	0.005	2	0.66	0.5227
	Fertilizer	0.02	2	3.23	**0.0571**
	Treatment x Fertilizer	0.02	4	1.68	0.1861
Total N	Treatment	829.7	2	2.17	0.1354
	Fertilizer	7395.7	2	19.38	**$9.76e^{-06}$**
	Treatment x Fertilizer	1455.8	4	1.90	0.1417

Note: Data is from a greenhouse study in which three fertilizer treatments (organic, inorganic, control) and three inoculant treatments (LEM, FLEM, water) were combined to generate a factorial of nine treatments that were applied to pots in which wheat was grown for 4 months. Rain event 2 occurred approximately two months after microbial inoculum application. Fifty mL of leachate was collected from soil columns and assayed for nitrogen content. Significant probabilities indicated by bold type.

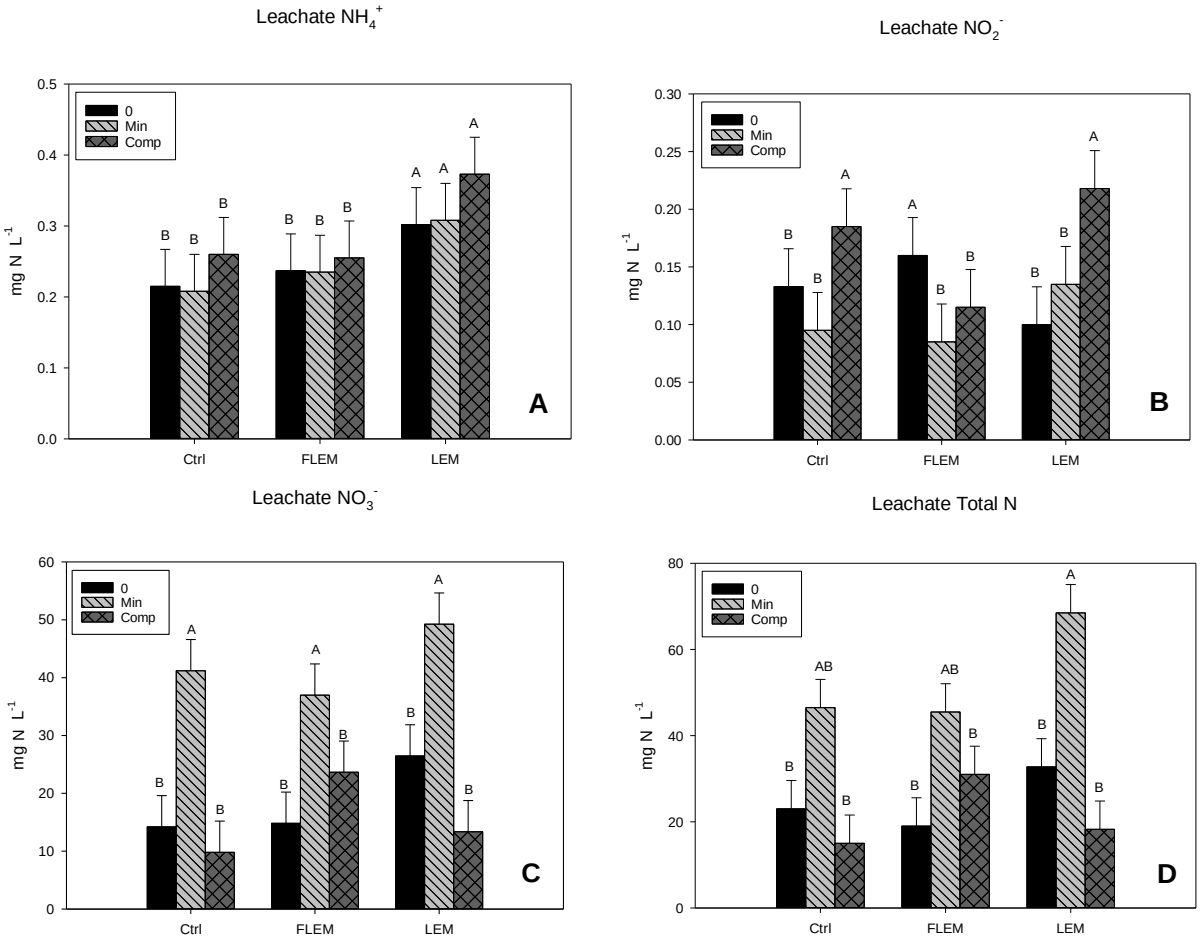

Figure 5. Rain event 2 nitrogen fraction concentrations in 50 mL of leachate collected from soil columns receiving different inoculum and fertilizer treatments. Data is from a greenhouse study in which three fertilizer treatments (organic (Comp), inorganic (Min), control (0)) and three inoculant treatments (LEM, FLEM, water) were combined to generate a factorial of nine treatments that were applied to pots in which wheat was grown for 4 months. Rain event occurred approximately two months after microbial inoculation. Graph **A** indicates NH_4^+ fraction, graph **B** indicates NO_2^- fraction, graph **C** indicates NO_3^- fraction and graph **D** indicates total nitrogen. Different letters indicate significant differences between microorganism treatments within fertilizer treatment according to pairwise comparisons using Tukey's test at p=0.05. Error bars indicate standard error of the mean.

Lower levels of nitrogen leached from the inoculated pot systems in the first rain event (Fig. 4) are most likely a result of increased soil microbial biomass (Fig. 6**A**). The nitrogen is rapidly utilized by the increased quantity of microorganisms in the soil and stored in their biological structures and products, sequestering it for later use through the process of

immobilization. This significantly limits immediate nitrogen losses through leaching in inoculated pots compared to control pots not treated with microorganism inoculant. This is consistent with the findings of previous studies (van Vliet, Bloem and de Goede, 2005), in which nitrogen is immobilized by soil microorganisms within the rhizosphere rather than leaching through the soil profile.

Decreased nitrogen leaching has the potential to impact surface and ground water quality in a significantly positive way by reducing the amount of nitrate introduced to bodies of water from nearby agroecosystems. Additionally, nitrogen fertilizer is a costly input for most agricultural systems, so the microbial fraction of the soil allows for more efficient use of such a financially important resource.

The second rain event displayed an increase in nitrogen leaching in the form of NH_4 from pot systems treated with LEM. Ney et al. (2019) showed that LEM has the ability to improve nitrogen mineralization. Total nitrogen, NO_2^- and NO_3^- only showed significant responses to fertilizer and not to microbial inoculant. There are a multitude of factors that could affect this, but time is again likely a significant factor. By the time of the second rain fall during the second month, the microbe communities have likely reached a more stable equilibrium in regard to mineralization and immobilization resulting in less N flux in the leachate.

3.3 Microbial Biomass and Soluble Organic Solids

Both LEM and FLEM inoculated soil showed significant increases in soil microbial biomass over controls ($p = 0.0003$) (Fig. 6**A**). Total soluble organic solid content in the top 5 cm of soil columns growing wheat and treated with FLEM inoculum was significantly greater ($p = 0.0291$) than LEM and control treatments (Fig. 6**B**). The FLEM by poultry manure treatments

displayed the most significant microbial biomass response compared to LEM and control treatments ($p = 0.0131$).

Soluble organic solid content provides insight for understanding the total relative amounts of organic molecules existing in the soil system including humic acid and fluvic acid, as well as deceased and living microbial components (Carletti et al., 2009). Greater levels of these compounds in the soil are associated with increased plant and soil health within agroecosystems.

Microbial biomass indicates the physical presence and relative quantity of live active microorganism populations within the soil which is an important distinction from soluble organic solids. This is important to note as the microbial fraction of soil acts as a storage system for nutrients, containing organic forms of major nutrients such as nitrogen (Malik et al., 2013) and plays a significant role in nutrient cycling.

Table 6. Soil microbial biomass and soluble organic solids ANOVA table.

Parameter	Effect	SS	DF	F	Prob F
Micro. Biomass	Treatment	929.3	2	11.65	**0.0003**
	Fertilizer	76.47	2	0.95	0.3974
	Treatment x Fertilizer	632.03	4	3.96	**0.0131**
Soluble Org. Solids	Treatment	0.03	2	4.11	**0.0291**
	Fertilizer	0.002	2	0.23	0.7899
	Treatment x Fertilizer	0.003	4	0.15	0.9575

Note: Data is from a greenhouse study in which three fertilizer treatments (organic, inorganic, control) and three inoculant treatments (LEM, FLEM, water) were combined to generate a factorial of nine treatments that were applied to pots in which wheat was grown for 4 months. Top 5 cm of soil was taken two months after inoculation and assayed for microbial biomass and soluble organic solids. Significant probabilities indicated by bold type.

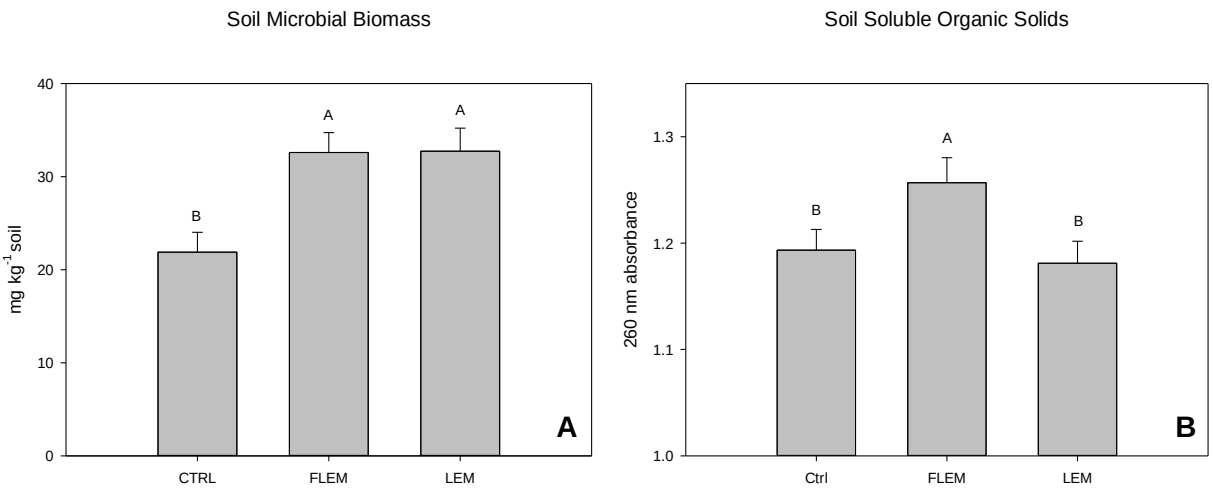

Figure 6. Soil microbial biomass and soluble organic solid responses to different inoculum treatments. Data is from a greenhouse study in which three fertilizer treatments (organic, inorganic, control) and three inoculant treatments (LEM, FLEM, water) were combined to generate a factorial of nine treatments that were applied to pots in which wheat was grown for 4 months. Top 5 cm of soil was taken two months after inoculation and assayed for microbial biomass and soluble organic solids. Graph **A** indicates soil microbial biomass levels and graph **B** indicates soluble organic solid levels. Different letters indicate significant differences between microorganism treatments within fertilizer treatment according to pairwise comparisons using Tukey's test at p=0.05. Error bars indicate standard error of the mean.

3.4 DNA Sequencing

Sequencing performed on 16s RNA in samples taken directly from fully mature liquid LEM and FLEM inoculum revealed unique and distinct differences in microbial community structure at the species and family levels.

In LEM, the main constituents at the species level were *Gluconacetobacter liquefaciens* (47%), *Gluconacetobacter tumulicola* (27%), *Lactobacillus paracasei* (10%), with the majority of the remaining 16% of the RNA reads consisting primarily of several more members of *Lactobacillaceae* (8%) and *Clostridaceae* (6%) and the final 2% being comprised of *Propionibacterium acnes*, *Roseomonas aerophile*, *Sphingomonas japonica*, *Achromobacter xylosoxidans*, and *Gluconacetobacter asukensis* (Fig. 7). Family level analysis displayed the same trend in regard to predominance in *Lactobacillaceae* and *Acetobacteraceae* (Fig. 8).

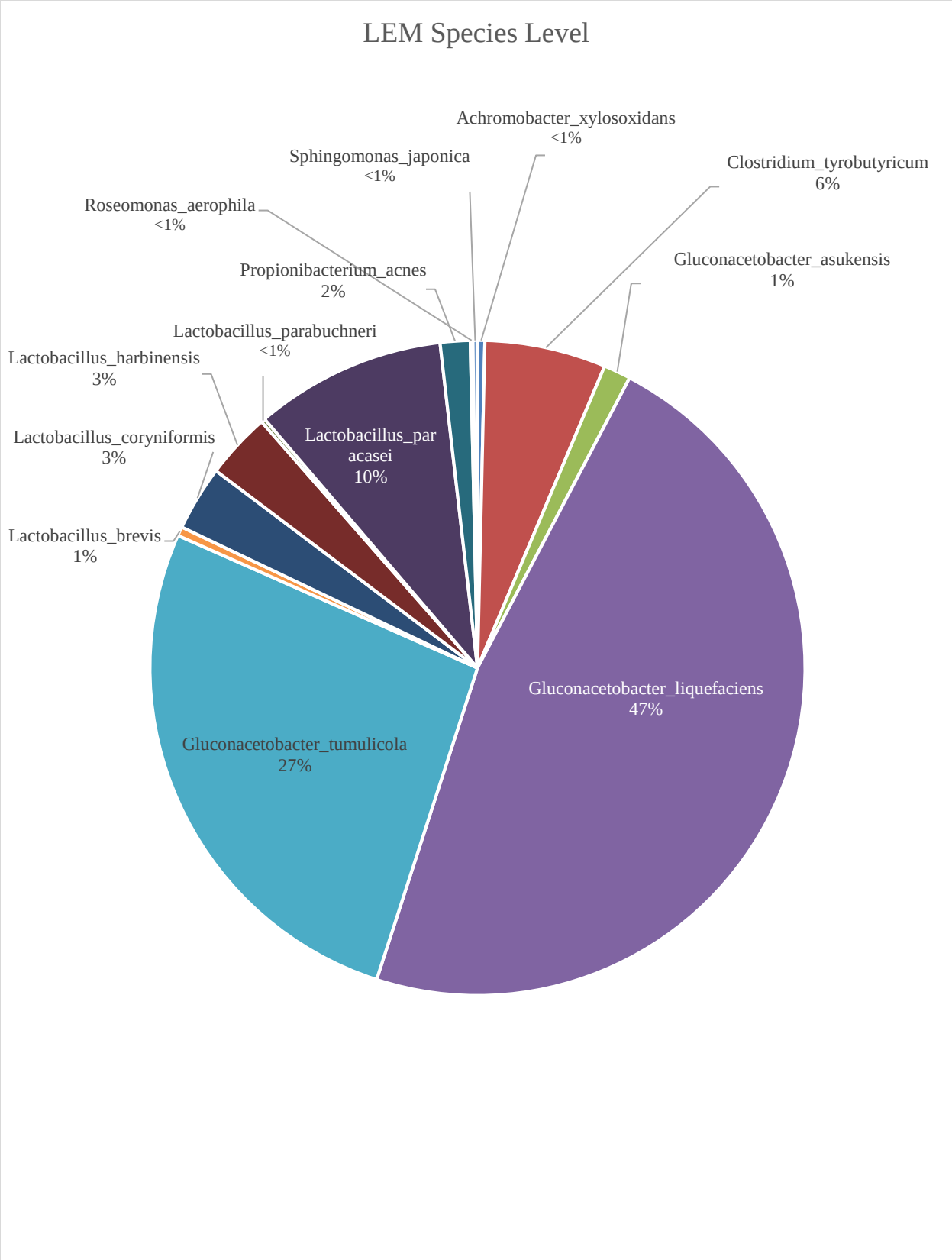

Figure 7. Species level percentages of 16s RNA reads from LEM inoculum.

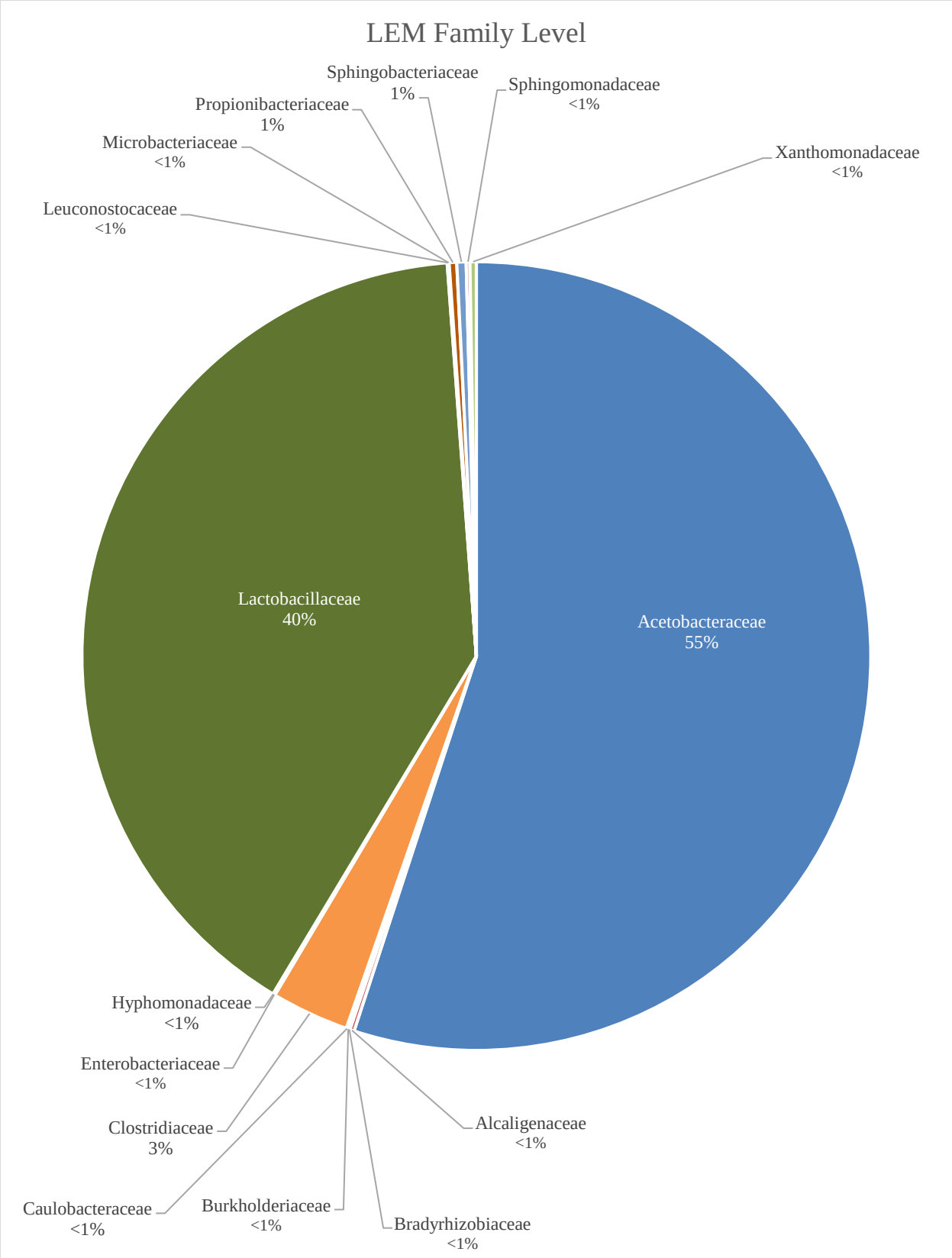

Figure 8. Family level percentages of 16s RNA reads from LEM inoculum.

Sequencing of 16s RNA on FLEM showed more diversity at the species and family level. The majority of the false inoculum was made up of Alcaligenes faecalis (25%), *Lactobacillus coryniformis* (21%), *Anaerosporobacter mobilis* (15%), *Propionibacterium acnes* (8%), *Lactobacillus concavus* (7%), and *Gluconacetobacter liquefaciens* (4%). The remaining 45% is comprised mostly of various members from *Lactobacillaceae*, *Acetobacteraceae*, and *Alcaligenaceae* (Fig. 9). Family level 16s RNA reads were dominated by *Lactobacillaceae* (24%), *Alcaligenaceae* (20%), *Acetobacteraceae* (7%), *Ruminococcaceae* (6%), *Enterobacteriaceae* (6%), *Lachnospiraceae* (5%), and *Propionibacteriaceae* (4%). The final 28% of the reads contain a wide variety of families in lesser quanities including notable families *Xanthomonadaceae* (2%), *Sphingobacteriaceae* (2%), *Streptococcaceae* (2%), *Rhizobiaceae* (<1%), and *Pseudomonadaceae* (<1%) (Fig. 10).

The microbial communities of FLEM and LEM display significant differences, with a higher diversity present in FLEM. However, in quantitative terms, LEM has a far higher richness of microorganisms (+ 50% in comparison to F-LEM), with a strong increase of *Lactobacillaceae* and *Acetobacteraceae*, and a decrease of other families, such as *Clostridiaceae, Alcaligenaceae, Enterobacteraceae and Ruminococcaceae*. *Lactobacillaceae, Acetobacteraceae* and *Enterobacteracea* are known to be plant growth promoting rhizobacteria (PGPR) involved in the solubilization of P at the rhizosphere level, thus having a potential beneficial effect also on plant P uptake. Several more families of microbes defined by previous literature as containing PGPR also appear in varying quantities in LEM and FLEM, such as *Clostridacea, Burkholderiaceae, Alcaligenaceae, Pseudomonadaceae, Flavobacteriaceae* and *Bradyrhizobiaceae* (Fig. 8 and Fig. 10) (Itelima et al., 2018; Çakmakçı et al., 2017; Kumari et al., 2019; Bhattacharyya, et al., 2016).

Members of *Acetobacteraceae* can oxidize a variety of different carbohydrate sources such as ethanol, sugars (including glucose and lactose, but also arabinose, fructose, galactose, mannose, ribose, sorbose and xylose), sugar alcohols, and organic acids. Thus, they are quite versatile in their ability to thrive in various substrates. This family contains obligate aerobes, and as such their process of breaking down and converting carbohydrates is known as oxidative fermentation, which is leveraged in the production of fermented foods such as vinegar and kombucha (Mamlouk and Gullo, 2013). Although production of LEM is generally done under anaerobic conditions, the significant presence of *Acetobacteraceae* in our inoculum shows that there was oxygen present near the end of the fermentation process. It is likely that the oxygen was introduced when the substrate was mixed before taking the samples and that these specific organisms were able to gain a significant foothold while the samples were waiting to be analyzed 3 weeks after the samples were taken. It is important to note, however, that *Acetobacteraceae* is still a highly functional group when considering agriculturally important soil microorganisms.

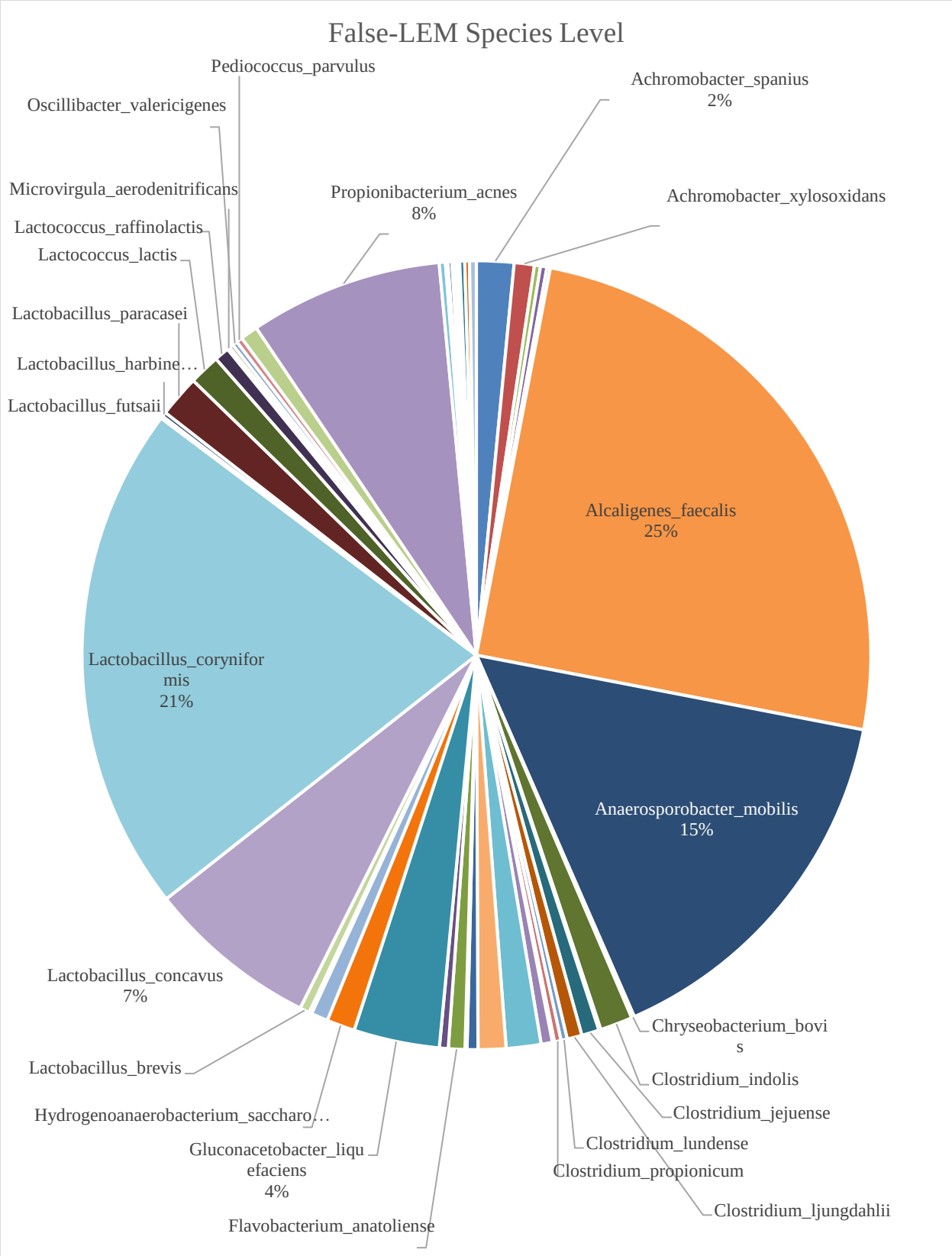

Figure 9. Species level percentages of 16s RNA reads from False-LEM inoculum.

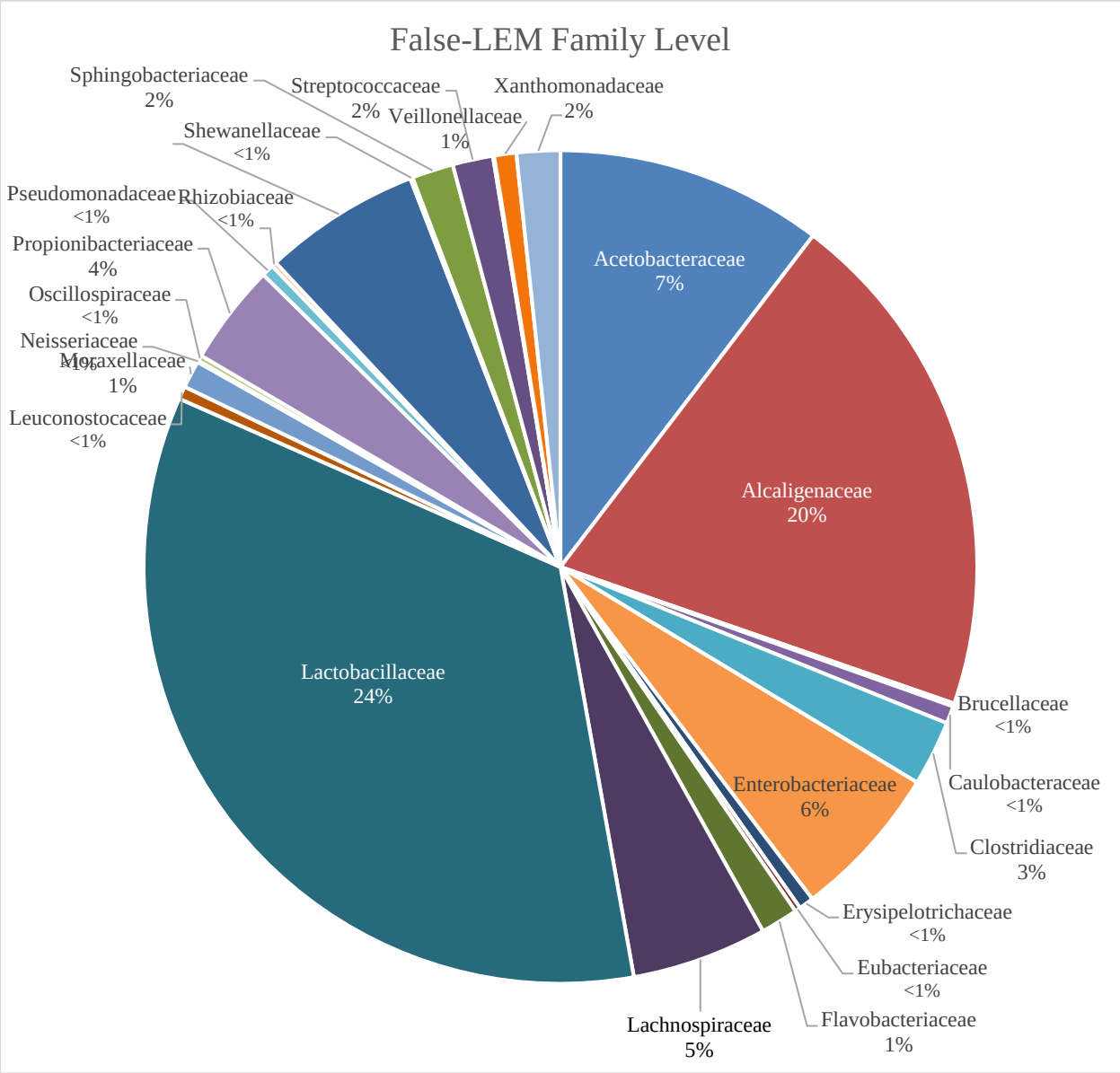

Figure 10. Family level percentages of 16s RNA reads from False-LEM inoculum.

While a diverse group of species are present within the LEM, the dominance of two to three species (Fig. 7 and Fig. 8) compared to FLEM (Fig. 9 and Fig. 10) is most probably a result of the LEM's increased maturity that is inherent in its production. The LEM and FLEM

inoculum consists of a two-phase fermentation: a solid phase fermentation followed by a liquid phase fermentation. The solid phase fermentation is intended to provide time and resources for the microorganism communities within the inoculum to become stabilized through competitive selection before the liquid phase, which differs from the solid fermentation because it is primarily for rapid expansion of the microbe communities. The LEM receives a dose of forest inoculum early in the solid phase of the production process while the FLEM does not. The introduction of the forest inoculum paves the way for higher selection pressure within the LEM fermentation vessel, allowing for members of *Lactobacillaceae* and *Acetobacteraceae* (Fig. 8) to gain more traction in the race to expand and proliferate. In comparison, no organisms within the FLEM fermentation vessel have a time advantage when it comes to competitive selection, so the communities that would have been overcome by *Lactobacillaceae* and *Acetobacteraceae* as seen in the LEM are still observed in FLEM which may give us insights as to which populations may flourish in aerobic soil environments. Both LEM and FLEM are dynamic populations of microorganisms growing in identical substrates, so it is likely that after more time the composition of the LEM and the FLEM microbial communities would begin to equilibrate and look more similar as the *Lactobacillaceae* and *Acetobacteraceae* communities also proliferated within the FLEM. Future research to study the effect of time as a variable in the production of locally sourced microbial inoculum will be needed to more fully understand the dynamics at play among the microorganisms during fermentation.

This study's intent was to quantify and elucidate crop and soil microbial community responses to LEM application through the use of proximal agronomic measures, leachate analysis and direct measures of soil microbial activity and presence. Attempting to quantify the influence of biological processes in agriculture is a significant challenge in modern

science due to natural variability in the organisms being studied and the lack of predictability associated with uncontrollable environmental factors inherent to the farms at which the studies are typically housed. This study was not immune to those challenges. With interaction between multicomponent living cultures and select plants, the variability is further compounded. Historical use of the method suggests that it may have more effect in highly weathered soils with little carbon, nutrients or biological activity, and it may be that in these areas there is a greater overall response. Notably, past research and reports highlight the importance of multi-year, season-by-season use of LEM for optimal reported effects. It may be that the populations of soils microorganisms build on each other after many seasons of application, which is a variable that the present study could not include. However, results collected in this study utilizing a decently productive and previously managed soil show increased total plant biomass in wheat treated with LEM. The significant increase of the microbial biomass with both F-LEM and LEM, indicates that the soil is responding to the application of the inoculates. The differences in soluble organic solids and on the microbial biomass in relation to the quantity and quality of fertilizers indicates that the structure of the soil microorganism population has been affected by the type of inoculant, with a differential utilization of the organic substrates with LEM or FLEM.

LEM + poultry manure treatments significantly reduced the loss of NO_2^- and NO_3^- through leaching in the first month after application and this, coupled with no signs of nitrogen deficiency using chlorophyll levels as a proxy, indicates that more nitrogen is retained in the rhizosphere to be used by the plant. Additionally, we have shown that LEM does not make nutrients unavailable to the plants in any significant way. Though the present study did not confirm previous study's findings of increased nutrient content in the reproductive tissue of the plants, it does show strong evidence that LEM and FLEM very significantly increased the

microbial biomass in the soil system, a distinct facet of soil and plant health. Significantly elevated levels of soluble organic solids were displayed by FLEM, which is possibly due to higher levels of species variability present in those particular cultures, as identified by the DNA sequencing data. Soluble solids analysis gives a wider representation of both living and deceased biological components present in the soil, so there is the possibility that FLEM inoculum had higher microbial turnover than the LEM inoculum, even though almost equal levels of microbial biomass were present at the soil level between the two treatments. That is to say, the FLEM likely had a higher ratio of dead microbes compared to living than the LEM. In either case, it is possible to conclude that more carbon is sequestered in the soil within the microbial bodies and humic compounds as a result of applying locally produced microbial inoculum, which can further optimize soil conditions and processes. Moreover, higher levels of carbon sequestration in the soil can lead to reduced levels of atmospheric carbon, an important function in fighting increasing global temperatures (Six, et. al., 2006). This leads to the indication that repeated use of LEM or LEM-like microbial cultures could be a great asset in not only small, marginal subsistence farms, but also on larger scale farms. The low cost, ready availability of components and relative ease of implementation highlight their potential gains.

4 Conclusion

The objectives of the present study were to methodically and scientifically investigate the effects of locally produced microorganism inoculum on key agronomic indices, nitrogen retention and microbial indicators. We found that nitrogen retention, plant biomass, leaf chlorophyll content, soil microbial biomass, and soil soluble organic solids were significantly elevated in systems receiving the microbial inoculum treatments. Improved retention and

stabilized provisioning of N in soil is an important microbial ecosystem service that can be strategically leveraged by farmers. The microbial fraction of the soil can serve to both increase agroecosystem resiliency by acting as a nutrient bank and to lessen leaching of costly nitrogen fertilizer from the system, ultimately resulting in added savings for growers and decreased environmental contamination. Based on our findings, in the container systems receiving locally produced microorganism inoculum treatments, nitrogen that would have otherwise been lost from the system was diverted to two other critical components- microbial biomass and plant growth. These results show that LEM has potential to increase plant, soil and environmental health within the first month of application.

Leveraging microorganisms and microorganism-mediated processes for use in agriculture will be a key facet in developing more efficient farms to keep up with increasing demand for food and fiber resulting from human population growth while adhering to global sustainability benchmarks. Local effective microorganism cultures show potential to be a viable path to this goal. Further research is needed to optimize the production of inoculants and to understand how local effective microorganisms mediate nutrient cycling, how they interact with plants and how their use over multiple seasons on the same soil might result in additive agronomic and soil health effects.

CHAPTER 3

General Conclusions

The present study set out to determine the possible effects of incorporating locally produced microbial inoculant (local effective microorganisms/LEM) into container-based cropping systems. LEM was used in the production of green bean (*Phaseolus vulgaris* cv. Valentino) and winter wheat (*Triticum aestivum*, L.) crops and was implemented together with varying fertilizer sources which included composted broiler litter, mineral fertilizer and no fertilizer. We chose three main response areas to serve as indicators to illustrate possible differences in soil microbial activity: basic agronomic indices, nitrogen retention within the soil system and key microbiological parameters. They were used to determine impacts of LEM as well as that of organic versus mineral fertilization. Through these observations and analyses of the data gleaned from our research, we greatly increased our understanding of LEM and additionally uncovered new questions to be answered in future research.

Through our trials incorporating LEM in conjunction with mineral and organic fertilizer (composted poultry manure), we discovered the ability of LEM to increase plant biomass in winter wheat, increase nitrogen retention within the cropping system, and increase soil microbial biomass. Additionally, we also found that FLEM elicited observable effects as well, resulting in increased leaf chlorophyll content, soil microbial biomass and soil soluble organic solids.

The primary goal of our research was to better understand how locally produced inoculum can be used in cropping systems to maximize its potential benefits to farmers. Based on our observations of LEM and FLEM's ability to positively affect agriculturally and environmentally significant parameters outlined above within the first month after application, we

can recommend that locally produced microbial inoculum be applied with an organic fertilizer source such as poultry manure. Along with recommendations for producers concerning the application of LEM, our research supports the possibility of microorganism cultures being produced on farms with cheap and readily available materials rather than in laboratory settings utilizing materials with greater barriers to entry.

In addition to providing valuable information about the benefits of locally produced microbial inoculum application and insights that will help producers maximize their associated benefits, our research has raised new questions. The influence of FLEM on soil microbial biomass, for instance, was an unexpected finding. FLEM was known to also introduce microorganisms to the cropping system but did not have the O horizon source material harvested from local forests like LEM did. It is important to understand if, and under what circumstances, FLEM might be more advantageous to apply in cropping systems compared to LEM. By manipulating variables such as soil moisture, soil type, time of application, repeated application and temperature, we could greatly improve our understanding of how and to what extend LEM versus FLEM application can improve soil and plant health.

Improved N retention in soils that received LEM application was another significant finding from this study. The increases in N retention in soils treated with LEM, however, were only consistent after the first month and dropped off after two months. Provisioning of nitrogen to plants is a critical soil microbial ecosystem service and greater understanding of how this can be prescribed to aid farmers in planning fertilization regimens is needed.

Though there is much more to learn about LEM production and use, the results from this study encourage continued research to develop and promote LEM as a method to sustainably leverage increased incremental gains from cropping systems, to reduce nitrogen

fertilizer losses and to bolster microbial communities within the rhizosphere. Based on our data, we conclude that LEM has the potential to be a useful tool to fortify the sustainability of agroecosystems and to invest in the healthful resiliency of that which is so foundationally important to life on earth- our soil.

REFERENCES

Abdu, N., 2006. Soil phosphorus extraction methodologies: a review. African Journal of Agricultural Research. Vol. 1(5) pp. 159-161.

Ahn, K., Lee, K.B., Kim, Y.J., and Koo, Y.M., 2014. Quantitative analysis of the three main genera in effective microorganisms using qPCR. Korean Journal of Chemical Engineering. Vol. 31(5) pp. 849-854.

Ait Barka E., Nowak J., Clement C. (2006). Enhancement of chilling resistance of inoculated grapevine plantlets with a plant growth-promoting rhizobacterium, Burkholderia phytofirmans strain PsJN. Appl. Environ. Microbiol. 72: 7246–7252.

Almario J., Kyselková M., Kopecký J., Ságová-Marečková M., Muller D., Grundmann G. L., et al. (2013a). Assessment of the relationship between geologic origin of soil, rhizobacterial community composition and soil receptivity to tobacco black root rot in Savoie region (France). Plant Soil.

Almario J., Prigent-Combaret C., Muller D, Mënne-Loccoz Y. (2013b). Effect of clay mineralogy on iron bioavailability and rhizosphere transcription of 2,4-diacetylphloroglucinol biosynthetic genes in biocontrol Pseudomonas protegens. Mol. Plant Microbe Interact. 26: 566–574.

Altieri, M.A., (1995). Agroecology: The Science of Sustainable 1434 Agriculture. Westview Press, Boulder, CO.

Arkhipova T. N., Prinsen E., Veselov S. U., Martinenko E. V., Melentiev A. I., Kudoyarova G. R. (2007). Cytokinin producing bacteria enhance plant growth in drying soil. Plant Soil 292: 305–315.

Badri D. V., Weir T. L., van der Lelie D., Vivanco J. M. (2009). Rhizosphere chemical dialogues: plant-microbe interactions. Curr. Opin. Biotechnol. 20: 642–650.

Bais H. P., Weir T. L., Perry L. G., Gilroy S., Vivanco J. M. (2006). The role of root exudates in rhizosphere interactions with plants and other organisms. Annu. Rev. Plant Biol. 57: 233–266.

Barea J. M., Pozo M. J., Azcón R, Azcón-Aguilar C. (2005). Microbial co-operation in the rhizosphere. J. Exp. Bot. 56: 1761–1778.

Baxter, J., and S. P. Cummings. 2008. The degradation of the herbicide bromoxynil and its impact on bacterial diversity in a top soil. J. Appl. Microbiol. 104:1605-1616.

Bekku, Y. et al., 1997. Examination of four methods for measuring soil respiration. Applied Soil Ecology. Vol. 5 pp.247–254.

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.

Bertin C., Yang X. H., Weston L. A. (2003). The role of root exudates and allelochemicals in the rhizosphere. Plant Soil 256: 67–83.

Bharti N., Yadav D., Barnawal D., Maji D., Kalra A. (2013). Exiguobacterium oxidotolerans, a halotolerant plant growth promoting rhizobacteria, improves yield and content of secondary metabolites in Bacopa monnieri (L.) Pennell under primary and secondary salt stress. World J. Microbiol. Biotechnol. 29: 379–387.

Bhattacharyya, P.N., D.K. Jha. (2012). Plant growth-promoting rhizobacteria (PGPR): emergence in agriculture. World J. Microbiol. Biotechnol., 28, pp. 1327-1350

Bhattacharyya, Goswami, and Bhattacharyya. (2016). Perspective of beneficial microbes in agriculture under changing climatic scenario: A review. Journal of Phytology 2016, 8: 26-41.

Bojović and Marković. (2009). CORRELATION BETWEEN NITROGEN AND CHLOROPHYLL CONTENT IN WHEAT (Triticum aestivum L.). Kragujevac J. Sci. 31 (2009) 69-74.

Bulgarelli D., Schlaeppi K., Spaepen S., Ver Loren van Themaat E., Schulze-Lefert P. (2013). Structure and functions of the bacterial microbiota of plants. Annu. Rev. Plant Biol. 64: 807–838.

Burns, R.G., DeForest, J.L, Marxsen, J., Sinsabaugh, R.L., et al. (2012). Soil enzymes in a changing environment: current knowledge and future directions. Soil Biology & Chemistry. Vol. 58 pp. 216-234.

Çakmakçı, Ramazan, et al. (2017) The Role of Soil Beneficial Bacteria in Wheat Production: A Review. IntechOpen: Wheat Improvement, Management and Utilization. DOI: 10.5772/67274

Cassán F., Perrig D., Sgroy V., Masciarelli O., Penna C., Luna V. (2009). Azospirillum brasilense Az39 and Bradyrhizobium japonicum E109, inoculated singly or in combination, promote seed germination and early seedling growth in corn (Zea mays L.) and soybean (Glycine max L.). Eur. J. Soil Biol. 45: 28–35.

Carletti, P., Vendramin, E., Pizzeghello, D., Squartini, A., et al. (2009). Soil humic compounds and microbial communities in six spruce forests as function of parent material, slope aspect and stand age. Plant Soil. 315: 47.

Chamam A., Sanguin H., Bellvert F., Meiffren G., Comte G., Wisniewski-Dyé F., et al. (2013). Plant secondary metabolite profiling evidences strain-dependent effect in the Azospirillum-Oryza sativa association. Phytochemistry 87: 65–77.

Chaparro J. M., Badri D. V., Bakker M. G., Sugiyama A., Manter D. K., Vivanco J. M. (2013). Root exudation of phytochemicals in Arabidopsis follows specific patterns that are developmentally programmed and correlate with soil microbial functions. PLoS ONE 8:e55731.

Chen C. R., Condron L. M., Davis M. R., Sherlock R. R. (2002). Phosphorus dynamics in the rhizosphere of perennial ryegrass (Lolium perenne L.) and radiata pine (Pinus radiata D. Don.). Soil Biol. Biochem. 34: 487–499.

Combes-Meynet E., Pothier J. F., Mënne-Loccoz Y., Prigent-Combaret C. (2011). The Pseudomonas secondary metabolite 2,4-diacetylphloroglucinol is a signal inducing rhizoplane expression of Azospirillum genes involved in plant-growth promotion. Mol. Plant Microbe. Interact. 24: 271–284.

Compant S., Clément C, Sessitsch A. (2010). Plant growth-promoting bacteria in the rhizo- and endosphere of plants: their role, colonization, mechanisms involved and prospects for utilization. Soil Biol. Biochem. 42: 669–678.

Contesto C., Desbrosses G., Lefoulon C., Bena G., Borel F., Galland M., et al. (2008). Effects of rhizobacterial ACC deaminase activity on Arabidopsis indicate that ethylene mediates local root responses to plant growth-promoting rhizobacteria. Plant Sci. 1–2: 178–189.

Cóndor Golec A.F., Lokare C., González Pérez P., 2007. Effective microorganisms: myth or reality? Rev Peru Biol. Vol. 14(2) pp. 315–319.

Couillerot O., Prigent-Combaret C., Caballero-Mellado J, Mënne-Loccoz Y. (2009). Pseudomonas fluorescens and closely-related fluorescent pseudomonads as biocontrol agents of soil-borne phytopathogens. Lett. Appl. Microbiol. 48: 505–512.

Daly, M. J., Stewart, D. P. C., 1999. Influence of Effective Microorganisms (EM) on Vegetable Production and Carbon Mineralization–A Preliminary Investigation. Journal of Sustainable Agriculture. Vol. 14 (2-3) pp. 15-25.

Davis, D.R, 2009. Declining fruit and vegetable nutrient composition: what is the eveidence? HortScience. Vol. 44(1) pp. 15-19.

Dempster, D.N., Jones, D.L., and Murphy, D.V., (2012). Clay and biochar amendments decreased inorganic but not dissolved organic nitrogen leaching in soil. Soil Research, 50, 216–221.

Desbrosses G. J., Stougaard J. (2011). Root nodulation: a paradigm for how plant-microbe symbiosis influences host developmental pathways. Cell Host Microbe. 10: 348–358.

Diver, S., 2001. Nature Farming and Effective Microorganisms, Rhizosphere II: Publications, Resource Lists and Web Links from Steve Diver. http://ncatark.uark.edu/~steved/Nature-Farm-EM.html

Dobbelaere S., Croonenborghs A., Thys A., Vande Broek A., Vanderleyden J. (1999). Phytostimulatory effect of Azospirillum brasilense wild type and mutant strains altered in IAA production on wheat. Plant Soil 212: 153–162.

Dodd I. C., Zinovkina N. Y., Safronova V. I., Belimov A. A. (2010). Rhizobacterial mediation of plant hormone status. Ann. Appl. Biol. 157: 361–379.

Doneche, B., G. Seguin, and P. Ribereau-Gayon. 1983. Mancozeb effect on soil microorganisms and its degradation in soils. Soil Sci. 135:361-366.

Drogue B., Combes-Meynet E., Mënne-Loccoz Y., Wisniewski-Dyé F., Prigent-Combaret C. (2013). "Control of the cooperation between plant growth-promoting rhizobacteria and crops by rhizosphere signals," in Vol. 1 and 2 Molecular Microbial Ecology of the Rhizosphere ed. de Bruijn F. J., editor. (NJ, USA: John Wiley & Sons, Inc.) 281–294.

Drogue B., Dore H., Borland S., Wisniewski-Dyé F., Prigent-Combaret C. (2012). Which specificity in cooperation between phytostimulating rhizobacteria and plants? Res. Microbiol. 163: 500–510.

du Jardin, Patrick, 2015. Plant biostimulants: Definition, Concept, Main Categories and Regulation. Scientia Horticulturae. Vol. 196 pp. 3-14.

Dutta S., Podile A. R. (2010). Plant growth promoting rhizobacteria (PGPR): the bugs to debug the root zone. Crit. Rev. Microbiol. 36: 232–244. El Mrabet, S., Ouhmane, L., El Mousadik, A., Msanda F., Abbas, Y., 2013. The effectiveness of arbuscular mycorrhizal inoculation and bio-

compost addition for enhancing reforestation with argania spinosa in morocco. Open Journal of Forestry. Vol. 4(1) pp. 14-23.

El Zemrany H., Cortet J., Lutz M. P., Chabert A., Baudoin E., Haurat J., et al. (2006). Field survival of the phytostimulator Azospirillum lipoferum CRT1 and functional impact on maize crop, biodegradation of crop residues, and soil faunal indicators in a context of decreasing nitrogen fertilisation. Soil Biol. Biochem. 38: 1712–1726.

El Zemrany H., Czarnes S., Hallett P. D., Alamercery S., Bally R., Jocteur-Monrozier L. (2007). Early changes in root characteristics of maize (Zea mays) following seed inoculation with the PGPR Azospirillum lipoferum CRT1. Plant Soil 291: 109–118.

Evans, J.R. (1983). Nitrogen and Photosynthesis in the Flag Leaf of Wheat (Triticum aestivum L.). Plant Physiol. 72, 297-302.

Fana, Ming-Sheng, Fang-Jie Zhaoa, Susan J. Fairweather-Tait, Paul R. Poulton, Sarah J. Dunhama, Steve P. McGrath. (2008). Evidence of decreasing mineral density in wheat grain over the last 160 years. Journal of Trace Elements in Medicine and Biology 22 (2008) 315–324.

Fester T., Maier W., Strack D. (1999). Accumulation of secondary compounds in barley and wheat roots in response to inoculation with an arbuscular mycorrhizal fungus and co-inoculation with rhizosphere bacteria. Mycorrhiza 8: 241–246.

Gaggia, F., Baffoni, L., Di Gioia, D., Accorsi, M., Bosi, S., Marotti, I., Biavati, B., and Dinelli, G., 2013. Inoculation with microorganisms of lolium perenne L.: evaluation of plant growth parameters and endophytic colonization of roots. New Biotechnology. Vol. 30(6) pp. 695-704.

Gans J., Wolinsky M., Dunbar J. (2005). Computational improvements reveal great bacterial diversity and high metal toxicity in soil. Science 309: 1387–1390.

García-Gutiérrez L., Zeriouh H., Romero D., Cubero J., de Vicente A, Pérez-Gracia A. (2013). The antagonistic strain Bacillus subtilis UMAF6639 also confers protection to melon plants against cucurbit powdery mildew by activation of jasmonate- and salicylic acid-dependent defense responses. Microb. Biotechnol. 6: 264–74.

Garvin, Welch, and Finely. (2006). Historical shifts in the seed mineral micronutrient concentration of US hard red winter wheat germplasm. Journal Sci Food Agric 86:2213–2220.

Giller, K.E., Beare, M.H., Lavelle, P., Izac, A.-M.N., Swift, M.J., 1997. Agricultural intensification, soil biodiversity, and agroecosystem function. Applied Soil Ecology. Vol. 6 pp. 3-16.

Grayston S. J., Wang S., Campbell C. D., Edwards A. C. (1998). Selective influence of plant species on microbial diversity in the rhizosphere. Soil Biol. Biochem. 30: 369–378.

Hartmann A., Schmid M., Tuinen D., Berg G. (2009). Plant-driven selection of microbes. Plant Soil 321: 235–257.

Hartz, T.K, J.P. Mitchell, and C. Giannini, (2000). Nitrogen and Carbon Mineralization

Dynamics of Manures and Composts. Hort. Sci. 35(2):209–212. Hemashenpagam N., Selvaraj T. (2011). Effect of arbuscular mycorrhizal (AM) fungus and plant growth promoting rhizomicroorganisms (PGPR's) on medicinal plant Solanum viarum seedlings. J. Environ. Biol. 32: 579–583.

Higa, T., (1998) Effective Micro-organisms. For Sustainable Agriculture and Healthy Environment. Jan van Arkel, Utrecht. 191 pp.

Higa, T., Parr, J.F., (1994). Beneficial and Effective Microorganisms for a Sustainable Agriculture and Environment. Vol. 1, International Nature Farming Research Center, Atami.

Hu, C., Qi, Y., (2012). Long-term effective microorganisms application promote growth and increase yields and nutrition of wheat in China. European Journal of Agronomy. Vol. 46 pp. 63-67.

Hu, C., Qi, Y., (2013). Effective microorganisms and compost favor nematodes in wheat crops. Agronomic Sustainable Development. Vol. 33 pp. 573-579.

Huang, N., Wang, W., Yao, Y., Zhu, F., Wang, W., & Chang, X., (2017). The influence of different concentrations of bio-organic fertilizer on cucumber Fusarium wilt and soil microflora alterations. Plos ONE. Vol. 12(2) pp. 1-17.

Indriani, N., Yuwariah, Y., Rochana, A., Susilawati, I., & Khairani, L., (2016). The role of Vesicular Arbuscular Mycorrhiza (VAM) and rock phosphate application on production and nutritional value of centro legumes (Centrosema pubescens), Legume Research: An International Journal, Vol. 39 pp. 987-990.

Irissarri, P., and Reinhold-Hurek, B., (2001). Azoarcus sp. strain BH72 as a model for nitrogen-fixing grass endophytes. Journal of Biotechnology Vol. 106 pp. 169-178

Itelima JU, Bang WJ, Onyimba IA, et al. (2018) A review: biofertilizer; a key player in enhancing soil fertility and crop productivity. J Microbiol Biotechnol Rep; 2(1):22-28.

Jaleel C. A., Manivannan P., Sankar B., Kishorekumar A., Gopi R., Somasundaram R., et al. (2007). Pseudomonas fluorescens enhances biomass yield and ajmalicine production in Catharanthus roseus under water deficit stress. Colloids Surf. B Biointerfaces 60: 7–11.

Javid, A., 2011. Effects of biofertilizers combined with different soil amendments on potted rice plants. Chilean Journal of Agricultural Research. Vol. 71(1) pp.157-163.

Javid, A., 2010. Beneficial microorganisms for sustainable agriculture. Sustainable Agriculture Reviews: Genetic Engineering, Biofertilisation, Soil Quality, and Organic Farming. Vol. 4. pp. 347-369.

Javaid, A., Bajwa, R., Anjum, T. 2008. Effect of heat-sterilization and EM (effective microorganisms) application on wheat (Triticum aestivum L.) grown in organic-amended sandy loam soil. Cereal Research Communications. Vol. 36(3) pp. 489-499.

Jilani, G., Akram, A., Ali, R.M., Hafeez, Y.F., Shamsi I.H., Chaudhry, A.N., Chaudhry, A.G., (2007). Enhancing crop growth, nutrients availability, economics and beneficial

Rhizosphere microflora through organic and biofertilizers. Annals of Microbiology. Vol. 57(2) pp. 177-183.

Jing Y. D., He Z. L., Yang X. E. (2007). Role of soil rhizobacteria in phytoremediation of heavy metal contaminated soils. J. Zhejiang Univ. Sci. B 8: 192–207.

Khaliq, A., Abbasi, M.K., Hussain, T., (2005). Effects of integrated use of organic and inorganic nutrient sources with effective microorganisms (EM) on seed cotton yield in Pakistan. Bioresource Technology. Vol. 97 pp. 967-972.

Kyselková M., Kopecký J., Frapolli M., Défago G., Ságová-Marečková M., Grundmann G. L., et al. (2009). Comparison of rhizobacterial community composition in soil suppressive or conducive to tobacco black root rot disease. ISME J. 3: 1127–1138.

Kumar B.L., Gopal D.S., (2015). Effective role of indigenous microorganisms for sustainable environment. 3 Biotech. Vol. 5(6), pp. 867–876.

Kumari, B., M. A. Mallick, Manoj Kumar Solanki, Anjali Chandrol Solanki, Amandeep Hora, and Wenfeng Guo. (2019). Plant Growth-Promoting Rhizobacteria (PGPR): Modern Prospects for Sustainable Agriculture. Plant Health Under Biotic Stress. Springer, Singapore.

Lavania M., Chauhan P. S., Chauhan S. V., Singh H. B., Nautiyal C. S. (2006). Induction of plant defense enzymes and phenolics by treatment with plant growth-promoting rhizobacteria Serratia marcescens NBRI1213. Curr. Microbiol. 52: 363–368.

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. doi:10.5423/PPJ.SI.02.2013.0021

Lorenz, T.C., (2012). Polymerase Chain Reaction: Basic Protocol Plus Troubleshooting and Optimization Strategies. Journal of Visualized Experiments. Vol. 63. 3998.

Lugtenberg B. J., Kamilova F. (2009). Plant-growth-promoting rhizobacteria. Annu. Rev. Microbiol. 63: 541–556.

Mahanta, K., Jha, D.K., Rajkhowa, D.J., Kumar, M., (2014). Isolation and evaluation of native cellulose degrading microorganisms for efficient bioconversion of weed biomass and rice straw. Journal of Environmental Biology. Vol. 35 pp. 721-725.

Malik, M.A., Khan, K.S., Marschner, P., Fayyaz-ul-Hassan. (2013). Microbial biomass, nutrient availability and nutrient uptake by wheat in two soils with organic amendments. J. Soil Sci. Plant Nutr. 13 (4), 955–966.

Mamlouk, D., & Gullo, M. 2013. Acetic Acid bacteria: physiology and carbon sources oxidation. Indian journal of microbiology, 53(4), 377–384.

Manero F. J., Algar E., Martin Gomez M. S., Saco Sierra M. D., Solano B. R. (2003). Elicitation of secondary metabolism in Hypericum perforatum by rhizosphere bacteria and derived elicitors in seedlings and shoot cultures. Pharm. Biol. 50: 1201–1209.

Mantelin S., Touraine B. (2004). Plant growth-promoting bacteria and nitrate availability: impacts on root development and nitrate uptake. J. Exp. Bot. 55: 27–34.

Mantelin S., Desbrosses G., Larcher M., Tranbarger T. J., Cleyet-Marel J. C., Touraine B. (2006). Nitrate-dependent control of root architecture and N nutrition are altered by a plant growth-promoting Phyllobacterium sp. Planta 223: 591–603.

Maramble B., Sangakkara U.R., (1998). Effect of EM on weed populations, weed growth, and tomato production in Kyusei nature farming. Proceedings of 4th Conference on Kyusei Nature Farming. Paris, France. pp. 211-216.

Mazzola M., Gu Y. H. (2002). Wheat genotype-specific induction of soil microbial communities suppressive to disease incited by Rhizoctonia solani anastomosis group (AG)-5 and AG-8. Phytopathology 92: 1300–1307.

Megali, L., Schlau, B., Rasmann, S., (2015). Soil microbial inoculation increases corn yield and insect attack. Agronomic Sustainable Development. Vol. 35 pp. 1511-1519.

McSorley, R., 2011. Overview of Organic Amendments for Management of Plant-Parasitic Nematodes, with Case Studies from Florida. Journal of Nematology. Vol. 43 pp.69–81.

Mikkelsen, R.L., Williams, H. M., and Behel Jr., A.D., 1994. Nitrogen leaching and plant uptake from controlled-release fertilizers. Fertilizer Research 37: 43-50.

Minorsky P. V. (2008). On the inside. Plant Physiol. 146: 323–324.

Moubayidin L., Di Mambro R., Sabatini S. (2009). Cytokinin-auxin crosstalk. Trends Plant Sci. 14: 557–562.

Müller-Stöver, D., Nybroe, O., Baraibar, B., Loddo, D., Eizenberg, H., French, K., & ... Christensen, S., (2016). Contribution of the seed microbiome to weed management. Weed Research. Vol. 56(5) pp. 335-339.

Ney et al. (2019) Rebuilding soil ecosystems for improved productivity in biosolarized soils. International Journal of Agronomy. vol. 2019, Article ID 5827585

Odum E. P., Barrett G. W. (2005). Fundamentals of Ecology, 5th Edn. Belmont, USA: Thomson Brooks/Cole.

Olsen, S. R., Cole, C. V., Watanabe, F. S., & Dean, L. A. (1954). Estimation of available phosphorus in soils by extraction with sodium bicarbonate. Circular. Vol. 939 (p. 19). Washington, DC: US Department of Agriculture.

Orr C. H., James A., Leifert C., Cooper J. M., Cummings S. P. (2011). Diversity and activity of free-living nitrogen-fixing bacteria and total bacteria in organic and conventionally managed soils. Appl. Environ. Microbiol. 77: 911–919.

Overvoorde P., Fukaki H., Beeckman T. (2011). Auxin control of root development. Cold Spring Harb. Perspect. Biol. 2.

Parniske M. (2008). Arbuscular mycorrhiza: the mother of plant root endosymbioses. Nat. Rev. Microbiol. 6: 763–775.

Pearse, A., (1980). Seeds of Plenty Seeds of Want: Social 1586 and Economic Implications of the Green Revolution. Oxford 1587 University Press, New York, p. 56.

Piccoli P., Bottini R. (1994). Effects of C/N ratio, N-content, pH, and incubation time on growth and gibberellin production by Azospirillum lipoferum. Symbiosis 17: 229–236.

Pothier J. F., Prigent-Combaret C., Haurat J., Mënne-Loccoz Y, Wisniewski-Dyé F. (2008). Duplication of plasmid-borne nitrite reductase gene nirK in the wheat-associated plant growth-promoting rhizobacterium Azospirillum brasilense Sp245. Mol. Plant Microbe Interact. 21: 831–842.

Prigent-Combaret C., Blaha D., Pothier J. F., Vial L., Poirier M. A., Wisniewski-Dyé F., et al. (2008). Physical organization and phylogenetic analysis of acdR as leucine-responsive regulator of the 1-aminocyclopropane-1-carboxylate deaminase gene acdS in phytobeneficial Azospirillum lipoferum 4B and other proteobacteria. FEMS Microbiol. Ecol. 65: 202–219.

Pothier J. F., Wisniewski-Dyé F., Weiss-Gayet M., Mënne-Loccoz Y., Prigent-Combaret C. (2007). Promoter-trap identification of wheat seed extract-induced genes in the plant-growth-promoting rhizobacterium Azospirillum brasilense Sp245. Microbiology 153: 3608–3622.

Pushpa, T., Sekaran, V., Basha, S., & Jegan, J., 2016. Investigation on Preparation, Characterization and Application of Effective Microorganisms (EM) Based Composts - An Ecofriendly Solution. Nature Environment and Pollution Technology. Vol. 15(1) pp. 153-158.

Ramaekers L., Remans R., Rao I. M., Blair M. W., Vanderleyden J. (2010). Strategies for improving phosphorus acquisition efficiency of crop plants. Field Crops Res. 117: 169–176.

Raimam, M.P., U. Albino, M.F. Cruz, G.M. Lovato, F. Spago, T.P. Ferracin, et al., 2007. Interaction among free-living N-fixing bacteria isolated from Drosera villosa var. villosa and AM fungi (Glomus clarum) in rice (Oryza sativa). Applied Soil Ecology. Vol. 35 pp. 25-34.

Richardson A. E., Baréa J. M., McNeill A. M., Prigent-Combaret C. (2009). Acquisition of phosphorus and nitrogen in the rhizosphere and plant growth promotion by microorganisms. Plant Soil 321: 305–339.

Richardson A. E., Simpson R. J. (2011). Soil microorganisms mediating phosphorus availability update on microbial phosphorus. Plant Physiol. 156: 989–996.

Roesch L. F. W., Camargo F. A. O., Bento F. M., Triplett E. W. (2007). Biodiversity of diazotrophic bacteria within the soil, root and stem of field-grown maize. Plant Soil 302: 91–104.

Roy-Bolduc, A., Hijri, M., 2011. The Use of Mycorrhizae to Enhance Phosphorus Uptake: A Way Out the Phosphorus Crisis. J Biofertil Biopestici (2),104.

Ryan, M.G., Law, B.E., 2005. Interpreting, measuring, and modeling soil respiration. Biogeochemistry. Vol. 73 pp. 3-27.

Saharan B. S., Nehra V. (2011). Plant growth promoting rhizobacteria: a critical review. Life Sci. Med. Res. 21: 1–30.

Selvaraj T., Rajeshkumar S., Nisha M. C., Wondimu L., Tesso M. (2008). Effect of Glomus mosseae and plant growth promoting rhizomicroorganisms (PGPR's) on growth, nutrients and content of secondary metabolites in Begonia malabarica Lam. Maejo Int. J. Sci. Technol. 2: 516–525.

Shaw L. J., Morris P., Hooker J. E. (2006). Perception and modification of plant flavonoid signals by rhizosphere microorganisms. Environ. Microbiol. 8: 1867–1880.

Shukla K. P., Sharma S., Singh N. K., Singh V., Tiwari K., Singh S. (2011). Nature and role of root exudates: efficacy in bioremediation. Afr. J. Biotechnol. 10: 9717–9724.

Simkin AJ, McAusland L, Headland LR, Lawson T, Raines CA. (2015). Multigene manipulation of photosynthetic carbon assimilation increases CO2 fixation and biomass yield in tobacco. Journal of Experimental Botany 66: 4075– 4090.

Singh, B., & Singh, K., 2016. Microbial degradation of herbicides. Critical Reviews in Microbiology. Vol. 42(2), pp. 245-261.

Six J., S. D. Frey, R. K. Thiet, and K. M. Batten (2006). Bacterial and Fungal Contributions to Carbon Sequestration in Agroecosystems. Soil Sci. Soc. Am. J. 70:555–569.

Smith K. P., Goodman R. M. (1999). Host variation for interactions with beneficial plant-associated microbes. Annu. Rev. Phytopathol. 37: 473–491.

Stepanova A. N., Alonso J. M. (2009). Ethylene signaling and response: where different regulatory modules meet. Curr. Opin. Plant Biol. 12: 548–555.

Tak H. I., Ahmad F., Babalola O. O. (2013). “Advances in the application of plant growth-promoting rhizobacteria in phytoremediation of heavy metals,” in Vol. 223, Reviews of Environmental

Contamination and Toxicology ed. Whitacre D. M., editor. (New York: Springer Science Business Media) 33–52.

Vacheron J., Desbrosses G., Bouffaud M.-L., Touraine B., Moënne-Loccoz Y., Muller D., et al. (2013). Plant growth-promoting rhizobacteria and root system functioning. Front. Plant Sci. 4: 356.

Vandermeer, J., 1997. Syndromes of production: an emergent 1662 property of simple agroecosystem dynamics. J. Environ. 1663 Manage. 51, 59–72.

van Vliet, P.C.J., Bloem, J., de Goede, R.G.M., 2005. Microbial diversity, nitrogen loss, and grass production after addition of effective microorganisms to slurry manure. Applied Soil Ecology. Vol. 32 pp. 188-198.

Veresoglou S. D., Menexes G. (2010). Impact of inoculation with Azospirillum spp. on growth properties and seed yield of wheat: a meta-analysis of studies in the ISI Web of Science from 1981 to 2008. Plant Soil 337: 469–480.

Walker V., Couillerot O., Von Felten A., Bellvert F., Jansa J., Maurhofer M., et al. (2012). Variation of secondary metabolite levels in maize seedling roots induced by inoculation with Azospirillum, Pseudomonas and Glomus consortium under field conditions. Plant Soil 356: 151–163.

Wang, Y., Zhao, X., Wang, L., Jin, S., Zhu, W., Lu, Y., & Wang, S., 2016. A five-year P fertilization pot trial for wheat only in a rice-wheat rotation of Chinese paddy soil: interaction of P availability and microorganisms. Plant & Soil. 399 (1/2), pp. 305-318.

Weil, R., Islam, K., Stine, M., Gruver, J., Samson-Liebig, S., 2003. Estimating active carbon for soil quality assessment: A simplified method for laboratory and filed use. American Journal of Alternative Agriculture. Vol. 18 pp. 3-17.

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.

Yang, Jungwook & Kloepper, Joseph & Ryu, Choong-Min. (2009). Rhizosphere bacteria help plants tolerate abiotic stress. Trends in plant science. 14. 1-4.

Zak, D.R, Holmes, W.E., White, D.C., Peacock, A.D., and Tilman D., 2003. Plant diversity, soil microbial communities, and ecosystem function: are there any links? Ecology. Vol. 84(8) pp. 2042-2050.

Zakaria, Z., Gairola, S., and Shariff, N. M., 2010. Effective Microorganisms (EM) Technology for Water Quality Restoration and Potential for Sustainable Water Resources and Management. International Congress on Environmental Modelling and Software. 142.

Zechmeister-Boltenstern, S., Keiblinger, K.M., Mosshammer, M., Penuelas, J., Richter, A., Sardans, J., Wanek, W., 2015. The application of ecological stoichiometry to plant-microbial-soil organic matter transformations. Ecological Monographs. Vol. 85(2) pp. 133-155.

Zhang H., Kim M. S., Krishnamachari V., Payton P., Sun Y., Grimson M., et al. (2007). Rhizobacterial volatile emissions regulate auxin homeostasis and cell expansion in Arabidopsis. Planta 226: 839–851.

Zhang H., Kim M. S., Sun Y., Dowd S. E., Shi H, Paré P. W. (2008). Soil bacteria confer plant salt tolerance by tissue-specific regulation of the sodium transporter HKT1. Mol. Plant Microbe Interact. 21: 737–744.

Zhang H., Sun Y., Xie X., Kim M. S., Dowd S. E., Pare P. W. (2009). A soil bacterium regulates plant acquisition of iron via deficiency-inducible mechanisms. Plant J. 58: 568–577.

Zheng, H., Zhenyu Wang, Xia Deng, Stephen Herbert, Baoshan Xing, 2013. Impacts of adding biochar on nitrogen retention and bioavailability in agricultural soil. Geoderma 206, 32–39.